

Regulation of colorectal cancer stem cell apoptosis and self-renewal by dependence receptors

Morgan Brisset

ORCID : 0000-0002-4665-0661

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Department of Clinical Pathology

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ABSTRACT :

The incidence of colorectal cancer (CRC) remains a significant global health concern, ranking among the most common types of cancer worldwide. Despite relatively good survival among patients with an early form of the disease, the prognosis for metastatic colorectal cancer (mCRC) remains grim, with drastically reduced survival rates once the disease has metastasized. This disparity in survival can be attributed to the limited efficacy of current treatment options, highlighting the urgent need for novel therapeutic approaches or strategies to enhance the effectiveness of existing treatments.

In this context, our project aimed to evaluate the implications of dependence receptors, specifically focusing on the UNC5B dependence receptor and its ligand Netrin-1, in modulating CRC and mCRC cells. Our study used a range of assays to characterize phenotypical characteristics, such as self-renewal using extreme limiting dilution assays, and transcriptomic assays such as RNA sequencing, and single-cell RNA sequencing on our patient-derived organoid model. Moreover, we assessed the therapeutical potential of targeting the UNC5B/Netrin-1 pathway using NP137, a neutralizing antibody for Netrin-1 currently undergoing clinical trials for solid tumours.

Our findings demonstrate that the UNC5B/Netrin-1 pathway plays a crucial role in modulating self-renewal and the induction of apoptosis in colorectal cancer stem cells. The inhibition of Netrin-1 by NP137 resulted in decreased self-renewal, increased cancer stem cell apoptosis and decrease of stemness-related genes in our patient-derived organoid cell lines.

Moreover, we demonstrate, *in vivo*, that targeting Netrin-1 with NP137 is an attractive and innovative therapeutic strategy that has the potential to enhance the efficacy of conventional chemotherapy in mCRC. Furthermore, our study reveals novel interactions between UNC5B and another dependence receptor, NOTCH3, which also contributes to the modulation of self-renewal in mCRC.

Overall, our research highlights the significance of dependence receptors in driving key cellular processes in CRC and mCRC, offering valuable insights into potential therapeutic targets and strategies. The identification of the UNC5B/Netrin-1 pathway implications

in the regulation of self-renewal and apoptosis in colorectal cancer stem cells is providing solid foundations for future investigations and the development of treatments preventing chemoresistance and recurrence for metastatic colorectal cancer.

Declaration

This declaration certifies that:

I. This thesis comprises only my original work towards the Ph.D. except where indicated in the Preface,

II. Due acknowledgement has been made in the text to all other material used,

III. The thesis is fewer than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Morgan Brisset

The University of Melbourne Centre for Cancer Research

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Preface

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All other intellectual and/or technical contributions are noted by authorships in the publication in Chapter 1.

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List of abbreviations

5-FU	5-fluorouracil
AGRF	Australian Genome Research Facility
AJCC	American Joint Committee on Cancer
ANOVA	Analysis of Variance
APAF1	Apoptotic Protease Activating Factor 1
APC	Adenomatous polyposis coli
BAK1	Bcl-2 homologous antagonist/killer
BAX	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology domain
BID	BH3 interacting-domain death agonist
BRAF	v-RAF murine sarcoma viral oncogene homolog B1
BSA	Bovine serum albumin
c-FLIP	Cellular FLICE-like Inhibitory Protein
CAR	Chimeric antigen receptor
Cas9	CRISPR-associated protein 9
CIBERSORT	Cell type Identification by Estimating the Relative Subsets of RNA Transcripts
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CMS	Consensus molecular subtype
CNV	Copy number variation

COAD	Colorectal adenocarcinoma
CoT	Colorectal primary tumour
CRC	Colorectal cancer
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRL	CULLIN-RING-Ligase
CSC	Cancer stem cell
CTC	Circulating tumour cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
COMMD	Copper metabolism MURR1 domain containing
PD-1	Programmed cell death protein 1
PD-L1	Programmed cell death ligand 1
DAPI	4',6-diamidino-2-phenylindole
DAPK	Death-Associated Protein Kinase
DCC	Deleted in Colorectal Carcinoma
DD	Death Domain
DED	Death Effector Domain
DE	Differential expression
DEG	Differentially expressed gene
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DISC	Death-Inducing Signaling Complex
DSCAM	Down-syndrome cell adhesion molecule

ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELDA	Extreme limiting dilution assay
EMT	Epithelial-mesenchymal transition
Eph4A	Ephrin type A receptor 4
ERK	Extracellular Signal-Regulated Kinase
FADD	Fas Associated via Death Domain
FASLG	FAS ligand
FACS	Fluorescence-activated cell sorting
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
FC	Flow cytometry
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FOLFIRI	5-FU/leucovorin/irinotecan
FOLFOX	5-FU/leucovorin/oxaliplatin
FOLFOXIRI	5-FU/leucovorin/oxaliplatin/irinotecan
GFP	Green fluorescent protein
GPI	Glycophosphatidylinositol
GSK3β	Glycogen Synthase Kinase 3 β
H&E	Haematoxylin & Eosin
HNPCC	Hereditary non-polyposis colorectal cancer
HRP	Horseradish peroxidase

HSC	Haematopoietic stem cell
IFN	Interferon
IL	Interleukin
KRAS	Kirsten rat sarcoma 2 viral oncogene homologue
LT	Liver metastasis from colorectal Tumour
LGR5	Leucine Rich Repeat Containing G Protein-Coupled Receptor 5
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
MLH1	MutL homolog 1
MMR	Mismatch repair
MSH2/3/6	MutS homolog 2/3/6
MSI	Microsatellite instability
MSigDB	Molecular Signatures Database
Mule	Mcl-1 ubiquitin ligase E3
NGF	Nerve Growth Factor
NCBI	National Centre for Biotechnology Information
NRAGE	Neurotrophin Receptor-interacting MAGE homolog
NT-3	Neurotrophin-3
NTR	Netrin-like domain
NTN1	Netrin-1
OS	Overall survival
PAM	Protospacer-adjacent motif
PBMC	Peripheral blood mononuclear cell

PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PDO	Patient-derived organoid
PDX	Patient-derived xenograft
PEI	Polyethyleneimine
PEST	Proline (P), Glutamate (E), Serine (S), Threonine (T)
PFS	Progression-free survival
PI3K	Phosphoinositide 3-kinase
PIKE-L	PI 3-Kinase Enhancer
PINK1	PTEN-induced kinase 1
PROM1	Prominin1
Ptch1	Patched-1
PUMA	p53-upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
p75NTR	p75 Neurotrophin Receptor
READ	Rectal adenocarcinoma
ROS	Reactive Oxygen Species
RP	Ribosomal Protein
RPKM	Reads per kilobase million
RPMI	Roswell Park Memorial Institute 1640
RT	Room temperature
scRNAseq	Single cell RNA sequencing

SCF	Stem Cell Factor
SD	Standard deviation
SDS	Sodium dodecyl sulfate
sgRNA	Synthetic guide RNA
sgSCR	Synthetic guide scramble control
SHH	Sonic Hedgehog
SMOC2	SPARC-Related Modular Calcium-Binding Protein 2
SOX4	SRY (Sex Determining Region Y)-Box 4
SNV	Single nucleotide variant
TBS-T	Tris-buffered saline with tween
TCGA	The Cancer Genome Atlas
TFF3	Trefoil Factor 3
TGF-β	Transforming growth factor- β
TIDE	Tracking of indels by decomposition
TME	Tumour microenvironment
TNM	Tumour-Node-Metastases
TRAIL	TNF-related apoptosis-inducing ligand
Trk	Tropomyosin Receptor Kinase
TP53	Tumour protein P53
ULA	Ultra-low attachment
UNC5H	Uncoordinated 5 Homologs
UNC-6	Uncoordinated-6
UMAP	Uniform manifold approximation projection

VCL	Vinculin
VEGF	Vascular endothelial growth factor
WEHI	Walter and Eliza Hall Institute of Medical Research
WT	Wildtype
ZEB	Zinc Finger E-Box Binding Homeobox
Zu-5	Zona-occludens/Unc-5 homology domain

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Chapter 1: Introduction

I. Colorectal Cancer (CRC)

A. Epidemiology of colorectal cancer

1. Incidence and mortality

Colorectal Cancer (CRC) is one of the most prevalent and lethal forms of cancer globally. Approximately 2 million of person develop CRC each year resulting in nearly one million deaths, positioning it as the third deadliest and fourth most frequently diagnosed cancer worldwide [1]. The highest incidence rates of colorectal cancer are observed in developed countries such as those in Europe, Australia, New Zealand, and Northern America [2].

Australia, for instance, reported 15 713 new cases and 5 326 deaths attributed to CRC in 2022, representing the fourth most common cancer and the second leading cause of cancer-related death in the country [3]. Although the prevalence of CRC is usually higher in developed countries, the ongoing industrialization and lifestyle modifications in developing countries are driving an increase in cases and further justifying a particular interest in the treatment of CRC.

2. Risk factors

Increased risk of CRC is notably associated with the Western lifestyle. Major risk factors for this disease include obesity, smoking, alcohol consumption, and sedentary behaviour [4, 5]. Furthermore, dietary habits play a crucial role, with high consumption of red processed meat and elevated sugar intake significantly increasing the likelihood of developing CRC [6, 7].

In addition to these lifestyle-associated risk factors, prolonged inflammation emerges as a key risk factor. Indeed, individuals with chronic inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, demonstrate higher rates of CRC compared to the general population [8]. Moreover, there is a growing recognition of the influence of the intestinal microbiome on CRC development, with bacteria producing metabolites, invading tissues, and influencing immune responses [9-12].

Approximately 2% to 5% of colorectal cancers originate within inherited syndromes, such as Lynch syndrome, familial adenomatous polyposis, MUTYH-associated polyposis, and specific hamartomatous polyposis conditions. These syndromes are characterized by a significantly elevated risk of developing colorectal cancer. Beyond these syndromes, up to one-third of colorectal cancers show an augmented familial risk, likely attributed to hereditary factors[13].

3. Clinical grading and staging

Colorectal Cancer (CRC) stages are categorized mainly by three distinct criteria, denoted as T, N, and M, each characterizing a specific facet of cancer progression [14]. The T stage delineates the depth of local tumour invasion, specifically the growth of primary neoplasm within the intestinal epithelium, submucosa, and adjacent tissues. The N stage relates to lymph node involvement, indicating the invasion of cancer cells into lymph nodes that have escaped the primary tumour. Lastly, the M stage is based on the spreading of secondary tumours to distant organs (metastases). These classifications facilitate the grouping of tumours into five stages commonly referred to as the five stages of Union Internationale Contre le Cancer (UICC), ranging from stage 0 (confined to the mucous membrane) to stage IV (metastasized tumour).

CRC development is marked by genomic instability, with two main pathways: chromosomal instability (CIN, 85%) and microsatellite instability (MSI, 15%) [15, 16]. CIN tumours result from mutations causing chromosomal aneuploidy and structural rearrangements, accompanied by mutations in specific genes critical for CRC initiation and progression [17]. MSI tumours arise from mismatch repair (MMR) gene inactivation, particularly MSH2, MLH1, MSH6, and PMS2, leading to errors in DNA replication at microsatellite sequences [18-20]. This deficiency accelerates genetic errors, creating a hypermutator phenotype or MSI tumours, whereas microsatellite stable tumours (MSS) have intact MMR. Epigenetic changes, such as MLH1 promoter methylation, cause sporadic MSI in most cases, with only about 3% having germline MMR mutations (Lynch syndrome) [21]. MSI is more common in stage II (20%) than stage III (12%) CRC [22]. MSI tumours are associated with right colon tumours, poor differentiation, abundant

tumour-infiltrating lymphocytes, diploid DNA content, and less aggressive behaviour, whereas CIN tumours show aneuploidy without any site preference [23]. Sporadic MSI CRC shares clinicopathological features with germline MSI CRC but differs in patient demographics and mutation patterns. Lynch syndrome (LS) patients, with germline mutations in DNA MMR genes (MLH1 or MSH2 mutations), have unique characteristics, increased risk for other cancers, and approximately 50% cumulative risk of developing CRC by the age of 70 years [24].

Another critical factor influencing cancer prognosis is the tumour differentiation grade, also referred to as histological grade. This grade plays a pivotal role in reflecting the tumour biology and its distinctive characteristics. However, the histology grading process has faced criticism due to potential subjectivity [25]. The grading is primarily based on the degree of gland formation, representing the resemblance to the original tissue, and ranges from well-differentiated (G1) to poorly differentiated (G3) or even undifferentiated (G4). Multiple multivariate analyses emphasize the significance of tumour differentiation grade as a stage-independent prognostic factor in colorectal cancer, where a higher tumour grade is associated with an unfavourable prognosis [26-28]. Additionally, the grade correlates with the frequency of lymph node metastasis in stage III colon cancer (**Table 1.1**). Tumour differentiation grade stands out as a crucial prognostic factor, showing significant correlations not only with the overall stage of the TNM system but also with each of its components [25].

		Definition	Distribution	Proportion stage IV at diagnosis
Differentiation grade		Gland formation in per cent	n (%)	n (%)
Well	G1	>95%	55 (4.4%)	3 (5.5%)
Medium	G2	>50%	914 (73.8%)	104 (11.4%)
Poor	G3	<50%	216 (17.4%)	34 (15.7%)
Undifferentiated	G4	<5%	54 (4.3%)	9 (16.7%)
Total			1239	150/1239 (12.1%)

Table 1.1 – Proportions of Stage IV tumours according to Tumours differentiation grade (Adapted from Compton, C. Colorectal Carcinoma: Diagnostic, Prognostic, and Molecular Features. Mod Pathol 16, 376–388 (2003). <https://doi.org/10.1097/01.MP.0000062859.46942.93>)

4. Diagnosis and treatment

The common indicators of Colorectal Cancers (CRCs) include the presence of blood in, or the unusual size of stool, rectal bleeding, abdominal pain, and anaemia [29]. CRC detection at any stage is possible through a faecal occult blood test, a procedure designed to identify small amounts of blood in faeces [30]. This test is used as a screening tool for high-risk groups and in > 50-year-olds from the general population in a lot of countries, including Australia [31]. However, the predominant method for establishing a definitive diagnosis involves endoscopic procedures, particularly colonoscopies and sigmoid endoscopies [32]. Indeed, the histopathological analysis of endoscopic specimens is vital for diagnosing and prognosing colorectal cancer. The pathology report, influenced by clinical information and specimen characteristics, provides crucial details such as the presence of mucosal or submucosal structures, intraepithelial neoplasia, or invasive growth. Grading and resection margin information is crucial for follow-up recommendations, as carcinoma risk and local recurrence rates depend on these factors [33]. Detection of invasive growth, like adenocarcinoma, includes details on invasion depth, lymph or blood vessel involvement, and resection margins. Overall, histopathological analysis guides the management and prognosis of CRC [33]. It is to be noted that the use of computed tomography (CT) and whole-body positron emission tomography (PET) is recommended when metastatic disease is suspected.

For localized CRC, surgical resection stands as the most common treatment, while a variety of interventions, both surgical and chemotherapeutic, are available for mCRC. Although surgical resection is occasionally employed for curative purposes in mCRC, chemotherapy is more commonly employed.

The treatment of colon cancer varies based on the stage of the disease, and a comprehensive approach often involves a combination of surgery, chemotherapy, and, in some cases, radiation therapy [34]. At stage I, the surgery remains the primary

treatment, aiming to remove the tumour and nearby lymph nodes. At stage II, surgery is the mainstay, and depending on specific factors, such as tumour characteristics, chemotherapy may be recommended to reduce the risk of recurrence. During stage III, treatment often involves surgery to remove the tumour and affected lymph nodes, followed by adjuvant chemotherapy to target any remaining cancer cells and reduce the risk of recurrence [34]. Stage I-III rectal cancer is treated similarly to colon cancer, although typically also includes neoadjuvant chemoradiation.

For mCRC, treatment recommendations include various modalities based on specific molecular and clinical characteristics. Doublet or triplet chemotherapy forms the backbone of systemic treatment in most cases of initially unresectable mCRC. This can be combined with either an anti-vascular endothelial growth factor antibody or epidermal growth factor receptor inhibitor (EGFRi) (depending on RAS/RAF mutation status and primary tumour location). Additionally, tumours characterised with deficient mismatch repair status are treated with PD1-inhibition rather than chemotherapy [35].

After the failure of 1st line chemotherapy, patients may receive either 2nd line chemotherapy or targeted therapies, depending on their tumour molecular profile. This may include BRAF inhibition plus EGFRi, KRAS G12C inhibition plus EGFRi, or Her-2 directed therapy for BRAFV600E mutant, KRASG12C mutant and HER2 amplified CRC, respectively. Definitive surgery, (e.g. liver resection for isolated liver metastases or cytoreductive surgery for peritoneal metastases) and stereotactic body radiation therapy may be considered in selected cases of mCRC, typically after a period of systemic treatment. Multidisciplinary team management and shared decision-making play a crucial role in tailoring treatment strategies for individual patients with mCRC [35].

Stage	Treatment
I	Surgery
II	Surgery + adjuvant chemotherapy depending on tumour characteristics
III	Surgery (Remove tumour and lymph nodes) + adjuvant chemotherapy

I-III Rectal Cancer		Like colon cancer + neoadjuvant chemoradiation
Metastatic Colorectal Cancer (mCRC)	1 st line treatment	Doublet or triplet chemotherapy with anti-VEGF antibody/surgery may be considered after systemic treatment
	Deficient mismatch repair status	1 st line treatment + PD1-inhibitor
	RAS/RAF mutated	1 st line treatment + EGFR inhibitor
	2 nd line treatment	BRAF inhibitor, KRAS G12c inhibitor, Her-2 directed therapy

Table 2.2 – Recapitulative tables of the most common treatment used in CRC according to tumour stage

Various molecules are employed in chemotherapy regimens, including 5-fluorouracil (5-FU), an anti-metabolite and uracil analogue that hinders DNA synthesis and inhibits cell division [36]; Oxaliplatin, a platinum-based agent exerting a cytotoxic effect by binding to DNA, RNA, and certain protein structures [37]; and Irinotecan, a molecule inhibiting cell division by acting on topoisomerase I [38]. Patients receive combinations of these three molecules, usually together with Leucovorin, an active metabolite of folic acid serving as an essential coenzyme for nucleic acid synthesis, to enhance the efficacy of 5-Fluorouracil [39]. These combinations are known as FOLFOX (Oxaliplatin, 5-FU, and Leucovorin), FOLFIRI (Irinotecan, 5-FU, and Leucovorin), and FOLFOXIRI (Oxaliplatin, Irinotecan, 5-FU, and Leucovorin) [40].

Targeted therapies in cancer aim to inhibit cell proliferation, migration, and differentiation, influencing both cancerous cells and the tumour microenvironment. Small molecules and monoclonal antibodies are integral to these therapies, with a focus on molecules penetrating cells to inactivate enzymes like cyclin-dependent kinases, proteasomes, and poly ADP-ribose polymerase. Monoclonal antibodies also target cell surface receptors, regulating downstream cell cycle progression and cell death. In CRC, cetuximab and bevacizumab were among the first FDA-approved targeted drugs, with numerous others entering the market. However, challenges persist in targeting various CRC-related pathways, such as Wnt/ β -catenin, Notch, Hedgehog, and TGF- β /SMAD, due

to complex downstream signalling and the need for more efficient clinical interference in diverse pathways that remain in preclinical or phase I trial stages. The dynamic landscape of CRC-targeted therapies underscores the ongoing efforts to address the intricate molecular mechanisms underlying cancer initiation, progression, and migration[41, 42].

EGFR antibodies, including cetuximab and panitumumab, are crucial components of targeted therapy in CRC. However, their efficacy is limited to patients without KRAS/NRAS mutations. In cases where these mutations are present, the activation of downstream signalling pathways renders EGFR inhibitors less effective. Therefore, molecular profiling of the tumour is essential to determine the suitability of EGFR antibody treatment [43]. For patients with KRAS/NRAS wild-type CRC, EGFR antibodies have demonstrated notable success. Additionally, targeted inhibitors like BRAF V600E inhibitors, such as vemurafenib and dabrafenib, have shown promise in addressing specific genetic alterations associated with CRC, offering a targeted therapeutic approach for patients with BRAF mutations. These precision medicine strategies underscore the importance of specific treatments based on the genetic and phenotypical profile of each patient's tumour [44].

B. Pathogenesis of colorectal cancer

1. Multi-step Tumorigenesis

The pathogenesis of CRC involves a gradual accumulation of genetic and epigenetic changes, following a multi-step tumorigenesis process. In this context, the accumulation of alterations in oncogenes and tumour suppressor genes drives the histological progression from normal epithelium to invasive carcinoma [45]. The process is initiated by genetic or epigenetic changes in tumour suppressors or oncogenes within a single cell, commonly identified as a stem or stem-like cell [46]. These changes confer growth and/or survival advantages to the cell, leading to clonal expansions. Subsequent advantageous genetic alterations result in additional clonal expansions, and as these clones accumulate new functional abilities over cell cycles, malignant cells emerge. A cell is characterised as fully malignant after acquiring the complete set of 'hallmark'

characteristics of cancer cells, including self-sufficiency in growth signals, insensitivity to growth inhibitory signals, escape from apoptosis, limitless replicative potential, angiogenesis, invasion, and metastasis abilities [41].

The colon and rectum are lined by a layer of glandular epithelium, which gives rise to most neoplasms. Early lesions originating from the epithelium are termed adenomas, typically small polyps with structural and cytological abnormalities referred to as 'dysplasia.' Adenomas cannot metastasize initially but progress to a significantly larger size (over 8 times). Eventually, tumour cells gain the ability to invade the submucosa and access the lymphatic system and blood vessels, enabling the metastasis of the primary tumour to distant sites and other organs. The defining feature that distinguishes a colorectal carcinoma from an adenoma is the submucosal invasion and, consequently, the metastatic potential of cancer cells [47].

2. Molecular subtypes of colorectal cancer

The current understanding of colorectal tumorigenesis involves several distinct genetic pathways, each originating from different precursors and corresponding to distinct genetic changes. The three currently characterized pathways are the conventional adenoma-carcinoma pathway, the sessile serrated pathway, and the Lynch syndrome pathway [14].

The conventional adenoma-carcinoma pathway, constituting the chromosomal instability sequence pathway, is the predominant way leading to CRC, accounting for approximately 70 to 90% of sporadic colorectal carcinomas [48]. This pathway is initiated by the formation of a uni-cryptal adenoma, a dysplastic lesion involving a single crypt, progressing to tubular adenomas, and subsequently to tubulovillous adenomas or villous adenomas as DNA changes occur [49]. The evolution from early adenoma to carcinoma is estimated to take 10-20 years, with not all adenomas progressing to carcinoma, and some experiencing spontaneous regression [49].

The molecular characterization of sporadic lesions along the conventional adenoma-carcinoma pathway reveals a consistent sequence of genetic and epigenetic changes in

tumour suppressor genes and oncogenes. Most tumours in this pathway are initiated by bi-allelic inactivation of the adenomatous polyposis coli (APC) tumour suppressor gene, considered the gatekeeper of colorectal carcinogenesis. APC mutation leads to aberrant activation of the Wnt pathway [50, 51]. Additional gene modifications, including KRAS mutations[52], activating mutations in PIK3CA[53, 54] [53, 54], and loss of chromosome 18q, contribute to further adenoma growth and progression [45]. SMAD2 and SMAD4 are probably the main tumour suppressor genes that are responsible for this loss, both having implications in the transforming growth factor- β (TGF- β) signalling pathway. Nevertheless, another gene could be targeted during this loss, the DCC (deleted in colorectal carcinomas) gene, this could be the reason why the loss of the 18q chromosome occurs more frequently than inactivating mutations in SMAD2 and SMAD4 [56].

DCC being a gene coding for a dependence receptor that has been shown to have several implications in tumorigenesis, especially in the context of colorectal cancer [55]. Dependence receptors are a class of cell surface receptors that play a dual role in cellular signalling, contributing to both cell survival and cell death based on the presence or absence of their respective ligands. Dependence receptors activate pro-survival signalling pathways when their specific ligands are present. However, in the absence of these ligands, they trigger a pro-apoptotic signalling cascade. This ensures that cells that do not receive the necessary extracellular signals to support their survival will undergo apoptosis, thereby preventing potential issues such as uncontrolled cell growth or the survival of cells in inappropriate environments [55]. The concept of dependence receptors is a key to apprehend this thesis project and is further developed in the review on page 48.

Finally, the loss of chromosome 17p, primarily attributed to TP53 mutations, marks the transition from late-stage adenoma to invasive carcinoma. Patients with familial adenomatous polyposis (FAP) tend to follow a similar pathway to CRC development.

The serrated neoplasia pathway represents the second most common genetic pathway for sporadic colorectal carcinomas, constituting 10 to 20% of cases. This pathway comprises two sub-pathways, sessile and traditional, each associated with different

precursor lesions: micro-vesicular hyperplastic polyp (MVHP), sessile serrated adenoma (SSA), sessile serrated adenoma with dysplasia (SSAD) for the sessile pathway, and traditional serrated adenoma (TSA) for the traditional pathway.

These precursor lesions exhibit epigenetic instability, particularly high rates of promoter methylation in cancer-related genes categorized as CpG island methylator phenotype (CIMP-H for high methylation or CIMP-L for low methylation). The serrated pathway is characterized by microsatellite-stable and -unstable cancers, with activating mutations in BRAF and KRAS genes. BRAF mutations, notably the V600E mutation, are prevalent in MVHP, SSA, and SSAD, considered early or initiating events in this pathway [57-59].

A subset of SSAs and SSADs display silencing of MHL1 by promoter methylation and are associated with Microsatellite Instability (MSI) [60]. Most serrated pathway cancers follow the sessile serrated pathway, progressing through a sequence of serrated lesions associated with BRAF mutation, CIMP-H, and MSI: MVHP, SSA, SSAD, and invasive carcinoma. TSAs are considered precursors of the traditional serrated pathway, characterized by KRAS mutation, methylation of the DNA repair enzyme O6-methylguanine-DNA methyltransferase (MGMT) promoter, and CIMP-L. The genetic characterization of TSAs is less comprehensive due to their rarity [53, 61].

The role of the Wnt signalling pathway in the serrated pathway remains unclear, with inconsistent studies on the significance of β -catenin in tumour progression. While APC mutations are rare in serrated pathway lesions, genetic and epigenetic alterations in other Wnt pathway-related genes, such as the SFRP gene family, could contribute to aberrant pathway activation [62-64].

Finally, Lynch syndrome arises from a germline mutation in mismatch repair genes, particularly MLH1, PMS2, MSH2, and MSH6, resulting in an elevated risk of CRC [65]. The syndrome is induced by MSI, triggered by the loss of the wild-type allele, leading to accelerated progression from conventional adenomas to invasive carcinoma [66]. While most microsatellites are in non-coding regions, some reside in the coding regions of cancer-related genes such as TGFBR2, IGF2R, BAX, or TCF4, contributing to tumours [67-

69]. At a genetic level, Lynch syndrome cancers are characterized by unmutated BRAF and the absence of CpG island methylator phenotype (CIMP).

Most Lynch syndrome cancers exhibit aberrant activation of the Wnt pathway. However, tumour profiling indicates that mutations in CTNNB1, the gene for β -catenin, are more common than APC mutations [70].

An additional classification into four molecular subtypes (consensus molecular subtypes [CMS] 1–4) was established in 2014 based on gene expression. Each CMS is defined by specific genes or pathways and has been characterized using various bioinformatic and multi-omic approaches to offer a comprehensive understanding of CRC subtypes. The MSI immune subtype (CMS1) is distinguished by hypermutated DNA, microsatellite instability, and robust immune activation. The canonical subtype (CMS2) is identified by an epithelial signature and activation of the WNT and MYC pathways. The metabolic subtype (CMS3) is characterized by metabolic dysregulations, while the mesenchymal subtype (CMS4) exhibits stromal invasion, angiogenesis, and transforming growth factor- β activation [71]. (A Schematic representation of 4 broad subtypes according to the morphology of precursor lesions and to their most commonly enriched genomic/methylation characteristics and transcriptomic features is available in the attached article: **“Notch receptor/ligand diversity: contribution to colorectal cancer stem cell heterogeneity”, page 3**)

3. Mechanisms of metastasis

All these pathways have different steps for the lesion to become malignant, but they all result in a similar phenomenon: Metastasis. Metastasis is crucial in the prognosis of CRC, indeed if CRC has mostly a good prognosis, approximately 20% of patients are diagnosed with stage IV or metastatic disease, with a five-year survival of barely 13% [72]. CRC mainly metastasizes to the liver and/or lungs, the liver being the predominant site of secondary tumour development, with 25 to 30% of patients diagnosed with CRC developing liver metastasis during the length of their disease[73]. There are two main reasons for the predominance of CRC metastasis in the liver: the first is that the liver is

the centre of filtration of blood coming from the whole abdominal tract, and the second is the presence of several types of cells that provide a rich niche for the development of metastatic cells [74]. Indeed, it is now widely accepted that for metastasis, cancer cells need to acquire specific characteristics needed for migration to distant sites, but these sites need to have a physiological predisposition for receiving these invasive cells. This is often compared to the analogy of the “seed and the soil”, the seed being the cancer cell having the phenotypic capabilities of metastasis and the soil being the distant tissue optimized for the survival and outgrowth of metastatic cells and tumours. Cancer metastasis is a complex process that requires various intra- and extracellular factors such as angiogenesis, the ability to invade distant tissue, the secretion of specific cytokines or the participation of non-cancerous cells in the tumoral microenvironment.

Angiogenesis, characterized by the formation and remodelling of new blood vessels, is, for instance, a crucial process implicated in tumour growth, cancer cell survival, and metastasis. It facilitates metastasis by increasing the number of tumour cells entering the bloodstream, providing nutrients for growth at metastatic sites, and limiting hypoxia-related injuries and cell death by increasing oxygen input [75]. This complex process involves various factors, including angiogenic factors, tumour-associated stromal cells, cytokines, growth factors, and extracellular matrix interactions.

For instance, the VEGF family, including VEGF-A and VEGF-C, plays a significant role in liver metastasis angiogenesis, enhancing endothelial cell proliferation and migration within primary tumours and metastases [76-79]. Additionally, there is crosstalk between VEGF signalling and other pro-angiogenic pathways, such as the angiopoietins or integrins, contributing to metastasis, angiogenesis, and lymphangiogenesis [80-83]. Consequently, targeted therapies like bevacizumab, a monoclonal antibody against the VEGF-A receptor, have been developed for mCRC treatment, trying to improve current treatment efficacy through inhibition of angiogenesis. These therapies are often used in combination with standard chemotherapy regimens [84].

The microenvironment plays a crucial role in regulating the development of CRC, impacting not only the initial stages of carcinogenesis but also the entire process of metastasis and the formation of secondary tumours. Various cell types within the

microenvironment contribute to these processes. Hepatic stellate cells, for instance, have been identified as having a substantial positive impact on liver pro-metastatic niches[85]. Fibroblasts, responsible for the synthesis of extracellular matrix (ECM) components, are associated with poor prognosis in CRC and mCRC [86]. Indeed, cancer-associated fibroblasts (CAFs) play a significant role in influencing colorectal carcinogenesis, enhancing invasiveness, and chemoresistance, and contributing to angiogenesis by secreting factors like VEGF-A and WNT2 [87-89].

The complex interactions between CAFs and tumour cells are implicated in the induction of several capital cellular processes such as epithelial-to-mesenchymal transition (EMT), activation of proliferative pathways, stemness-related signalling, and metabolic reprogramming, further emphasizing their impact on multiple stages of CRC progression and cancer-associated hallmarks [89]. Additionally, CAFs facilitate the migration of metastatic cancer cells by assisting in their adhesion during travel from primary tumour sites to the liver. Once at the metastatic site, CAFs modulate the metastatic niche by altering ECM composition and architecture through the secretion of proteins like Matrix Metalloproteinases (MMPs) and lysine oxidase, influencing tumour growth and metastasis [90]. The heterogeneity of CAFs in colorectal cancer poses a challenge for targeted therapy, as studies have identified distinct subtypes with different gene expressions and functions [91]. Dysregulation of ECM by CAFs plays a pivotal role in colorectal cancer progression, impacting tumour growth, metastasis, and response to chemotherapies [92-94]. Notable differences in ECM composition between primary tumours and liver metastasis are observable, highlighting the complexity and importance of ECM modulation [95]. These alterations are considered by scientists for the development of new effective therapeutic molecules in CRC and mCRC.

In recent years, the significance of the immune microenvironment in CRC has gained increasing recognition. The immune system, responsible for maintaining body integrity, plays a crucial role in controlling abnormal cell proliferation and homeostasis maintenance. As seen previously, chronic inflammatory conditions, like bowel inflammatory diseases, directly impact the risk of developing CRC. The late stages of tumorigenesis involve a diverse population of immune cells, including innate immune cells (granulocytes, mast cells, and various macrophages) and adaptive immune cells (T

and B lymphocytes). Studies on mCRC have revealed that, similarly to ECM composition, distinct immune landscapes were found within CRC metastases, with distinct subsets of immune cells. The infiltration and activation levels of these immune cells directly influence tumorigenesis and resistance to chemotherapies [96].

An illustration of the impact of this subset on clinical features could be the enhanced activation of cytotoxic, helper, and memory T-cells in tumour tissue, which is known to be associated with improved prognosis [97, 98], whereas the presence of B cells and innate immune cells like neutrophils, mast cells, and immature dendritic cells is linked to poor prognosis [99]. Certain immune subtypes, such as mast cells, contribute to not only immunosuppression but angiogenesis, tumour invasion as well [100]. This immunosuppressive activity is mediated by the secretion of cytokines, including IL-10 and IL-2 interleukin by regulatory T cells, as well as the expression of immune checkpoint inhibitor proteins like PD-L1, PD-L2, and CTLA-4, inhibiting the suppressive effects of effector T-cells [101].

These findings have led to the development of small-molecule inhibitors or monoclonal antibodies targeting immune cells and checkpoints as adjuvants for mCRC chemotherapies. These therapies aim to enhance the anti-tumoral activities of T-cells and inhibit checkpoint inhibitor functions. Despite the success of these innovative therapies in various cancer types, their efficacy in mCRC has been limited, demonstrating the challenges in translating these advancements to specific cancer contexts [102-104].

Finally, the phenotypical state of the cells greatly influences the metastatic process. Indeed, the progression from the primary site to the formation of metastasis is marked by notable phenotypic plasticity that occurs within the tumour. This refers to the ability of disseminated tumour cells to undergo temporary and reversible transformations, allowing them to adapt to constantly changing environmental conditions [105]. One of the most common plasticity-related phenomena in colorectal cancer cells is EMT, which is a crucial process in carcinogenesis, where cancer cells shift from an epithelial to a more invasive and migratory mesenchymal phenotype. This complex phenomenon involves changes in cellular morphology and alterations in cell-to-cell or cell-to-matrix

adhesions [106]. Some of the key transcription factors driving EMT include the ZEB, Snail, and Twist families, their expression resulting in decreased E-cadherin and increased vimentin expression [107]. Recent findings identify SOX2, OCT4, and Nanog, primarily known for their roles in stem cells, as regulators of EMT, highlighting the insidious link between these two phenotypes [108-110].

EMT regulation in CRC cells relies heavily on intracellular pathways like TGF- β , Wnt, AKT, and MEK as well [111-114]. Moreover, as discussed previously, EMT is regulated by the tumour microenvironment. Elements such as CAFs and ECM greatly influence the phenotypic switch of cancer cells towards a mesenchymal phenotype through the production of MMPs, MMP inhibitors, ECM components, growth factors and cytokines [115].

EMT not only enhances cancer cell invasiveness but also contributes to resistance to treatments, particularly to certain chemotherapeutical molecules. For instance, studies reveal correlations between EMT-related features such as invasiveness, and resistance to drugs like oxaliplatin and 5-fluorouracil [116]. Indeed, EMT-related proteins, such as Snail and TGF- β , have been implicated in promoting chemoresistance [117, 118]. Conversely, negative regulators of EMT, such as particular miRNAs for instance, restore sensitivity to chemotherapy [119]. In addition to its effect on molecules targeting DNA, EMT impact extends to immune-based and targeted therapies, which are less efficient on mesenchymal-transitioned cancer cells [120, 121]. While the exact mechanisms linking EMT and chemoresistance remain unclear, it is recognized that the association between EMT and the stem cell phenotype plays a significant role in this phenomenon.

4. The biology and heterogeneity of colorectal cancer stem cells

(It is to be noted that this section is based on the publication: Brisset M, Mehlen P, Meurette O, Hollande F. Notch receptor/ligand diversity: contribution to colorectal cancer stem cell heterogeneity. *Front Cell Dev Biol.* 2023 Oct 4. doi:10.3389/fcell.2023.1231416)

a. CSCs: the seeds of metastasis

Cancer stem cells (CSCs) are a key aspect of cancer cell hallmark, a cell being recognised as cancerous once it acquires the ability to replicate indefinitely, a well-known characteristic of CSCs. These cells exhibit some of the phenotypic characteristics of non-pathological stem cells and can initiate and maintain tumour growth. This subset of tumoral cells exhibits particular properties such as higher self-renewal, replicative immortality due to telomerase activity, differentiation potential and tumour induction capabilities [122]. They are also characterized by the expression of some specific markers like CD44, CD133, CD24, SMOC2, EpCAM, LGR5 and the ALDH detoxifying enzyme, and by the expression of stemness-related transcription factors such as NANOG, SOX2 or KLF4. These cells can also be recognised and characterized by their phenotypical characteristics such as their sphere- and organoid-forming ability that relates to their tumour-inducing abilities [123]. CSCs may also exhibit an aberrant activation of pathways such as NOTCH, Hedgehog and Wnt, increasing their proliferative activities and capabilities [124].

CSCs are believed to be responsible for CRC neoplasm initiation, originating from a normal stem cell and transforming into a tumorigenic cell through the accumulation of mutations resulting in survival and proliferative advantages. Indeed, colorectal CSCs are for most derived from differentiated intestinal cells or intestinal stem cells (ISCs) that accumulated enough DNA modifications to become malignant[125].

CSCs are ideal “seeds” for the metastasis of CRC cells to distant organs like the liver or lungs. Their mesenchymal and undifferentiated cell features allow them to adapt to new microenvironments physiologically different from the site of the primary tumour. In addition, the heterogeneity born from the asymmetric division of colorectal CSCs further enhances the ability of metastases to develop despite the new microenvironmental selection.

The CSCs' heterogeneity is illustrated by the variability in the traits they're displaying. Indeed, features related to CSC seem uncoupled events enhancing sphere-forming abilities may, for instance, have a negative impact on the expression of markers normally attributed to stemness, on or resistance to chemotherapies[126]. An illustration of the

independence of these CSC-related characteristics would be that the knockdown of expression of genes such as AHNK2 influences sphere-forming abilities, though this invalidation does not enhance the expression of stemness markers such as CD133 [127]. Whereas knockdown of genes like HLA-B, CCDC92 and PLIN4 enhance CD133 expression without impacting cells' self-renewal abilities [127].

Another point of discord upon recognition of CSC-like cells is the expression of the EpCAM (epithelial cellular adhesion molecule) marker. Indeed, in several studies, a direct link between EpCAM expression and stemness-like features such as colony formation, self-renewal, tumour-inducing abilities, metastatic capability, or resistance to chemotherapeutical molecules, is observed [128-130]. Nevertheless, other studies are inclined toward the opposite conclusion, suggesting that CRC cells that express low levels of EpCAM are more motile, invasive, resistant to chemotherapies, and highly tumourigenic [131].

These differences among the observations of colorectal CSCs are mainly due to their heterogeneity. An instance of this heterogeneity is seen in the fact that a majority of circulating CRC cells are not expressing Lgr5, though these CSCs form distant metastases in which Lgr5-expressing stem cells arise. CSC plasticity seems to occur independently of microenvironmental factors and is necessary for metastasis outgrowth. Furthermore, these results demonstrate the major role of CSC plasticity in metastatic formation [132].

There is, in colorectal neoplasms, the existence of a CSC phenotypic heterogeneity that results in a different subset of co-existing CSCs. In primary CRC tumours, for instance, two different CSC populations form an equilibrium: the LGR5+ crypt-base columnar stem cells (CBCs) and the LGR5 negative regenerative stem cells (RSCs). CSCs exhibiting either CBC, RSC, or both cell markers display self-renewal capabilities whereas cells without expression of any CSC markers have limited tumourigenic capacity. Nevertheless, despite comparable self-renewal abilities, CBC stem cell phenotype appears to rely heavily on ligand-independent Wnt signalling activation (such as APC), whereas RSC exhibits increased activity of KRAS, YAP, TGF β , and inflammatory pathways (such as IFN- γ). This heterogeneous and dynamic CSC population is believed to be vital to allow an adaptive response to selective pressures, promote tumour outgrowth and resist chemotherapy.

b. CSCs and chemoresistance

CSCs represent one of the main factors fuelling tumour resistance and recurrence, representing a challenge in CRC treatment strategies [133]. Conventional therapies such as radiotherapies or chemotherapies, demonstrate their efficacy against differentiated and proliferative CRC cells but show limited efficacy against stem cells [133, 134]. The resistance of CSCs to chemotherapeutic molecules is due to several of their features, involving mechanisms such as high DNA-repair capabilities upon damages induced by chemotherapies [135]. Consequently, drugs targeting DNA replication and DNA-related processes, such as cisplatin, oxaliplatin, methotrexate, doxorubicin, and daunorubicin, display poor efficacy against CSCs [135]. The modulation of cell cycle checkpoints, the elevated expression of DNA damage repair proteins, the adapted metabolism, and the efficient scavenging of reactive oxygen species (ROS) originating from treatments collectively contribute to CSCs resistance to molecules targeting DNA [136-141].

In addition, the quiescence of CSCs appears to be another factor explaining their chemoresistance capabilities, as some cytotoxic drugs target highly proliferating cells [142-144]. Post-treatment, quiescent CSCs resume their cell cycle activity, contributing to tumour regrowth through the activation of proliferative signalling pathways [145]. While the mechanisms linked to CSC quiescence in CRC are not fully understood, the expression of transcription factors such as c-MYC, ZEB-2, and HMGA-1 has been determined as critical factors for CSC entering a quiescent G0 cell-cycle phase in a reversible way [145-147].

CSCs display an adapted and flexible metabolism, driven by epigenetic and genetic changes linked to tumour development, tumoral heterogeneity, and tumoral recurrence [148]. Their metabolic adaptability allows them to switch between glycolysis and oxidative metabolism (OXPHOS) to adapt to microenvironmental stress nutrient deprivation and [149]. CSCs rely less on ATP and glucose-dependent lipid synthesis, impacting directly their quiescence and resistance to cytotoxic agents [150]. For instance, 5-FU, which inhibits thymidine synthesis, demonstrates limited efficacy against CSCs due to their ability to switch to high levels of oxidative phosphorylation [151, 152].

Proteins such as aldehyde dehydrogenases (ALDH) and ATP Binding Cassette (ABC) transporters, respectively belonging to the ALDH1 family and the ABC transporter family, contribute significantly to CSCs resistance to chemotherapeutical molecules. The members of the ALDH1 family, notably ALDHA1, ALDHA3 or ALDHB1, are highly expressed in normal stem cells and are considered as a CSC marker in CRC [153, 154]. The ALDH enzymes can detoxify aldehydes formed by ROS and protect cells from molecules increasing ROS levels [155]. ABC transporters are a family of large transmembrane proteins that rely on ATP hydrolysis to excrete metabolites, foreign bodies, and toxic substances from cells [156]. In colorectal CSCs the ABCB1, ABCC1, and ABCG2 ABC-transporters are highly expressed and actively reject antitumoral drugs outside of the cell, decreasing the intracellular concentration of harmful molecules, resulting in multi-drug resistance [157, 158].

The complex regulatory signals and factors within the microenvironment significantly contribute to shaping the stem cell phenotype, influencing their behaviour and responses to therapeutic interventions. Understanding this bidirectional relationship between CSCs and their surrounding environment is crucial, as it could influence potential therapeutical strategies aimed at modulating CSC behaviour and sensitizing them to chemotherapy treatment (A schematic representation of treatment response and recurrence potential by different CRC cells depending on their self-renewal potential is available in the attached article: **“Notch receptor/ligand diversity: contribution to colorectal cancer stem cell heterogeneity”**, page 5).

C. CSCs modulation by tumoral microenvironment

Scientists studying stemness have long focused on cellular intrinsic characteristics of the cell. Nevertheless, several studies are now integrating the intrinsic features of CSCs with other sources of phenotypic plasticity and variability like the tumoral microenvironment [160-162].

The microenvironment is indeed a key regulator of CSCs-induction and maintenance in CRC tumours. The specific localization of cells within the tumour is, for instance, a determinant parameter in the pluripotency potential of CRC cells, clones responsible for

tumour outgrowth in CRC predominantly originating from locations in the outer tumour sections [163]. Cells from outer tumour regions display higher clonogenic capabilities. The enhanced contacts of CRC cells with microenvironment components such as CAFs in these outer regions would influence favourably clonogenic abilities. This is partly due to the release in the ECM of differentiation-inhibiting proteins such as osteopontin [163] or Netrin-1 [164, 165]. ECM influence stemness through biochemical and biophysical aspects as well such as the stiffness of the cell's extracellular environment[166].

These studies demonstrate that CSCs' functional characteristics such as their pluripotency are not fully linked to intrinsic factors such as the expression of stemness-related genes. It also underlines the importance of the microenvironment as much as cell-autonomous features, in determining CSC functionality.

CSC modulation by the microenvironment involves various signals and factors that regulate the equilibrium between stemness and differentiation. Among these factors, the hepatocyte growth factor (HGF), and bone morphogenetic protein (BMP) play key roles in the modulation of CSC fate [167]. These factors act as positive regulators, allowing the maintenance of CSCs as well as their expansion. HGF is well known for its mitogenic properties, contributing to the self-renewal capability of CSCs. Similarly, BMP, a member of the transforming growth factor-beta superfamily, exhibits a similar ability to positively regulate the CSC pool.

Conversely, the CSC microenvironment also includes differentiation-inducing factors, driving them away from their pluripotent state. This delicate equilibrium between positive and negative modulators of the CSC microenvironment reflects the dynamic nature of CSC behaviour, ultimately determining whether stem cells remain pluripotent or undergo differentiation. The molecular modulation of these signalling pathways is one of the keys needed to understand CSC biology complexity and unlock the therapeutical potential of strategies targeting the CSC subpopulation.

While understanding the interplay between CSCs and their surroundings provides valuable insights, targeting the vulnerabilities of these cells becomes a crucial aspect of therapeutic strategies [165]. The dependence receptor concept offers a possibility to target this cell subset, these receptors being characterized by the active triggering of

apoptosis in the absence of their ligands. In mCRC, exploring how dependence receptors influence the behaviour of CSCs may pave the way for targeted treatments that disrupt the dependence of CSCs on specific signalling pathways, potentially sensitizing them to therapeutic interventions, offering new perspectives for precision medicine approaches in the challenging landscape of mCRC.

II. Dependence Receptors: new targets for cancer therapy

Morgan Brisset, Melodie Grandin, Patrick Mehlen, Agnes Bernet, Frédéric Hollande

This Chapter includes the manuscript entitled “*Dependence Receptors: new targets for cancer therapy*”. Brisset M., et al EMBO Mol Med. 2021 Nov 8;13(11):e14495. doi: 10.15252/emmm.202114495. Epub 2021 Sep 20.” The candidate contributed intellectually to the development of this review manuscript, including drafting and revising the manuscript, together with the assistance of the other authors Melodie Grandin and Frederic Hollande. Agnes Bernet and Patrick Mehlen advised on the scientific aspects of the review. Overall, the candidate contributed approximately 50% to the preparation of the work for this publication.



Dependence receptors: new targets for cancer therapy

Morgan Brisset^{1,2}, Mélodie Grandin^{1,2} , Agnès Bernet³, Patrick Mehlen³ & Frédéric Hollande^{1,2,*}

Abstract

Dependence receptors are known to promote survival and positive signaling such as proliferation, migration, and differentiation when activated, but to actively trigger apoptosis when unbound to their ligand. Their abnormal regulation was shown to be an important feature of tumorigenesis, allowing cancer cells to escape apoptosis triggered by these receptors while promoting in parallel major aspects of tumorigenesis such as proliferation, angiogenesis, invasiveness, and chemoresistance. This involvement in multiple cancer hallmarks has raised interest in dependence receptors as targets for cancer therapy. Although additional studies remain necessary to fully understand the complexity of signaling pathways activated by these receptors and to target them efficiently, it is now clear that dependence receptors represent very exciting targets for future cancer treatment. This manuscript reviews current knowledge on the contribution of dependence receptors to cancer and highlights the potential for therapies that activate pro-apoptotic functions of these proteins.

Keywords apoptosis; cancer hallmarks; treatment resistance; tumor progression

Subject Categories Autophagy & Cell Death; Cancer; Signal Transduction

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The dependence receptor paradigm

For a long time, the accepted paradigm regarding transmembrane receptors was solely that their activation by specific ligands could lead to the initiation of signaling cascade transduction, resulting in various outcomes such as cell proliferation, motility, or cell survival. According to this model, the absence of ligand-receptor binding was simply thought to result in the absence of intracellular signal transduction. However, the dependence receptor paradigm, first reported by Rabizadeh *et al* (1993) with a study of the p75NTR receptor (Rabizadeh *et al*, 1993), has changed our way of understanding molecular signaling downstream from transmembrane

receptors. Indeed, dependence receptors (DRs) share a common feature: They actively trigger opposite outcomes depending on the availability of their ligand. These receptors, such as classical receptors, activate positive signaling in the presence of their ligands, but they also actively trigger apoptosis in their absence (Fig 1).

To date, more than twenty DRs have been identified within structurally divergent receptor families. Simply put, their shared property is not structural but a paradoxical pro-survival/pro-cell death function. Several DRs belong to the class of tyrosine kinase receptors. Indeed, receptors such as c-MET (Tulasne *et al*, 2004), ALK (Mourali *et al*, 2006), TrkA and TrkC (Nikolopoulos *et al*, 2010), RET (Bordeaux *et al*, 2000), EphA4 (Furne *et al*, 2009), EphB3 (Tsenkina *et al*, 2015), or even insulin receptor and insulin-like growth factor 1 receptor (Boucher *et al*, 2010) and more recently c-KIT (Wang *et al*, 2018) have been characterized as DRs. Receptors involved in major developmental signaling pathways such as hedgehog (SHH receptors patched-1) (Thibert *et al*, 2003) and CDON (cell adhesion associated, oncogene regulated) (Delloye-Bourgeois *et al*, 2013) or NOTCH (NOTCH3 receptor) are also part of this class of receptors. The p75NTR neurotrophin receptor (Rabizadeh *et al*, 1993), the Dickkopf-related protein 1 (DKK1) receptor Kremen1 (Causeret *et al*, 2016), and the GPCR receptor latrophilin (Jackson *et al*, 2016) also exhibit this feature. DRs also include transmembrane receptors binding netrin-1 (a laminin related protein involved in axon guidance), such as deleted in colorectal carcinoma (DCC), uncoordinated 5 homologs (UNC5H (A-D)) and neogenin (Llambi *et al*, 2001; Arakawa, 2004; Matsunaga *et al*, 2004; Mille *et al*, 2009), although the latter may not display any real dependence toward Netrin-1 but rather toward the repulsive guidance molecule (RGM). Finally, even some integrin subtypes such as $\alpha 5\beta 3$ and $\alpha 5\beta 1$ (Stupack *et al*, 2001; Stupack, 2005) have been shown to actively trigger apoptosis when they are unbound.

Despite their divergent structure, most DRs regulate a small number of related biological processes: neuronal development and embryonic morphogenesis. Moreover, in many cases, their ligand-dependent activation results in the stimulation of common signaling pathways, including PI3K/AKT and MAPK/ERK. These similarities may suggest that DRs act in a coordinated manner to modulate physiological functions such as neuronal development or embryonic vasculogenesis.

1 Department of Clinical Pathology, Victorian Comprehensive Cancer Centre, The University of Melbourne, Melbourne, Vic., Australia

2 University of Melbourne Centre for Cancer Research, Victorian Comprehensive Cancer Centre, Melbourne, Vic., Australia

3 Apoptosis, Cancer and Development Laboratory, Centre de Recherche en Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Centre Léon Bérard, Université de Lyon, Lyon, France

*Corresponding author. Tel: +613 8559 7023; E-mail: frederic.hollande@unimelb.edu.au

Glossary

Angiogenesis

Formation of new blood vessels.

Apoptosis

Programed cell death.

Autocrine/paracrine ligand

Molecule secreted by a given cell and acting on the cell itself (autocrine) or on neighboring cells (paracrine).

Chemoattractant

Substance capable of attracting motile cells that respond to it.

Colorectal carcinoma

Bowel cancer.

Dimerization, oligomerization

Process of joining two (di-) or more (oligo-) individual subunits of a protein, individually named monomers. Joined subunits can be the same (homodimerization) or different (heterodimerization).

Extracellular matrix

Network of proteins and other molecules that surround and support cells and tissues in the body.

Feedback loop

Control mechanism whereby an agent (e.g., protein) acts to regulate the process that controls its own production or activity.

Immune checkpoint inhibitors

Molecules used to block a type of inhibitory interaction between immune cells and their target, often used to restore adequate immune response against tumor cells.

Ligand pleiotropy

Ability of secreted molecules to modulate several, sometimes unrelated effects, e.g., by binding to different receptor types.

Metastatic spread

Dissemination of cancer cells from their organ of origin to distant organs throughout the body.

Mitochondria

Specific structure (organelle) found within most cells and where biochemical processes that enable cell respiration and energy production occur.

Morphogenesis

In the context of an animal or human, generation of tissue organization and shape.

Neutralizing antibody

Antibody molecule with the ability to block the function of its target protein.

Phenotypic switch

Rapid change in cell behavior/characteristics and/or structure.

Pluripotency markers

Genes and/or proteins that demonstrate characteristically high expression in stem cells that have the potential to give rise to all cells of the adult body.

Tyrosine kinase

Protein with the ability to drive the transfer of phosphate molecules onto tyrosine amino acids located within itself and/or other target proteins, leading to modifications in their structure/location/behavior.

The dependence function of DRs: ligand-independent activation of apoptotic signals

Conversely, multiple DRs also show similarities in the way they trigger apoptosis. For example, in most cases, DR monomerization in the absence of ligand appears to initiate the apoptotic cascade through activation of various pro-apoptotic protein partners (Negulescu & Mehlen, 2018). However, there are some exceptions to this paradigm. For instance, it was recently reported that withdrawal of DKK1 triggers the oligomerization of Kremen1, rather than its monomerization (Sumia *et al*, 2019).

The next step of ligand-independent DR activation of apoptotic pathways frequently involves cleavage of an intracellular receptor moiety, a process that remains only partially understood. This initial step of DR activation appears heavily dependent on pro-apoptotic proteases, including but not restricted to caspases. In some cases, “non-apoptotic” proteases such as calpains, serine proteases, or lysosomal proteases have been shown to induce DR cleavage, with cleavage products sometimes initiating caspase-dependent downstream signaling (Huai *et al*, 2010). Another mechanism appears to rely on the previously reported finding that non-apoptotic cells have a baseline level of caspase activation, which plays a role in regulating differentiation during tissue development (Svandova *et al*, 2018). Such level of caspase activity is insufficient to trigger apoptosis but may be sufficient to initiate DR cleavage upon ligand withdrawal (Negulescu & Mehlen, 2018), suggesting that changes in DR oligomerization and/or conformation may be sufficient for them to reach a caspase-sensitivity threshold. Accordingly, DR amino acid sequences often contain caspase-like cleavage sites juxtaposed to an aspartate (Bredesen *et al*, 2005), a well-known feature of caspase cleavage sites (Bratton & Cohen, 2001). Intracellular region within DR frequently exhibits unique a caspase-like cleavage site, as seen in NOTCH3, DCC, neogenin, PTCH-1, ALK, EphA4, EphB3, c-KIT, CDON, as well

as in the UNC5H receptor family, while multiple cleavage sites have been reported for other DRs such as RET, MET, and TrkC (Negulescu & Mehlen, 2018; Wang *et al*, 2018). In contrast, integrin isoforms previously identified as DRs do not exhibit any recognized cleavage site, even though β -integrins induce apoptosis through recruitment and downstream activation of caspase-8 (Stupack *et al*, 2001). Similarly, no cleavage sites were identified for p75NTR, latrophilin, IR, IGF-1R, suggesting that early ligand-independent signaling downstream from these receptors may not involve their cleavage.

Interestingly, caspases responsible for DR cleavages involve both “initiator” (caspases 2, 8, 9, 10, and 12) and “effector” caspases (caspases 3, 6, and 7). For instance, upon withdrawal of their respective ligand, the intracellular regions of UNC5B or c-KIT are cleaved by caspase-3, UNC5D can be cleaved by caspase-2 or 3, while EphB3 can be cleaved by caspase-8 or 9 (Abbaspour Babaei *et al*, 2016; Tsenkina *et al*, 2020; Zhu *et al*, 2021). Moreover, studies reveal that DCC can interact with caspase-3 or caspase-9 depending on its conformation, suggesting implication of one of these caspases in DCC cleavage (Forcet *et al*, 2001).

DR cleavage by caspases leads to their conformational modification, most frequently resulting in the exposure of a dependence domain of these receptors that remains anchored to the extracellular membrane. In contrast, the dependence domain of a few DRs (c-MET, RET, TrkC, c-Kit, and UNC5H receptors) is released in the cytoplasm (Bordeaux *et al*, 2000; Tulasne *et al*, 2004; Wang *et al*, 2018; Zhu *et al*, 2021). Exposure or release of dependence domains leads to the recruitment and activation of apoptotic partners, most frequently caspases. DRs such as c-KIT, DCC, RET, TrkC, CDON, Notch3, PlexinD1, or PTCH-1 trigger apoptosis through activation of caspases 3 and 9, whereas p75 NTR acts through caspase 2 activation.

Thus, DRs appear to play a major role in the amplification of caspase signaling. Indeed, on the one hand some level of

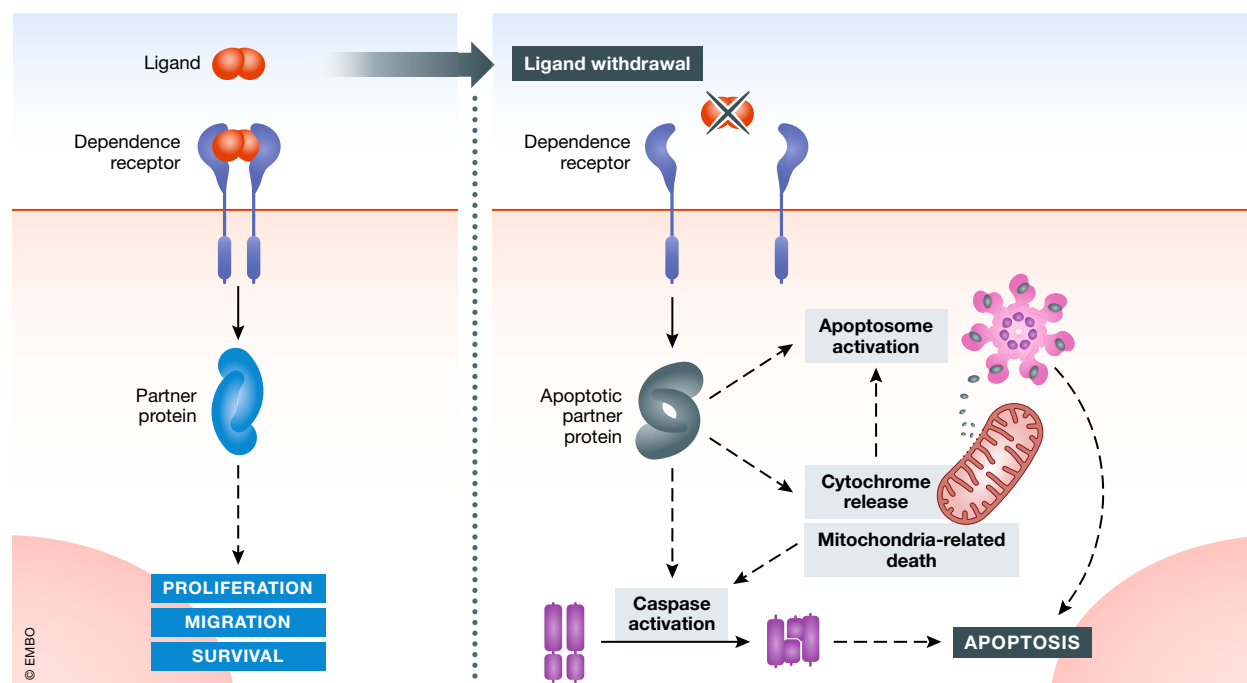


Figure 1. Dependence receptor paradigm.

Schematic representation of DR triggering: positive signaling in the presence (Top) and negative signaling in the absence of ligand (Bottom) (Created with BioRender.com).

pre-existing caspase activation appears required to initiate pro-apoptotic signaling from several DRs following withdrawal of their ligand, through cleavage of these receptors. In turn, most of these receptors also activate caspases and thereby contribute to the emergence of pro-apoptotic signaling “waves”. DRs act as an intermediate between “initiator” and “effector” caspases, and their ligand-independent function could also synergize with other effectors to amplify pro-apoptotic signals. An example of such positive feedback loop involves p53 which, beyond its direct role in promoting apoptosis, also mediates upregulation of UNC5B and thereby leads to enhanced sensitivity to Netrin-1 deprivation [34].

It is also noteworthy that apoptotic cascades triggered by some DRs do not always involve downstream recruitment of caspases but rather involve formation of pro-apoptotic complexes with various other protein partners. The nature of recruited partners may not correlate with receptor structure, as exemplified by the UNC5H receptor family. Indeed, contrary to what structural homology among members of this family may suggest, these receptors trigger different apoptotic complexes involving distinct apoptotic partners. For example, upon Netrin-1 withdrawal, monomerization of UNC5A activates the c-Jun apoptotic pathway through its interaction with the neurotrophin receptor-interacting MAGE homolog NRAGE (Williams *et al*, 2003). In contrast, UNC5B monomerization leads to PP2A (protein phosphatase 2A) recruitment and to the subsequent dephosphorylation and activation of DAPK1 (death-associated protein kinase 1) (Llambi *et al*, 2005), a mechanism inhibited by CIP2A (cellular inhibitor of PP2A) in the presence of Netrin-1. UNC5D is cleaved by caspases 2 or 3, and the resulting intracellular

fragment interacts with E2F1 (E2F transcription factor 1) into the nucleus to activate transcription of pro-apoptotic genes (Zhu *et al*, 2013). Finally, although pro-apoptotic mechanisms downstream from UNC5C remain less characterized, UNC5C intracellular cleavage by γ -secretase upon Netrin-1 deficiency has been observed in Alzheimer disease and may also occur in other models (Chen *et al*, 2021).

In addition, pro-apoptotic cascades triggered by several other DRs involve direct modulation of mitochondrial function. For example, NT-3 withdrawal induces an internal cleavage of TrkC, followed by translocation of the cleaved intracellular portion into mitochondrial membrane via the protein Cobra 1 (cofactor of BRAC1). In turn, this leads to permeabilization of the mitochondria, release of cytochrome C and eventually to cell death via apoptosome activation (Ichim *et al*, 2013). The mechanism for c-MET-induced cell death also involves mitochondrial membrane permeabilization. Upon HGF withdrawal, MET is cleaved to yield a 40 kD intracellular fragment called p40MET (Foveau *et al*, 2007). This fragment localizes to mitochondria-associated membrane (MAM) regions of the endoplasmic reticulum and triggers a calcium transfer from the endoplasmic reticulum toward the mitochondria. Massive Ca^{2+} entry into the mitochondria leads to mPTP (mitochondrial permeability transition pore) opening and to the release of mitochondrial components that trigger cell apoptosis (Duplaquet *et al*, 2020). PlexinD1 interacts with the nuclear receptor NR4A1 (nuclear receptor subfamily 4 group A member 1) to induce cell death through cytochrome-c release and caspase-9 activation (Luchino *et al*, 2013). Finally, ALK cleavage by caspase-3 or another caspase facilitates apoptosis,

possibly by regulating the activity of BCL-2 family members (Mourali *et al*, 2006).

Recent studies into mechanisms that underpin c-MET function in the absence of ligand have unraveled unexpected complexities in its regulation of cell death. First, multiple cytoplasmic cleavage products from c-MET have been described (Fernandes *et al*, 2019) and a 10 amino acid small c-terminal cleavage product was found to inhibit apoptosis by blocking active site of caspase-3 in hepatocytes (Ma *et al*, 2014). Second, the implication of c-MET in necrosis, a caspase-independent cellular death, has also been reported, with calcium stress leading to cleavage of c-MET by both ADAM-family membrane metalloproteases and calpains to generate a fragment similar to p40MET but unable to trigger apoptosis (Fernandes *et al*, 2019). Whether similar alternative pathways exist for other DRs to control cell death remains unanswered at this point.

Linked to their role in the regulation of apoptosis, a function for DRs in tissue homeostasis has been identified, with DR ligands being produced by cells contributing to the stem cell niche. In this context, DR signaling contributes to promoting stem cell maintenance and tissue growth when their ligand is present, but negatively regulates cell numbers through apoptotic signaling when ligand expression drops. This process can control tissue/organ size. For instance, c-MET regulates neural stem cell compartment expansion and self-renewal (Nicoleau *et al*, 2009), whereas Notch3 and its ligand Jagged-1 control differentiation of progenitors in the upper airways (Mori *et al*, 2015).

This ability of DRs to switch from positive to negative signaling is highly relevant for their function in morphogenesis, as the ability

to induce cell death in the absence of ligand could be a way to foster normal organ development. However, this property of DRs can also have major pathological implications, especially in cancer. Indeed, the continuing proliferation of cancer cells may be limited by DRs, as an increasing number of cells in a growing tumor will compete for available ligand.

Collectively, these observations indicate that anomalies in the apoptotic pathways linked to DRs may confer a survival advantage to tumor cells. Accordingly, it is therefore not surprising that multiple DR pathway alterations have been reported in cancer (Fig 2), and alterations of DR signaling have been found to contribute to multiple steps in the tumorigenic process, as detailed below.

Dependence receptors and tumorigenesis

The negative (or apoptotic) pathway downstream from various DRs is frequently altered in cancer, thus preventing apoptosis and favoring tumor growth. For example, genes that encode DRs such as DCC, neogenin, UNC5B, UNC5C, or TrkC are repressed in multiple cancer types (Bernet *et al*, 2007; Luo *et al*, 2013; Forrest *et al*, 2016; Kong *et al*, 2016; Xing *et al*, 2021), while cancer cells can also escape DR-mediated apoptosis through autocrine overexpression of ligands including DKK1 (Huang *et al*, 2014), Netrin-1 (Kefeli *et al*, 2017), NT-3 (Louie *et al*, 2013), Sema3E (Yong *et al*, 2016), or HGF (Baykal *et al*, 2003). Alternatively, cancer cells can also stimulate the secretion of dependence ligands by cancer-associated fibroblasts

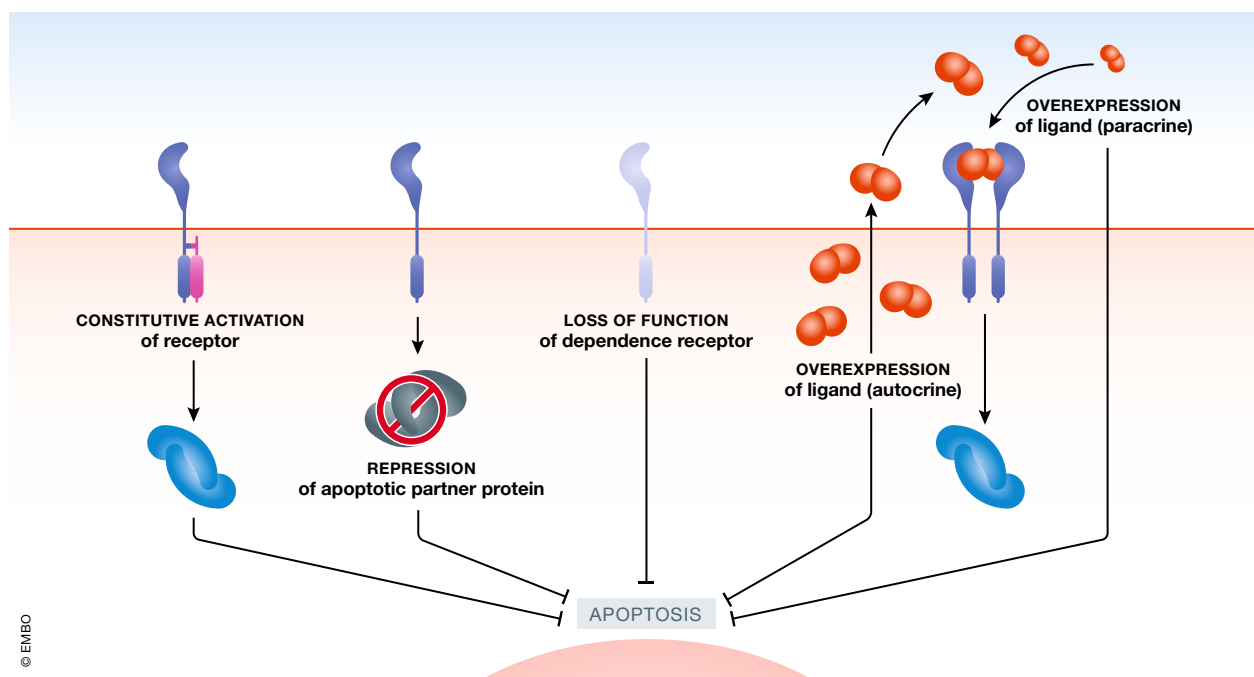


Figure 2. Mechanisms of dependence receptor-triggered apoptosis escape in cancer.

Schematic representation of ways to escape DR-triggered apoptosis: Constitutive activation of the receptor; repression of the DR apoptotic protein partner; DR loss of function; and autocrine or paracrine ligand overexpression (Created with BioRender.com).

(CAFs), ensuring that pro-apoptotic signaling by corresponding DRs is restrained (Sung *et al.*, 2019).

In addition, overexpression of DRs such as PlexinD1 (Blanc *et al*, 2011), NOTCH3 (Park *et al*, 2006), c-KIT (Jones *et al*, 2007), c-MET (Baykal *et al*, 2003; Zhang *et al*, 2017), PTCH-1 (Kang *et al*, 2013), or TrkC (Jin *et al*, 2007) has been reported in several cancer types, in contexts where their ligands are also expressed. Indeed, joint overexpression of DRs and their ligand often promotes aggressive tumor progression, as was, for example, shown for HGF and c-MET in NSCLC (Navab *et al*, 2009) or gastric cancer (Toiyama *et al*, 2012), suggestive of a positive selection for ligand-induced rather than ligand-independent signaling. Altogether, these observations suggest that silencing of the ligand-independent pro-apoptotic DR signaling combines with “positive” ligand-driven DR signaling to confer a selective advantage to cancer cells, resulting in the promotion of multiple stages of tumorigenesis (see Fig 3 and Table 1).

Negative selection pressure on pro-apoptotic DR signaling pathways in cancer

In view of the pro-apoptotic activity initiated by DRs in the absence of ligand, it is somewhat unsurprising that this function, and by

extension the occurrence of high receptor-to-ligand ratios that would foster the activation of ligand-independent signaling, can be negatively selected in human cancer. Indeed, prior studies have identified multiple mechanisms of DR gene genetic invalidation (as in the case of DCC in colorectal cancer) (Fearon & Vogelstein, 1990), epigenetic downregulation (e.g., for UNC5H family receptors) (Thiébaud *et al*, 2003), or sporadic mutations leading to selective loss-of-pro-apoptotic function (e.g., for TrkC in colorectal cancer) (Ichim *et al*, 2013). Other mechanisms of DR pro-apoptotic pathway abrogation include the constitutive activation of positive signaling by the ALK receptor through gene fusion with EML4 (Wei *et al*, 2018) or the epigenetic alteration of pro-apoptotic signaling cascade proteins such as DAPK1 (apoptotic partner of UNC5B) (Grandin *et al*, 2016).

A causal relationship between DR inactivation and tumor progression was, for example, demonstrated in the case of UNC5C, which is downregulated via LOH and epigenetic silencing in colorectal cancer, whereby *in vivo* UNC5C inactivation increased tumor progression and decreased apoptosis in an APC1638N spontaneous intestinal tumors mouse model. Negative pressure on pro-apoptotic function can also result from constitutive ligand overexpression in

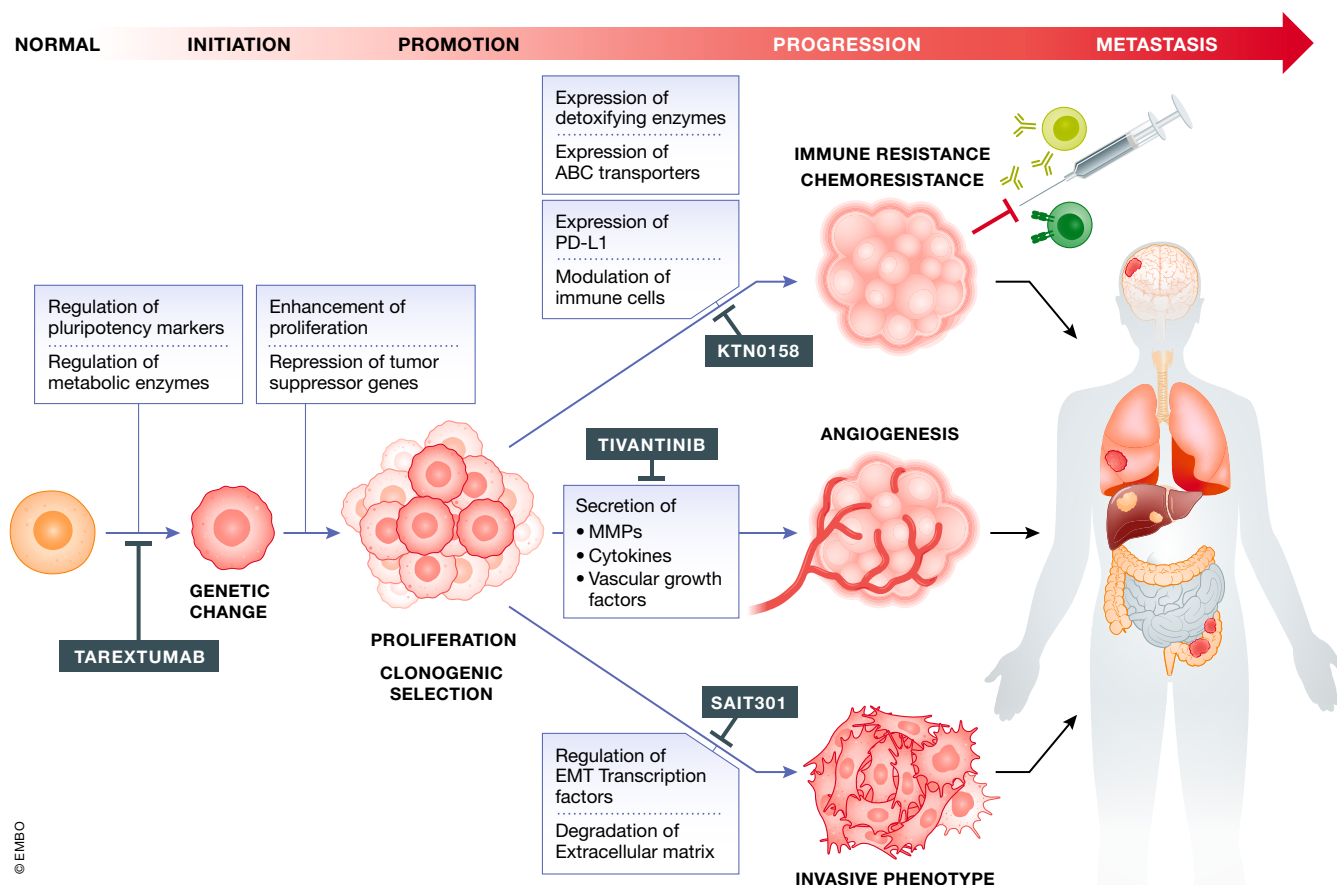


Figure 3. Involvement of dependence receptors in tumorigenesis and examples of drugs targeting them.

Impact of Dependence Receptors on different stages of tumorigenesis. Blue boxes highlight DR effects, while red boxes highlight examples of inhibitors that target them (Created with BioRender.com).

Table 1. Examples reflecting the implications of DR in tumorigenesis.

	Ligand/Dependence receptor implicated	Effect on cancer cells	Cancer type	References
Initiation (Stemness)	c-MET	Sox2, c-Myc, Nanog, Klf4, Oct4 expression ALDH expression	Glioblastomas	Li, Y., <i>et al</i> Proceedings of the National Academy of Sciences, 2011b. 108(24): p. 9951–9956
		Sox2, Nanog, Oct4 expression, hexokinase-2 expression	Pancreatic cancer	Yan, B., <i>et al</i> Experimental Cell Research, 2018. 371(1): p. 63–71
	Netrin-1/Unc5B	CAFs secretion of pro-stemness interleukins	Colon cancer and lung cancer	Sung, P.J., <i>et al</i> Cancer Res, 2019. 79(14): p. 3651–3661
	Notch3	Oct4, Nanog, Klf4, Rex1, Sall4, Rlf1, NAC1 expression	Ovarian cancer	Park, J.T., <i>et al</i> The American journal of pathology, 2010. 177(3): p. 1087–1094
		β -catenin inhibition, Nanog expression	Hepatocellular carcinomas	Zhang, Q., <i>et al</i> Oncotarget, 2015. 6(6): p. 3669–3679
	c-KIT	Lgr5, CD44, OLFM4, BMI-1, β -catenin expression	Colorectal cancer	Siemens, H., <i>et al</i> Oncotarget, 2013. 4(9)
Promotion (Proliferation)	Notch3	Cell cycle progression, Hif-2 α regulation	Renal cancer	Han, Q., <i>et al</i> Oncology letters, 2020. 20(6): p. 379–379
	P75NTR	P53 repression	Skin, breast, bone and brain cancers	Zhou, X., <i>et al</i> eLife, 2016. 5: p. e15099.
Progression (Angiogenesis)	c-MET	PI3K pathway activation	Breast cancer	Garcia, S., <i>et al</i> Int J Oncol, 2007. 31(1): p. 49–58
	TrkA	MMP2, NOS, VEGF expression	Breast cancer	Romon, R., <i>et al</i> Molecular cancer, 2010. 9: p. 157–157
	ALK	VEGF, VEGFR2, MMP2, MMP9 expression	Neuroblastoma	Di Paolo, D., <i>et al</i> Molecular Therapy, 2011. 19(12): p. 2201–2212
	c-KIT	IL-3, CXCL12, VEGFA production by mesenchymal cells	Breast cancer	Li, W., H. Xu, and C. Qian, BioMed Research International, 2017. 2017: p. 7407168
Progression (EMT)	c-MET	MMP1, MMP2, MMP9 expression E-cadherin repression	Colon cancer	Kermorgant, S., <i>et al</i> Carcinogenesis, 2001. 22(7): p. 1035–1042
		STAT3/Zeb1 activation	Gastric cancer	Cheng, Y., <i>et al</i> Translational Oncology, 2018. 11(2): p. 487–497
	Unc5B/Neogenin	MAPK pathway activation	Medulloblastoma	Akino, T., <i>et al</i> Cancer Research, 2014. 74(14): p. 3716–3726
	Neogenin	Zeb1 upregulation, PI3K/Akt activation	Gastric cancer	Qu, H., H. Sun, and X. Wang, Cellular Physiology and Biochemistry, 2018. 48(4): p. 1457–1467
	P75NTR	MMP12 and cadherin-11 upregulation	Glioblastomas	Berghoff, J., <i>et al</i> Molecular and Cellular Neuroscience, 2015. 69: p. 41–53
Chemo- and immune resistance	c-MET	ALDH expression	Glioblastomas	Li, Y., <i>et al</i> Proceedings of the National Academy of Sciences, 2011b. 108(24): p. 9951–9956
	P75NTR	Mitotical quiescence	Esophageal squamous cell carcinoma	Yamaguchi, T., <i>et al</i> Int J Oncol, 2016. 48(5): p. 1943–1954
	Notch3	ATP-binding cassette expression	Ovarian cancer	Park, J.T., <i>et al</i> The American journal of pathology, 2010. 177(3): p. 1087–1094
		PD-L1 expression	Breast cancer	Fatmah, A., <i>et al</i> Oncolimmunology, 9:1, https://doi.org/10.1080/2162402X.2020.1729299

the tumor environment (e.g., Netrin-1) (Fitamant *et al*, 2008), which enables consistent ligand/receptor engagement.

Role of DRs in tumor initiation

Activation of these receptors may provide a facilitating environment for the initiation of tumorigenesis. Cancer stem cells (CSCs), a subpopulation of cancer cells that exhibit long-term self-renewal ability, are essential contributors to tumor initiation and

maintenance of tumor growth (Prasetyanti & Medema, 2017), and a plethora of studies demonstrates the importance of DR activation in promoting their stem-like cell behavior. For instance, c-MET plays an important role in maintaining stemness in pancreatic cancer cells (Li *et al*, 2011a). Its activation results in enhanced expression of pluripotency markers NANOG, OCT4, and SOX2, through activation of YAP and potentiation of the Warburg effect (Yan *et al*, 2018). In fact, the activation of multiple DRs has been

shown to regulate the expression of pluripotency markers. For example, in ovarian cancer, the activation of c-KIT enhances tumor initiation *in vivo* through activation of PI3K/AKT and Wnt / β -catenin/TCR pathways. The crosstalk between cancer cells and cells from their microenvironment, such as cancer-associated fibroblasts (CAFs), also promotes plasticity and self-renewal through DR activation. For instance, in colon and lung cancers, cancer-associated fibroblasts (CAFs) secrete Netrin-1 and take part in a two-way signaling process with cancer cells, which involve the secretion of stemness-promoting cytokines such as interleukin-6 (IL-6), resulting in an increased stemness phenotype of cancer cells (Sung *et al*, 2019). These studies and multiple others (Huang *et al*, 2009; Park *et al*, 2010; Kimura *et al*, 2011; Li *et al*, 2011b; Xu *et al*, 2020) highlight the involvement of DRs in tumor initiation through the regulation of pluripotency markers, metabolic enzymes, and other important features of CSCs.

DR contribution to tumor progression

Tumor promotion involves a phase of clonal expansion. During this step, tumor cells undergo sequential genetic modifications that drive malignant transformation and enable escape from tumor suppressors. Therefore, during this step of tumorigenesis, a prevalent aspect is proliferation of tumor cells, and DR activation has major implications in the promotion of this hallmark. Thus, NOTCH3 was shown to promote the proliferation of renal cancer cells through regulation of cell cycle progression and of hypoxia-inducible transcription factor 2- α (HIF-2 α) expression (Han *et al*, 2020). Another example is p75NTR activation, which enhances the clonogenic capabilities of cancer cells by inactivating p53 through activation of MDM2-mediated ubiquitylation (Zhou *et al*, 2016). These are representative illustrations of the combined DR involvement in promoting proliferation and escaping tumor suppressor gene-driven apoptosis, supporting transformation of a preneoplastic lesion into a malignant one and subsequently promoting tumorigenesis.

Angiogenesis constitutes another major aspect of tumor progression. The formation and remodeling of new blood vessels and capillaries are essential to compensate the tumor core hypoxia that often accompanies uncontrolled proliferation of cancer cells. Pathways triggered by DRs are critical for different aspects of angiogenesis modulation, including the secretion of vasculogenesis-promoting cytokines, of protein involved in extracellular matrix remodeling such as matrix metalloproteinases (MMPs), and in the expression of vascular growth factors such as VEGF and its receptors. These factors can be produced by cancer cells themselves, as exemplified by the secretion of several MMPs and of VEGF in neuroblastoma cells expressing ALK (Di Paolo *et al*, 2011). They can also be secreted by endothelial cells surrounding the tumor, as demonstrated by the increased levels of MMP2, NOS, and VEGF in endothelial cells following activation of signaling pathways such as PI3K and ERK downstream of NGF-activated TrkA, leading to enhanced invasion, cord formation, and monolayer permeability in a breast cancer model (Romon *et al*, 2010). Interestingly, Netrin-1 can also function as a survival factor for endothelial cells by blocking UNC5B apoptotic activity. Thus, Netrin-1 not only promotes tumor progression directly but may also enhance the development and survival of new vessels within the tumor (Mehlen & Guenebeaud, 2010). Moreover, Netrin-1 also plays a role in vasculogenic mimicry (VM) (Zhang *et al*, 2018) (Zuazo-Gaztelu & Casanovas,

2018). Importantly, VM and neo-angiogenesis promote metastatic spread, as newly formed vessels provide a route by which tumor cells can leave the primary tumor site and invade other sites in the body (Folkman, 2002; Takeda *et al*, 2002).

Involvement of DRs in metastasis development

The development of distant metastases is a key feature of aggressive tumor progression and has important implications for patient outcome. Multiple studies report the role of dual signaling from DRs in regulating the metastatic spread of tumor cells, with DR-related cell death hindering tumor cell migration to and colonization of sites where ligands are absent, and overexpression of DR ligands conversely providing a selective advantage during metastasis development. It follows that escaping DR-induced apoptotic death is an important step to foster metastatic dissemination of tumor cells as well as their survival at metastatic sites. For instance, the overexpression of Netrin-1 was reported to confer a survival advantage to metastatic cells, acting through the inhibition of DCC and UNC5H receptors apoptotic activities. This likely explains why Netrin-1 is overexpressed in metastatic breast cancer whereas its expression in non-metastatic tumors is lower (Fitamant *et al*, 2008). Similarly, co-expression of c-MET and its ligand HGF was shown to predict peritoneal dissemination in gastric cancer (Toiyama *et al*, 2012).

Besides the previously discussed role of angiogenesis as a facilitator of metastatic dissemination, tumor cells themselves often undergo a phenotypic switch that enables them to acquire invading capacities. Indeed, epithelial-to-mesenchymal transition (EMT), a well-described phenotypic switch involved in metastasis development, is one of the fields where DR regulation has been the most studied across various cancer types. DR-driven activation of PI3K and ERK pathways activates critical EMT transcription factors such as ZEB, TWIST, and SNAIL. For instance, the activation of TrkA in head and neck carcinomas activates STAT3, which in turn increases the expression of Twist1, Snail1, and Snail2, thereafter leading to decreased E-cadherin expression and increased levels of vimentin and N-cadherin (Lin *et al*, 2020). These transmembrane and cytoskeleton proteins are major regulators of cellular adherence and morphology, and their modulation is an essential step in the EMT process. Moreover, DRs also contribute to the metastasis process by modulating extracellular matrix composition through increased secretion of MMPs, as mentioned earlier, thus facilitating migration of cells. An additional example is the activation of c-MET receptors by HGF, which results in enhanced production by cancer cells of MMP1, MMP2, and MMP9, enzymes that can degrade extracellular matrix proteins. In addition to its effects on E-cadherin and MMPs, it has been found that c-MET regulates EMT in cancer cells through the activation of hBvR (human biliverdin reductase), an ERK nuclear transporter required for MAPK signaling (Lerner-Marmarosh *et al*, 2008; Liu *et al*, 2017). This impact of c-MET on EMT has a drastic impact on cancer cell invasiveness and metastatic abilities, and is reflected in the upregulation of both c-MET and HGF in circulating tumor cells (CTCs), as shown, for example, in hepatocellular carcinoma (Ogunwobi *et al*, 2013). In addition, EMT has been linked to increased chemotherapeutic resistance of cancer cells through enhancing of cancer cell survival, cell fate transition, and upregulation of drug resistance-related genes (Wang *et al*, 2016), suggesting that DRs may have a role in regulating this process.

DRs and treatment response

Indeed, numerous studies show a link between the expression of DRs in cancer cells and resistance to different types of treatments. Correlations between expression of c-MET and resistance to cisplatin and ionizing radiations in several types of cancer (De Bacco *et al*, 2011; Sun & Wang, 2011), or between NOTCH3 expression and enhanced cisplatin resistance (Park *et al*, 2010), are good illustrations of their implications. This role may directly result from their effect on cell cycle and on the expression of detoxifying enzymes such as aldehyde dehydrogenase (ALDH) and ATP-binding cassette (ABC) transporters, transmembrane proteins that mediate drug export out of the cell (Park *et al*, 2010; Liu *et al*, 2018; Prager *et al*, 2019). Another reason underlying the correlation between DR expression and resistance to chemotherapies could be the positive impact of these receptors on angiogenesis, since high vascular density was shown to associate with a decreased response to chemotherapies (Mattern, 2001).

A role for DRs in immune escape?

In addition, besides resistance to chemotherapies, DRs may also be implicated in the ability of tumor cells to escape immune surveillance in several cancer types. The correlation between NOTCH3 and PD-L1 expression in breast cancer, and especially in breast cancer stem cells, is a good illustration of this. Indeed, PD-L1 expression is modulated by NOTCH3 via the activation of mTOR signaling (Mansour *et al*, 2020). In addition, DRs have a significant impact on the migration and maturation of immune cells. For instance, neogenin has been described as an important regulator of neutrophil migration and an activator of inflammation in a lung injury model (Mirakaj *et al*, 2012). In the context of cancer, the activation of NGF-TrkA signaling axis is an important step to produce IL-10 in tumor-associated macrophages (TAMs) (Ley *et al*, 2013). These examples among others illustrate the modulatory effect of DRs on immune cells and their involvement in tumor immune escape.

Thus, from tumor initiation to the promotion of metastasis development and the avoidance of immune cell regulation, DRs display multiple implications along multiple steps of tumorigenesis (Table 1), highlighting their potential as a therapeutic target in cancer.

Dependence receptors and clinical treatments

Targeting dependence receptor pathways: a promising avenue for cancer therapy

In view of their multi-faceted role in the regulation of cancer hallmarks, DRs are emerging as very promising potential targets in oncology (Fig 3). Blocking interactions between these receptors and their ligands not only would trigger apoptotic death of cancer cells, but could also prevent metastatic spread, immune escape, and resistance to chemotherapy by inhibiting positive signaling from DRs responsible for EMT, stemness, angiogenesis, and immunomodulation. A lot of molecules targeting these receptors are being evaluated in clinical trials. Even though the dependence function has not directly been considered in most of these trials, results from some do highlight therapeutic potential. As discussed above, such molecules could have an impact on all step of tumorigenesis, from the tumor initiation to the metastatic dissemination.

We have seen that DRs can have an impact on stemness features in some cancer cells and that their activation supports the potential of tumor-initiating cells. Preventing their activation could therefore have a significant impact on post-treatment recurrence. For instance, Tarextumab, a Notch2/Notch3 monoclonal antibody, was shown to inhibit tumor growth and to decrease tumor-initiating cell frequency in preclinical studies. Unfortunately, the phase II clinical trial evaluating the efficacy of Tarextumab on metastatic pancreatic cancer (NCT01647828) did not show any improvement in patient survival (Hu *et al*, 2019), but its efficacy is still being tested for other cancer types (NCT01277146). Thus, targeting DRs may not only diminish cancer stem cell plasticity but potentially inhibit interactions between CSC and niche cells, including CAFs, thereby minimizing cancer recurrence if used in combination with conventional chemotherapies.

Since DRs can modulate early steps of the tumorigenesis process, targeting them could also be used as an innovative early-stage therapy, preventing key events that foster tumor growth such as angiogenesis. For instance, current clinical trials evaluate the combination of anti-angiogenic drugs and c-MET inhibitors to enhance anti-angiogenic killing of cancer cells, as exemplified by the combination of pazopanib and ARQ 197, also called tivantinib, a highly selective c-MET inhibitor, in renal cancer (NCT01468922). The same principle was followed in a clinical trial combining bevacizumab (Avastin), an anti-angiogenic drug already used in several cancer types and INC280, a highly potent and selective c-MET inhibitor (NCT02386826). Based on preclinical results demonstrating a synergistic effect between c-MET and VEGFR inhibitors in mice (Harshman & Choueiri, 2013; Cascone *et al*, 2017), these phase I clinical trials show the potential benefits of targeting c-MET, and more generally, DR-associated angiogenesis. Indeed, INC280 has already been approved by the United States FDA (Food and Drug Administration) for the treatment of NSCLC (East Hanover).

The impact of therapies targeting DRs on angiogenesis, such as the combinations described above, could also decrease metastatic spread. Since most DRs also have a direct impact on invasion and migration of cancer cells, their targeting could potentiate this anti-metastatic effect, for example, by preventing EMT. SAIT301, a humanized monoclonal antibody targeting the c-MET extracellular domain alpha chain, provides a good illustration of this potential. It was shown to provide significant benefit in a phase I trial (NCT02296879) and to reduce invasion and migration of nasopharyngeal cancer cells (Bhatia *et al*, 2017). Migration and invasion of cancer cells were also inhibited by another monoclonal antibody targeting c-MET, YYB-001 (Kim *et al*, 2019). A phase I clinical trial with this antibody has just been completed for colorectal cancer (NCT04368507) (Kim *et al*, 2020), reflecting the potential of therapies targeting DRs for preventing metastatic spread.

As discussed above, DRs have also been shown to modulate interactions between tumor and immune cells, usually promoting the ability of tumor cells to evade the immune system. Consequently, targeting them could be an innovative way to decrease immune resistance of cancer cells and enhance the efficiency of immune checkpoint inhibitors, when used in combination. DR antagonists could also be used as single therapies against hematopoietic cancer cells. For example, the anti-c-KIT antibody KTN0158 (also called CDX-0158) demonstrated a good efficacy against malignant mast cells in preclinical studies (London *et al*,

2017). This antibody is now undergoing phase I clinical trial (NCT02642016) and was shown to enhance the efficacy of immune checkpoint inhibitors such as CTLA-4 or PD-L1 antibodies (Garton *et al*, 2017). This synergistic effect between KTN0158 and checkpoint inhibitors has been attributed to SCF-mediated control of mast cell development. Indeed, both SCF and KIT are expressed by several pro-inflammatory cells such as mast cells, regulating their migration through activation of PI3K/AKT signaling. SCF acts as a chemoattractant to mast cells and is responsible for their infiltration in tumors. Mast cells activated by SCF participate in tumor immunosuppression, increasing regulatory T cells number and enhancing the suppression of other T and NK cells in tumors, having a major pro-tumoral effect. This is the result of enhanced production of IL-17 and other inflammatory cytokines by SCF-stimulated mast cells. The presence of SCF-activated mast cells results in higher levels of immunomodulatory cytokines and transcriptional regulators such as IL-10, TGF- β , and Foxp3, within tumors (Huang *et al*, 2008). This study elegantly exemplifies how using drugs targeting DRs could enhance immunotherapy efficacy.

Finally, while DRs represent promising therapeutic targets in multiple cancer types, analysis of their expression could also enable the development of prognostic and/or predictive biomarkers in oncology. For example, Netrin-1 quantification in human fluids was shown to be a promising biomarker and a predictor of tumor recurrence in bladder, gastric, and lung cancers (Kefeli *et al*, 2012; Yıldırım *et al*, 2016; El-Gamal *et al*, 2020), and to correlates with poor prognosis in patients with brain metastasis (Harter *et al*, 2014). Another example is the quantification of c-MET expression, which provides predictive information on VEGFR tyrosine kinase inhibitor sensitivity in non-small-cell lung cancer (Cascone *et al*, 2017).

Dependence receptors: how to target them efficiently

We have seen that DRs are promising targets for therapy in oncology, firstly because of their implication throughout all phases in tumorigenesis and secondly, because this kind of drug would not only prevent these cancer-associated features but also lead to apoptosis in cancer cell harboring these receptors. We can then speculate that preventing these ligand-DR associations could be an efficient way of treating tumors. Nevertheless, since the DR concept is relatively new, it is important to discuss how pathways regulated by these proteins can be targeted most efficiently.

First, the decision of whether to target the ligand or its receptor has important implications, with several factors to be considered. For example, ligand availability and abundance, as well as the affinity of a ligand for its receptor and its binding dynamics, are critical variables to take into consideration (Kim & Cochran, 2017). A key parameter to consider for this strategy is the possible ligand pleiotropy. For example, Netrin-1 being a ligand for several DRs (DCC and UNC5H family receptors), targeting it offers an effective way to treat several types of cancers where one or several of its receptors is expressed. Conversely, SHH is a ligand for two DRs (PTCH-1 and CDON), but also acts through binding to several other non-DR receptors, suggesting that targeting only some of its receptors may allow a better control of subsequent biological effects. Moreover, the reported ability of some drugs targeting cell surface receptors to induce receptor downregulation, leading to a decreased presence of receptors at the membrane, should also be taken into consideration. Indeed, this phenomenon was reported to promote the therapeutic

efficacy of a treatment with non-DR receptors (Kim & Cochran, 2017) but could decrease efficacy of treatments targeting DRs, since a diminution of their expression at the membrane would lead to decreased apoptotic signaling. Therapeutic targeting of DRs therefore requires careful consideration of the number of different ligands and receptors (DRs and non-DRs) involved, of the ligand bioavailability, and of the nature of downstream pathways implicated in a specific cancer type to achieve optimal efficiency.

Possible interactions between signaling pathways activated by different DRs may make their targeting more complex but could also reveal systems of cellular dependencies that are amenable to medical targeting. Ligand-dependent DR signaling seems to mostly coordinate the activity of a small number of signaling pathways (ERK/MAPK, PI3K/AKT, Wnt, or TGF- β), implying that the net result of individual DR activation may vary depending on the cellular context of other similarly acting receptors. It is therefore plausible that the concomitant targeting of several of these DRs could have synergistic effects and may result in greater efficacy than the targeting of individual ones. Moreover, in view of the reported interaction and coordinated activation of similar signaling pathways by multiple DRs, targeting of several of these receptors concomitantly may preclude the emergence of resistance to drugs targeting single DRs. For example, targeting p75NTR could help bypass the observed resistance to tyrosine kinase inhibitors targeting TrkA or TrkC since it is involved in their regulation and seems to cooperate with them to induce several types of cellular responses (Barker & Shooter, 1994; Hantzopoulos *et al*, 1994; Jung *et al*, 2003).

Many existing drugs such as tyrosine kinase inhibitors in fact target DRs. Examples include the ALK and c-MET inhibitor Crizotinib, the c-KIT inhibitor Axitinib, as well as Entrectinib and Merestinib, which inhibit Trk receptors. However, these molecules act on DRs without preventing ligand binding. When considering the DR paradigm, it is therefore clear that these drugs inhibit positive signaling pathways triggered by these receptors, but that they are not expected to mimic the effects of ligand withdrawal and therefore to actively trigger apoptotic pathways within cancer cells. We argue that, in the case of DRs, alternative targeting approaches that result in abrogation of receptor activation, while inducing pro-apoptotic signaling by the “ligand-starved” receptor, should provide much more significant therapeutic benefit. Such is the case for monoclonal antibodies that inhibit survival pathways triggered by these receptors by preventing binding of their ligands. Such molecules are currently being developed and tested such as ficlatuzumab (NCT03422536), KTN0158 (London *et al*, 2017)(NCT02642016), or NP137 (Cassier *et al*, 2019b) which, respectively, inhibit the binding between HGF and c-MET, SCF and c-KIT, or Netrin-1 and its receptors.

DRs responding to Netrin-1, particularly DCC and UNC5B, have been studied extensively in cancer, with validation of efficacy of a therapeutic molecule that inhibits interactions between Netrin-1 and its receptors in this context. For example, NP137, a humanized neutralizing monoclonal antibody against Netrin-1, is currently undergoing phase Ib/II clinical trials for the treatment of gynecological cancers (NCT04652076) and phase 1b trials in advanced or metastatic solid tumors (NCT02977195)(Cassier *et al*, 2019a). Interestingly, despite the involvement of Netrin-1 and its DRs in numerous pathways, no obvious toxicity has been observed in phase 1 trials (Cassier *et al*, 2019b).

Beyond their implication across multiple stages of tumorigenesis, DRs are also attractive targets because their mutation as well as the expression level of themselves and/or their ligands can predict sensitivity to their inhibitors. This opens the door for a precision oncology approach, whereby tumors that exhibit a high expression of DRs and their ligands may be eligible for treatment with relevant molecules targeting these receptor/ligand interactions. For instance, Larotrectinib (or LOXO-101), a drug which targets all three Trk receptors, is now approved for treatment of several types of cancer after several clinical trials (NCT02122913, NCT02637687, and NCT02576431) (Hong *et al*, 2020). This molecule was developed for the treatment of cancers carrying NTRK gene fusions, regardless of cancer type (Roviello *et al*, 2020). The use of Larotrectinib suggests that specific targeting of some DRs driven by improved molecular tumor profiling should allow better and more effective treatments for patients.

While DRs represent interesting targets for cancer therapy, additional information must be gathered to document potential side effects for this type of treatment. Because these receptors are generally expressed at low levels in adults, side effects of molecules targeting them may be low, as already shown for the anti-Netrin-1 antibody mentioned above. Nevertheless, considering their implication in neuronal development, a special attention should be paid to possible neurological side effects during development of these new drugs. It is also likely that cancer cell plasticity will enable the appearance of some degree of resistance to this type of treatment. This could, for example, occur via inhibition of pro-apoptotic signaling, for instance through repression of DAPK1 to disable UNC5B-dependent apoptotic signaling in the absence of ligand (Grandin *et al*, 2016).

DRs: latest advances and translational impact

Since the field of DR study is relatively new, recent and exciting discoveries in this area not only include additional mechanistic characterization of their role in various physio-pathological settings, but also include the novel identification of additional receptors that exhibit a DR function. Notably, Wang *et al* recently observed that SCF withdrawal triggers apoptotic signaling through c-KIT intracellular cleavage by caspases, resulting in apoptosome activation (Wang *et al*, 2018). Considering the broadly reported involvement of c-KIT in several cancer types and in cancer stem cell regulation, this first identification of c-KIT as a DR should have important repercussions for researchers aiming to develop molecules targeting this receptor (Stankov *et al*, 2014; Abbaspour Babaei *et al*, 2016), since DR characteristics of c-KIT should be considered to fully exploit the therapeutic potential of this receptor.

Several elegant studies have also brought additional light on signaling mechanisms that drive the ligand-independent activation of apoptotic pathways by DRs, with the description of mitochondria permeabilization triggered by p40MET (Duplaquet *et al*, 2020), of EphB3 cleavage by Dral/FHL-2 and caspase-8 or 9 (Tsenkina *et al*, 2020), and of UNC5C cleavage by active δ -secretase all constituting important advances in the field. Findings from these studies not only further strengthen the concept of DR but also enable a better understanding of the DR signaling network, an essential step toward the rational design of new therapeutic molecules efficiently targeting these receptors. Along with these studies, the recent demonstration by Sumia *et al* that homo- and heterodimerization of Kremen1 is

Pending issues

- i Determine whether the full scope of dependence receptor has yet been discovered.
- ii Characterize the extent and biological significance of cross-signaling events between different dependence receptors.
- iii Establish how the initial activation of cleavage unfolds for most dependence receptors.
- iv Evaluate optimal targets for clinical translation (ligand vs receptor, single vs multitarget...)
- v Optimize targeting of dependence receptors so as to concomitantly inhibit positive signaling and promote pro-apoptotic signals.

required to trigger apoptotic signaling in the absence of Dickkopf1 binding and that the Kremen2 paralogue receptor can act as an inhibitor of Kremen1-induced cell death through direct competition with Kremen1, illustrates the underlying complexity of DR apoptotic signaling, and highlights an additional layer of DR regulation via direct interaction with neighboring transmembrane molecules.

Finally, an important recent development in the field has been the preclinical validation and clinical evaluation of NP137, a first-in-class monoclonal antibody targeting netrin-1 and the first molecule designed to fully exploit the therapeutic potential of a DR (Cassier *et al*, 2019b). Following promising data from a phase I clinical trial of NP137 as single agent in patients with advanced refractory solid tumors, this antibody is currently assessed in additional trials, including in combination with the anti-PD-1 antibody pembrolizumab for patients with locally advanced/metastatic uterine tumors (NCT04652076), known to exhibit frequent Netrin-1 overexpression. Beyond their direct impact on the survival of treated patients, results from these trials will represent an important step toward a better understanding of neutralizing monoclonal antibody as possible tools of choice to target DR, through the inhibition of ligand binding and the resulting activation of pro-apoptotic DR signaling.

Conclusion

We have sought here to define, explore, and analyze the concept of DRs and their implication in tumorigenesis. Indeed, through modulation of pathways such as ERK/MAPK or PI3K/AKT, activation of DRs influences tumorigenesis from initiation to metastasis. Altogether, studies involving DR pathways highlight the targeting of these receptors as an innovative and interesting approach for future cancer therapies. Indeed, preventing their activation could decrease the tumorigenic potential of cancer cells by limiting pro-tumoral features such as proliferation, angiogenesis, and/or chemoresistance, as well as by preventing ligand binding to induce apoptotic cell death.

Nevertheless, before accessing the full therapeutic potential of these receptors, a better understanding of the pathways they transduce within cancer cells and of their interactions, direct or indirect, is needed. Moreover, an important amount of preclinical and clinical studies is required to define what is the best way to target these receptors for which tumor type and to further characterize potential side effects. These studies represent an essential step toward unraveling the real potential of dependence receptor targeting for the benefit of cancer patients.

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Conflict of interest

Patrick Mehlen and Agnès Bernet are shareholders of Netris Pharma, which develops a netrin-1 antibody for clinical use. The other authors declare no conflict of interest.

For more information

- i F. Hollande lab: <https://mdhs.unimelb.edu.au/centre-for-cancer-research/our-research/Tumour-Heterogeneity-in-Metastatic-Cancer>
- ii P. Mehlen lab: <https://www.crcl.fr/en/teri-department/apoptose-cancer-et-developpement/>

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III. Unc5B & Netrin-1: the star couple of dependence receptors

The UNC5B receptor, like the DCC receptor, is one of the first dependence receptors to have been identified and characterized. For this reason, it is part of the prototypical dependence receptors, laying the foundations for a description of the mechanisms and functions of dependence receptors. This receptor shares the same ligand as DCC, namely Netrin-1, a protein whose functions are described in the literature, as summarized here.

A. Netrin-1

The first characterization of a member of the Netrin proteins family dates from 1992 when UNC-6 (Uncoordinated-6) was identified in *Caenorhabditis elegans* for its involvement in axon guidance and neuronal migration [168]. Subsequently, numerous netrin proteins were identified in multiple species including netrin-1, a protein widely described in the literature, especially in the context of cell migration and axonal growth in the embryonic nervous system [169]. Confirming a hypothesis formulated in the late 19th century by Santiago Ramon y Cajal, asserting the existence of diffusible factors enabling axonal guidance during development. Two homologues of this protein family, netrin-1 and netrin-2, were first purified by M. Tessier Lavigne and his team in 1994. Isolated from chicken embryos, they decided to name it "Netrin," derived from the Sanskrit term "netr," meaning "the one who guides," in reference to its status as a chemotropic factor [170].

1. Netrin-1 structure

Netrins are highly conserved throughout evolution, although there are species-specific forms. In mammals, there are three secreted netrins, netrin-1, 3, and 4, and two other forms of membrane-associated netrins, linked by a GPI (glycophosphatidylinositol) anchor, netrin G1 and G2.

The netrin family belongs to the superfamily of laminin-type proteins, whose members are composed of approximately 600 amino acids forming three major protein domains. The N-terminal end consists of a VI domain and a V domain, itself composed of three EGF (Epidermal Growth Factor) motifs. These domains are homologous to the gamma

chains of laminins for netrins 1 and 3, and to the beta chains of laminins for netrins 4, G1, and G2 [170]. In the case of secreted netrins, these domains are associated with a C-terminal end, called the "C Domain" or Netrin-like domain (NTR). This domain does not have homology with laminins but, in addition to the VI and V domains, would facilitate various interactions with the extracellular matrix [169]. In the case of netrin-1, the laminin domains located at the N-terminus are necessary for interaction with its transmembrane receptors UNC5B and DCC. These structural characteristics make netrin-1 a secreted molecule capable of interacting with the extracellular matrix and surrounding cells to fulfil diverse functions.

2. The Netrin-1, an "attracting" ligand

Netrin-1 was initially extensively described for its role in the development of the nervous system as an axon guidance factor. Thus, netrin-1 controls the attraction, repulsion, or elongation of axons towards specific sites, as well as synaptogenesis, ensuring the proper development of the neuronal network [171]. During tissue development and maintenance, netrin-1 also plays a role outside the nervous system by regulating cell adhesion and morphogenesis of branched organs such as the mammary gland [172] or the lung [173]. This protein has also been described as having functions in angiogenesis [174] immune regulation [175], and cellular pluripotency regulation [176, 177]. Finally, as seen earlier, netrin-1 is also involved in the tumoral context, where it promotes the proliferation, migration, and survival of tumour cells [178-180].

The range of these functions is highly diverse, sometimes even with opposing effects, and a single molecule would not be sufficient to achieve such finely regulated effects. Netrin-1, therefore, operates in tandem with a repertoire of receptors ensuring the diversity and specificity of the signals it transmits. Although UNC5B and DCC are the prototypical receptors associated with this ligand, netrin-1 is described to interact with other receptors and co-receptors. These receptors are all single transmembrane domain receptors, including UNC5B homologs UNC5A, C, and D, the Down-syndrome cell adhesion molecule (DSCAM) receptor [181], the amyloid precursor protein (APP) [182], and certain integrins [183], the adenosine receptor A2b [184] (Corset et al., 2000), and Neogenin [185].

Netrin-1 is, therefore, a pleiotropic protein with complex mechanisms of action, involving multiple receptors and bivalent signalling pathways, generating keen interest within the scientific community. Paradoxically, the signalling pathways induced by UNC5B, both with and without netrin-1, are still poorly understood.

B. Netrin-1 receptors: The light on the UNC5 family

Such as the Netrin family, the UNC5 receptor was identified in *Caenorhabditis elegans* as a transmembrane axon guidance receptor [186], predicted to interact with the Netrin-1 ortholog, UNC6. Subsequently, four homologs were described in mammals, named UNC5H1, 2, 3, and 4 (corresponding to UNC5A, B, C, and D in humans) [187]. In addition to Netrin-1, UNC5 receptors are also described to interact with Netrin-3 [188], Robo4 [189], Draxin [190], and FLRT2 and 3 [191].

1. Unc5B: Structure and physiological roles

These receptors belong to the immunoglobulin family of proteins and have implications for motility and cellular adhesion regulation [192-194]. Their extracellular structure is constituted of two immunoglobulin (Ig) domains binding Netrin-1 and two thrombospondin type I domains [195]. Their cytoplasmic tail is composed of a ZU5 domain, also found in proteins that are important for the linking of membrane-bound proteins to the cytoskeleton [196], and for tight and adherent junction formation [197]. In addition, these proteins possess a death domain (DD) and a DCC binding domain [198]. Further crystallography studies have demonstrated that, for Unc5B, the absence of Netrin-1 triggers conformation modification of its ZU5-UPA-DD domain allowing the interaction of the ZU-5 domain with both the DD and UPA [199]. Wang and al. predicted that this conformation would be responsible for the inhibition of UNC5B angiogenic activity. They further showed that the apoptotic activity of UNC5B was inhibited when expressed at physiological levels. Nevertheless, when overexpressed, its apoptotic activity is enhanced. Indeed, in the absence of Netrin-1, UNC5B monomerization occurs, which triggers the activation of Death-associated protein kinase DAPK1 through its

dephosphorylation by PP2A (protein phosphatase 2A) [200]. In the presence of Netrin-1, DAPK1 dephosphorylation is inhibited by CIP2A (Cancerous Inhibitor of PP2A).

In addition to its dependence on receptor functions, UNC5B signalling is associated with numerous functions including brain development, angiogenesis, and intestinal homeostasis [201]. Its most known feature is the modulation of axonal guidance in association with DCC and Netrin-1. In these interactions, the DCC/Unc5B complex is induced by netrin-1 and results in long-range repulsion whereas the Unc5B receptor alone triggers axonal repulsion [198]. Since Netrin-1 has been directly linked with angiogenesis through modulation of the PI3K/AKT/eNOS pathway, particularly in the context of vascular endothelial cells, a particular insight has been brought to study Unc5B in this context [202]. It has been noticed for instance, that mesenchymal stem cells synthesize pro-angiogenic factors, including non-canonical such as Netrin-1, to enhance the growth of endothelial cells and so, tubule formation [203]. This effect is probably the result of UNC5B or UNC5C activation since they're the only Netrin-1 receptors expressed by the endothelial cells of the umbilical vein used in the study. Nevertheless, the results of these different studies seem to indicate a negative impact of UNC5B on angiogenesis. Indeed, the fact that UNC5B and Netrin1 knockdown exacerbate sprouting vessels in zebrafish and mice, by negatively regulating filopodial extension in the vascular system, resulting in endothelial cell migration inhibition, supposes an inhibitory effect of the Netrin-1/UNC5B axis in angiogenesis [204].

UNC5B has been identified as a key player in promoting the migration of Schwann cells, a type of glial cells crucial for maintaining peripheral nerve fibres. Its positive impact on cell migration is achieved by influencing p38 and PI3K-Akt signalling pathways[205]. Moreover, the influence of Netrin-1 and Unc5B activation has been observed in renal proximal tubular epithelial cells. This activation, in turn, leads to an increase in the phosphorylation of AKT and ERK significantly associated with the migration and proliferation of renal tubular cells. The intricate interplay between Netrin-1, UNC5B, and downstream signalling pathways highlights the multifaceted roles of these molecular components in diverse cellular contexts [206].

In epidermal stem cells (ESCs) Netrin-1 treatment prevented UV-B-induced oxidative stress, mitochondrial dysfunction, and loss of ESC capacities by maintaining the

expressions of key markers (integrin β 1 and Krt19), with these effects being dependent on UNC5b and potentially involving the Wnt/ β -catenin signalling pathway [207]. The findings suggest that the netrin-1/Unc5B axis could be an important regulator of stem cells' responses to various stresses.

It is interesting to note that UNC5B signalling occurs through homodimerization but can also take place through heterodimerization of UNC5B with other netrin receptors [171]. This allows, through its ligands, a diverse range of signalling pathways. The downstream signalling induced by the receptor in the presence of the ligand, beyond a few key partners, remains poorly documented, and no specific canonical signalling pathway for the UNC5B receptor has been identified to date.

2. Unc5B: regulator of stemness and EMT in cancer

In a physio-pathological context such as cancer, Unc5B seems to modulate various features as well. Several studies investigated the implication of Netrin-1 and Unc5B in cancer cell plasticity in particular. Indeed, there is some indication that Netrin-1, the ligand of DCC and UNC5 receptors, contributes to the EMT phenotype of cancer cells. Indeed, high levels of Netrin-1 are a promising biomarker of muscle invasion in bladder cancer[208] and correlate with poor prognosis in patients with brain metastasis in the same cancer type [209]. Moreover, Netrin-1 has been shown to induce EMT through the activation of PI3K/AKT and ERK pathway in NSCLC (Non-Small Cell Lung Cancer)[210], leading to E-cadherin down-regulation and N-cadherin up-regulation. Moreover, it has been found that Netrin-1 promotes hepato-carcinoma cell metastasis by negatively regulating BVES (Blood vessel epicardial substance) expression via AKT activation [211]. However, it is not fully understood if Unc5B is the Netrin-1 receptor influencing EMT in cancer cells. Each of the Netrin-1 receptors seems to have different effects on cell motility, invasiveness and EMT. For instance, UNC5B inhibition in ovarian cancer was studied using a tumour-bearing assay in mice [212]. Knockdown of UNC5B resulted in reduced tumour proliferation, evidenced by decreased size and weight. Silencing UNC5B led to decreased expression in ovarian cancer tissues. Additionally, the inhibition correlated with molecular changes, including an upregulation of E-cadherin and a

downregulation of vimentin, suggesting a role in reversing epithelial-mesenchymal transition (EMT). Moreover, oleanolic acid (OA) demonstrates inhibitory effects on ovarian cancer cells by targeting UNC5B, leading to decreased proliferation, migration, and inhibition of EMT [212]. In other cases, Unc5B interact with other Netrin-1 receptors to modulate migration and EMT. For example, in medulloblastoma cells, UNC5B and Neogenin cooperate to enhance invasiveness via MAPK activation [213].

Increased netrin-1 expression was observed in B-ALL, particularly in patients with high- and intermediate-risk stratification. Netrin-1 demonstrated an anti-apoptotic effect in B-ALL cells and co-IP results revealed its interaction with the receptor Unc5B [214]. Interference with UNC5B abolished netrin-1's anti-apoptotic effects, and further analysis identified the activation of the FAK-MAPK signalling pathway, which was found to be downstream of Unc5b and implicated in the anti-apoptotic effect of netrin-1.

In both skin squamous cell carcinoma (SCC) and human endometrial carcinomas (ECs), netrin-1 emerges as a significant factor in tumorigenesis, particularly in promoting EMT and metastasis [215, 216]. The use of NP137 in mouse models demonstrated efficacy in reducing tumour progression and inhibiting EMT. A Phase I trial involving 14 patients with advanced EC further supported NP137's potential, with stable disease observed in 57.1% and an objective response in 7.1% of patients. Notably, the combination of NP137 with carboplatin-paclitaxel outperformed carboplatin-paclitaxel alone in the EC mouse model[215].

Furthermore, single-cell and bulk-RNA sequencing analyses revealed that NP137 administration modulates the tumour microenvironment, influencing the proportion of different EMT tumour cell states. Genetic knockdown experiments confirmed that NP137's impact on EMT is mediated through disruption of the netrin-1–UNC5B signalling axis EMT in a cellular autonomous manner, irrespective of the tumour microenvironment [216].

Netrin-1 receptors are less studied in the context of CSCs, but the available data so far supports their possible involvement in the regulation of these cells. In cooperation with Neogenin, the Unc5B receptor seems to regulate the pluripotency of embryonic stem cells (ESCs) by co-regulating the Wnt and MAPK pathways in both mouse and human

ESCs [217]. Through these two receptors, Netrin-1 activates Fak kinase to modulate Gsk3 α/β and thus stabilize β -catenin, while reducing ERK activity by increasing phosphatase activity of the Ppp2r2c-containing Pp2a complex. Moreover, in colorectal and lung cancer, UNC5B and Netrin-1 expression correlates with a cancer-associated fibroblasts (CAFs) signature, a signature defined by the expression of 131 genes extracted from TCGA data. In both cancer types, CAFs secrete netrin-1, which contributes to an interplay between cancer cells and CAFs that fosters an increased stemness phenotype of cancer cells [164]. In these studies, it appears that Netrin-1 could modulate the production of stemness-promoting cytokines such as IL-6 (Interleukine-6) by CAFs.

3. Unc5B : other pathophysiological features

In the pathological context of cancer, UNC5B seems to inhibit angiogenesis similarly to in the physiological context. Indeed, Netrin-1 overexpression reduces the growth of vessels expressing UNC5B and delays tumour angiogenesis in prostate cancer cells[218]. But Netrin-1 is not the only ligand of UNC5B, indeed, Robo4, a tumour endothelial marker present in the vascular network of different tumour types, has also been described as a UNC5B activator. UNC5B interaction with Robo4 preserves vascular integrity, preventing VEGF signalling in endothelial cells by recruiting pSrc to UNC5B and so, inhibiting the Src activation by VEGF[219]. Moreover, UNC5B-mediated signalling has also been described as a specific and important component of foetal-placental angiogenesis[220].

There are several clues indicating that Netrin-1 regulates the tumoral immune microenvironment through its receptors as well. First, Netrin-1 is secreted by macrophages and inactivates their migration through the UNC5B receptor, chemokines CCL2 and CCL19 modulation and activation of Rac1 GTPase[221]. Indeed, Netrin-1 was shown to inhibit the migration of several immune cell types such as lymphocytes, monocytes and granulocytes, potentially via its receptor UNC5B (strongly expressed by these cell types) [222]. In the model of atherosclerotic plaques, UNC5B activation by Netrin-1 was shown to promote the survival of macrophages under hypoxic

conditions[223]. In the model of kidney ischemia, activation of UNC5B receptor of CD4 T cells by Netrin-1 led to suppression of Th1, Th2 and Th17 responses and decreased cytokines linked to these responses' levels, notably IL-2, IL-6, IL4, IL-10, IL-13, IL-17, IFN- γ and TNF- α .

Some mechanisms in which the Unc5B receptor controls cell biology are not fully understood. There are several explanations for this. First, the concept of dependence receptor is not considered in a vast proportion of these studies, making it hard to distinguish between the positive or negative pathways triggered by this receptor. Moreover, Netrin-1 seems to have a dose-dependent effect represented by a “bell shape curve”, making the concentration used in the experiment crucial for a good understanding of their effect. Moreover, some dependence receptors are involved in the regulation of other dependence receptors or of their partners. For instance, as previously discussed, UNC5B and Neogenin can collaborate in several cancer types. It will then, in further studies, become crucial to better understand the interplay between dependence receptors in both positive and negative signalling.

IV. An overview of Notch3 dependence receptor

A. Notch pathway in colorectal cancer stem cells

Morgan Brisset, Patrick Mehlen, Olivier Meurette, Frédéric Hollande

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EDITED BY

Julie Pannequin,
Centre National de la Recherche
Scientifique (CNRS), France

REVIEWED BY

Cecilia Sahlgren,
Åbo Akademi University, Finland
Adam F. Odell,
York St John University, United Kingdom

*CORRESPONDENCE

Frédéric Hollande,
✉ frederic.hollande@unimelb.edu.au

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Notch receptor/ligand diversity: contribution to colorectal cancer stem cell heterogeneity

Morgan Brisset^{1,2,3}, Patrick Mehlen³, Olivier Meurette³ and
Frédéric Hollande^{1,2*}

¹Department of Clinical Pathology, Victorian Comprehensive Cancer Centre, The University of Melbourne, Melbourne, VIC, Australia, ²Centre for Cancer Research, The University of Melbourne, Melbourne, VIC, Australia, ³Cancer Cell Death Laboratory, Centre de Recherche en Cancérologie de Lyon, INSERM U1052-CNRS UMR5286, Centre Léon Bérard, Université de Lyon, Lyon, France

Cancer cell heterogeneity is a key contributor to therapeutic failure and post-treatment recurrence. Targeting cell subpopulations responsible for chemoresistance and recurrence seems to be an attractive approach to improve treatment outcome in cancer patients. However, this remains challenging due to the complexity and incomplete characterization of tumor cell subpopulations. The heterogeneity of cells exhibiting stemness-related features, such as self-renewal and chemoresistance, fuels this complexity. Notch signaling is a known regulator of cancer stem cell (CSC) features in colorectal cancer (CRC), though the effects of its heterogenous signaling on CRC cell stemness are only just emerging. In this review, we discuss how Notch ligand-receptor specificity contributes to regulating stemness, self-renewal, chemoresistance and cancer stem cells heterogeneity in CRC.

KEYWORDS

colorectal neoplasms, neoplastic stem cells, self-renewal, chemoresistance, posttreatment recurrence, tumor heterogeneity

1 Introduction

Cancer is a dynamic disease. During disease progression, tumor heterogeneity generally increases, resulting in the diversification of cell populations within the tumor bulk, each with different phenotypes and distinct sensitivities to chemotherapy (Stanta and Bonin, 2018). This heterogeneity was first thought to emerge solely as a result of expansion and genomic diversification of mutated cells. Indeed, clonal evolution, *i.e.*, genetic and epigenetic changes occurring over time in individual cancer cells, confers a selective advantage to some cells that outgrow others, partly explaining intra-tumoral heterogeneity (Greaves et al., 2012; Stanta and Bonin, 2018). However, this heterogeneity also depends on cancer stem cells (CSCs) present within the tumor. Like normal stem cells differentiating into phenotypically different lineages with limited proliferative potential, CSCs experience epigenetic changes to form phenotypically diverse non-tumorigenic cancer cells that compose the tumor bulk (Shackleton et al., 2009). The cancer stem cell concept was first proposed as an alternative to the clonal evolution of cancer cells to explain intra-tumoral heterogeneity (Nowell, 1976). This model emphasizes the hierarchical organization of cancer cells into phenotypically distinct tumorigenic and nontumorigenic populations. This hierarchical organization results in different subpopulations of cells within the tumor bulk displaying distinct levels or absence of tumor initiating potential. It is now accepted that both clonal

evolution and cancer stem cells co-exist to shape intra-tumor heterogeneity (Lyne et al., 2021).

Following chemotherapeutic treatment, sites of recurrence reflect the survival and amplification of drug-refractory cells. CSCs are likely drivers of this process, as intrinsic chemoresistance of CSCs may result in their survival and subsequently lead to their proliferation following treatment cessation (Abdullah and Chow, 2013). Thus, CSC-related heterogeneity fuels chemoresistance of tumor cells and thus recurrence (Zhang A. et al., 2022). Consequently, a precise understanding of the mechanisms underlying this chemoresistance and recurrence via tumor heterogeneity is necessary for the development of effective therapies. Indeed, the contribution of CSCs to intra- and inter-tumor heterogeneity significantly limits the efficacy of cytotoxic and targeted therapies in multiple solid malignancies, such as esophageal, liver, lung, pancreatic, or colorectal cancer (Khatib et al., 2020; Biswas and De, 2021), resulting in low survival rates for patients with advanced disease that cannot be cured via surgical means.

Inter- and intra-tumoral heterogeneity emerge from a combination of tumor cell-intrinsic events and of extrinsic, often microenvironment-driven factors. Intrinsic events include the progressive appearance of new subclones carrying *de novo* mutations (genomic drift) as well as residual epigenetic programming of differentiation pathways, leading to the potential presence of cells varying differentiation states (CSCs, progenitors, specialized differentiated cells) within genetically homogenous subclones. Extrinsic factors include microenvironmental niches and local or systemic regulators of signaling pathways such as the Wnt or Notch pathways (Borovski et al., 2011; Vermeulen et al., 2010; Crea et al., 2009). Along with the regular acquisition of new mutations, these pathways contribute to shaping further heterogeneity within solid tumors, including within CSCs themselves, which can differ according to cell cycle status, surface marker expression, response to chemotherapies, or differentiation potential (Magee et al., 2012; Birbrair, 2019; Tang, 2012). Colorectal cancer (CRC) is a highly heterogeneous disease, providing an ideal background to observe and characterize CSC heterogeneity.

In this review, we will summarize the current knowledge about CSC heterogeneity in colorectal tumors, describe its impact on chemoresistance and post-treatment recurrence, then discuss the relationship between Notch pathway activation, Ligand/Receptor specificity and CSC heterogeneity in colorectal cancer.

2 Colorectal cancer

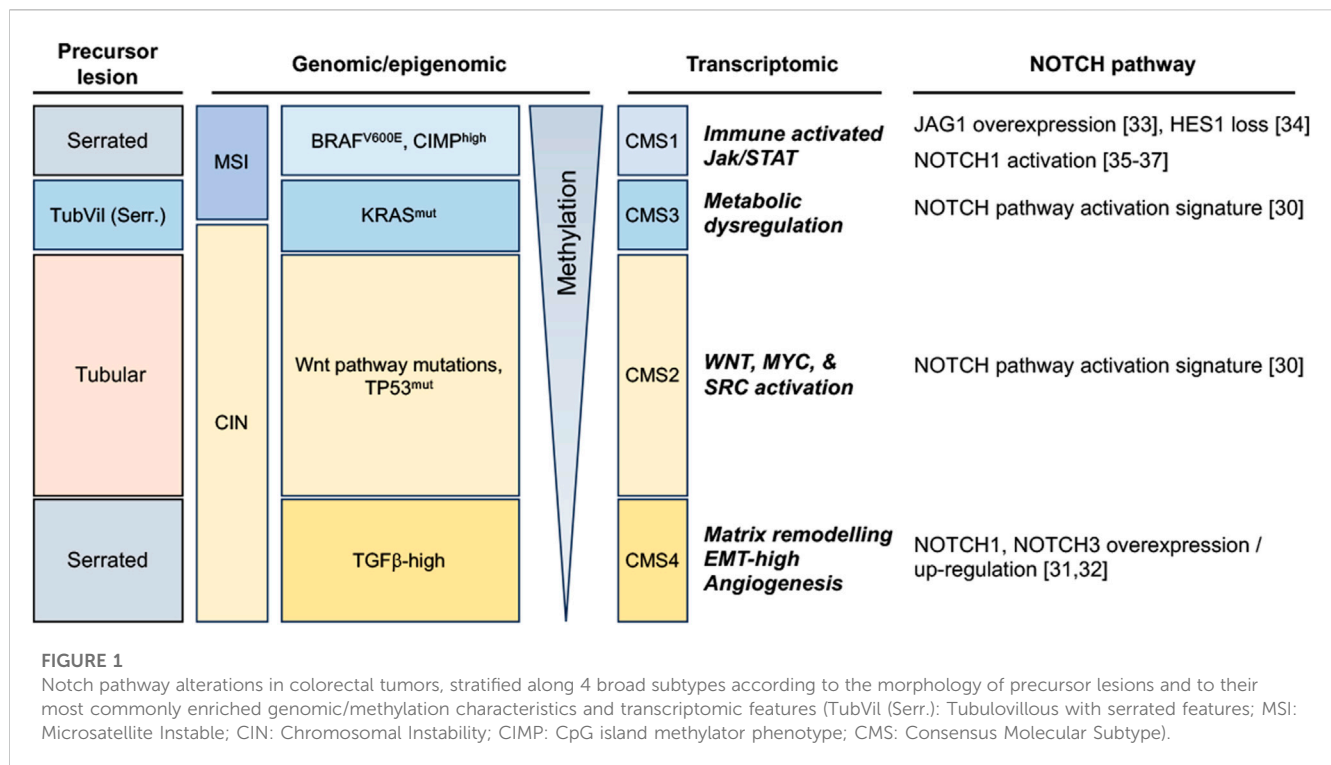
Colorectal cancer is the most commonly diagnosed and the third deadliest cancer worldwide, with almost 2 million new cases and a mortality reaching a million cases each year (Rawla et al., 2019). Main risk factors for this disease include age, family history, inflammatory bowel disease, as well as hereditary syndromes (e.g., Familial Adenomatous Polyposis, Lynch syndrome) (Brenner et al., 2014). Tumor stage, lymph node involvement, and the presence of distant metastases remain the main prognostic factors and guide therapeutic decisions (Lbianca et al., 2013).

Colorectal display significant inter-tumoral heterogeneity, underpinned by the existence of different sequences of genomic/

epigenomic alterations in different patients, and reflected at the macroscopic level by the emergence of different precursor lesions (summarized in Figure 1). Broadly, preneoplastic colonic lesions (adenomas) can develop along conventional or serrated carcinogenesis pathways. The former manifests itself through the initial development of Tubular, Villous, or Tubulovillous adenomas, while the latter can take either a Traditional or Sessile Serrated appearance, with a small subset displaying pathological features of both (Langner, 2015; Thanki et al., 2017) (Figure 1). Colorectal carcinoma emerging from the conventional carcinogenesis pathway are often driven by activating mutations of genes controlling the Wnt signaling pathway, most frequently through bi-allelic inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene, leading to aberrant Wnt pathway activation (Jen et al., 1994; Näthke, 2004). These tumors later develop additional driver mutations, often in genes encoding the KRAS GTPase and the TP53 tumor suppressor, the latter alterations often driving transition from late-stage adenoma to invasive carcinoma (Baker et al., 1990; Naccarati et al., 2012). Conversely, serrated carcinogenesis manifests along two distinct subsets that display significantly different genomic/epigenomic features (Kambara et al., 2004; Yang et al., 2004; Rosenberg et al., 2007). Sessile Serrated lesions emerge from mutations in DNA mismatch repair (MMR) genes that enable microsatellite instability (MSI-High), are enriched for *BRAF*^{V600E} mutations, and usually exhibit a high CpG island methylator profile (CIMP), resulting in high DNA methylation in resulting tumors. In contrast, traditional serrated adenomas usually display low methylation, a stable microsatellite profile (MSS), may exhibit mutations in KRAS or BRAF, but are mostly characterized by their high level of TGFβ pathway activation (Nguyen et al., 2020; Kim and Bodmer, 2022) (Figure 1).

More recently, a more detailed molecular classification has emerged from the integration of genomic and transcriptomic features of colorectal tumors, resulting in the stratification of this disease into four consensus molecular subtypes (CMS 1–4) (Guinney et al., 2015). Genes and pathways that characterize each CMS have significantly improved our understanding of previously described CRC subtypes and further highlight the inter-tumoral heterogeneity of CRC. Along this classification, CMS1 are characterized by hypermutated DNA, microsatellite instability (MSI-H) and a strong immune activation; CMS2 represents a canonical subtype characterized by chromosomal instability (CIN), by a strongly epithelial signature and an activation of Wnt and Myc pathways; CMS3 is characterized by metabolic dysregulations and an enriched KRAS mutation profile; and CMS4 represents a mesenchymal subtype, emerging mostly through the traditional serrated pathway and characterized by stromal invasion and angiogenesis and an activation of the transforming growth factor-β (TGFβ) (Guinney et al., 2015) (Figure 1).

Considering the important role of Wnt signaling in colorectal cancer and the well-documented crosstalk between Wnt and other developmental pathways, the Notch pathway has also been a subject of intense investigation in this cancer. Altered expression or functional activation of Notch receptors and ligands have indeed been reported to play a significant role in colorectal cancer progression, and data is progressively emerging that identifies which CRC subtypes may be more dependent on this pathway



(Figure 1). Indeed, it appears that NOTCH1 and NOTCH3 receptors are upregulated in the CMS4 subtype (Jackstadt et al., 2019; Koch and Radtke, 2020). NOTCH3 is associated with tumor staging, lymph node and distant metastasis in this subtype, whereas NOTCH1-signaling correlates with poor prognosis and drives metastasis. Furthermore, both CMS2 and CMS3 subtypes exhibit a Notch pathway activation transcriptomic signature (Guinney et al., 2015). The CRC serrated pathway exhibit Notch pathway dysregulation as well, notably single cell analysis revealed overexpression of the *JAG1* ligand and of *MYC* (Notch target) compared to normal tissue (Zhou et al., 2023). Interestingly, the *HES1* effector has been shown to be completely loss between the progression from hyperplastic polyp to Sessile serrated adenoma/polyp (Cui et al., 2016). Besides, it has been shown that the Bone Morphogenetic Protein (BMP) non-canonical activation of NOTCH1 pathway was participating in epithelial-to-mesenchymal transition (EMT) and led to poorer prognosis in mesenchymal-subtype of colorectal cancer (Irshad et al., 2017), a subtype featuring EMT and MSI high signatures (Kim et al., 2021; Singh et al., 2021).

This increased understanding may have future implications on colorectal cancer treatment (Vinson et al., 2016; Tyagi et al., 2020), particularly in the case of treatment refractory and metastatic tumors.

3 CRC therapy

The most common treatment for local CRC remains surgical resection, and though this may also be used for some metastatic colorectal cancers (mCRC), chemotherapeutic interventions are more frequent. Different molecules are used as chemotherapies:

5-fluorouracil (5-FU), an anti-metabolite and uracil analogue preventing DNA synthesis and inhibiting cell division (Longley et al., 2003); Oxaliplatin, a platinum-based anti-neoplastic, that exerts a cytotoxic effect by binding to DNA, RNA and some protein structures (Martinez-Balibrea et al., 2015); and Irinotecan a molecule that inhibits cell division by acting on topoisomerase I (Fujita et al., 2015). Patients receive different combinations of these three molecules, as well as Leucovorin, an active metabolite of folic acid and an essential coenzyme for nucleic acid synthesis that enhances the efficacy of 5-FU (Moran, 1989). These combinations are called FOLFOX (a combination of Oxaliplatin, 5-FU and Leucovorin), FOLFIRI (a combination of Irinotecan, 5-FU and Leucovorin) and FOLFOXIRI (a combination of Oxaliplatin, Irinotecan, 5-FU and Leucovorin) (Fakih, 2015). Another class of treatments is under development, namely, targeted therapies. Indeed, several monoclonal antibody-targeted therapies are recommended for mCRC, such as antagonists of the epidermal growth factor receptor (EGFR): cetuximab and panitumumab, or of the vascular endothelial growth factor A (VEGFA): bevacizumab. Those molecules respectively aim at preventing proliferative signaling cascades and at helping to reduce angiogenesis, two important hallmarks of cancer growth (Hanahan and Weinberg, 2011). Unfortunately, most patients with advanced CRC eventually die from the disease, despite initial response to these therapies, due to post-treatment tumor recurrence (Song et al., 2018). Effective treatment and patient stratification play crucial roles in managing colorectal cancer and improving patient outcomes. Treatment approaches include surgery, chemotherapy, targeted therapy, immunotherapy, and radiation therapy, which are selected based on tumor stage and molecular characteristics (Benson et al., 2021; Van Cutsem et al., 2016). While advancements in treatment have led to improved survival rates, tumor recurrence remains a significant

challenge and a leading cause of mortality in colorectal cancer (Tarantino et al., 2015). Recurrence patterns may involve local, regional, or distant metastasis (Weiser, 2018). To address this, personalized treatment strategies are employed, which may include adjuvant chemotherapy, targeted therapies, and close surveillance to facilitate early detection of recurrence (Grothey et al., 2018). Molecular biomarkers, such as *RAS* and *BRAF* gene mutations, guide treatment decisions and help identify patients at higher risk of recurrence (Douillard et al., 2013). Although most adjuvant chemotherapies still appear to have limited effect on recurrence (Kaiser et al., 2006), patient stratification based on molecular profiling and genetic testing enables tailored treatment plans and may provide benefit in the future (Thanki et al., 2017; Sveen et al., 2018). Overall, a multidisciplinary approach, close follow-up, and innovative therapies are essential in mitigating the mortality outcomes associated with tumor recurrence in colorectal cancer (Labianca et al., 2013).

Resistance and recurrence of CRC seems to be linked to specific cell subpopulations. These cells are defined as tumorigenic and able to reproduce tumoral heterogeneity indicating their self-renewal abilities (De Angelis et al., 2019). The characterization of these subpopulations in CRC and the events modulating them is therefore a key objective to enhance overall survival of CRC patients and prevent recurrence after treatment. A crucial factor to understanding CSC dynamics and homeostasis is to decipher the interactions between them and their neighboring environment. Indeed, like normal stem cells, CSCs are influenced by external signals, involving interactions with components of the tumor microenvironment and pathways implicated in cellular development, such as Wnt and Notch (Leedham et al., 2005; Ye et al., 2014). An increasing number of studies indicate that the Notch pathway is involved in modulating stemness in normal and cancer cells (ElShamy and Duhé, 2013; Dejana et al., 2017). The aim of this review is thus to present current knowledge on the impact of Notch signaling on colorectal cancer stem cells and its subsequent implication in resistance to CRC treatment and recurrence.

4 Role of CSCs in colorectal cancer heterogeneity

As described previously, CSCs are a major determinant of cancer heterogeneity, representing the source of differentiation processes and phenotypic heterogeneity within genomically homogenous clones. In CRC, CSCs exhibit some of the phenotypic characteristics of non-pathological stem cells and are able to initiate and maintain tumor growth. They play a key role in the metastatic process, in resistance to chemotherapies and in the post-treatment relapse of CRC. This subpopulation of cancer cells is characterized by specific properties such as self-renewal, tumor-inducing and sphere forming abilities, limitless replication due to telomerase activity, and multi-lineage differentiation potential (Prasetyanti and Medema, 2017; Zhou et al., 2017). The expression of some specific stemness markers like CD44, CD133, CD24, EpCAM, LGR5 and the ALDH detoxifying enzyme, or the expression of stemness-related genes such as NANOG, SOX2 or KLF4 can be used to enrich or identify this cell subtype. CSCs may also exhibit an aberrant activation of several developmental

pathways such as Notch, Hedgehog and Wnt pathways (Cleophas et al., 2017).

Colorectal CSCs are ideal “seed” cells for metastasis of CRC to distant tissue. Their limitless replication and pluripotency enable them to form tumors that are suitable to new microenvironments, often very different from the primary tumor site. In addition, the heterogeneity originating from the asymmetric division of CSCs facilitates the development of metastases despite new microenvironmental conditions. The complexity of the CSC microenvironment is due to numerous signals and factors promoting or inhibiting the stem cell phenotype. Factors such as hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), bone morphogenetic protein (BMP) positively regulate the maintenance of the CSC pool, whereas other factors will trigger their differentiation (Zeuner et al., 2014).

It is worth noting that different characteristics of CSCs seem to be regulated independently from one another, and events enhancing sphere forming abilities may negatively impact stem cell-related gene expression, or resistance to chemotherapies (Ying et al., 2015). For instance, i) knocking down the expression of genes such as *AHNAK2* influences sphere formation without affecting the expression of biomarkers like CD133, while ii) knockdown of genes like *HLA-B*, *CCDC92* and *PLIN4* influences CD133 expression without changing self-renewal abilities, and iii) genes like *ALK* and *ALMS1* were reported to influence both traits (Zhang X. et al., 2022). Another example of this phenotypic heterogeneity among CSC-like cells is the expression of the EpCAM (epithelial cellular adhesion molecule) marker. In several studies, we observed a direct link between its expression and stemness-like characteristics, including colony formation, self-renewal, tumorigenicity, invasion, metastasis or chemoresistance (Liu et al., 2014; Xiang et al., 2017; Leng et al., 2018). However, other studies described the opposite, as they found that CRC cells expressing low levels of EpCAM were more motile, invasive, chemoresistant, and highly metastatic (Sacchetti et al., 2021).

In 2022, Liu et al. attempted to decipher the inter-tumoral heterogeneity of cancer stemness by performing an unsupervised clustering based on 26 published stemness signatures. Doing so, they revealed two different clusters of patients (Liu et al., 2022); one with low stemness and the other with high stemness properties. The cluster displaying greater stemness properties possessed a higher proportion of advanced tumors and was characterized by worse overall survival and relapse-free survival. The two clusters exhibited several other genetic and phenotypic differences. Genetically, the stemness-high cluster displayed more copy number deletions, whereas the stemness-low cluster possessed a greater mutational burden. Phenotypically, the stemness-low cluster exhibited a proliferation-related phenotype and abundant immune infiltration as well as predominant mutations in multiple oncogenes and tumor suppressors, such as *TP53*, *SYNE1*, *MUC16*, and transcription enrichment of *PIK3CA* and *GFPT1*, *PTMAP9*, *MOGAT3*, and *DPM3* genes, while the stemness-high cluster was significantly associated with mesenchymal and differentiation features as well as overexpression of *S100A12*, *PGM5*, *FUT6*, *SEMA3C*, and *ADAM33* genes. The difference in stemness level between these clusters could be explained by different proportions of CSCs within these tumors, although they presented distinct genomic alterations in multi-omics analyses, suggesting the

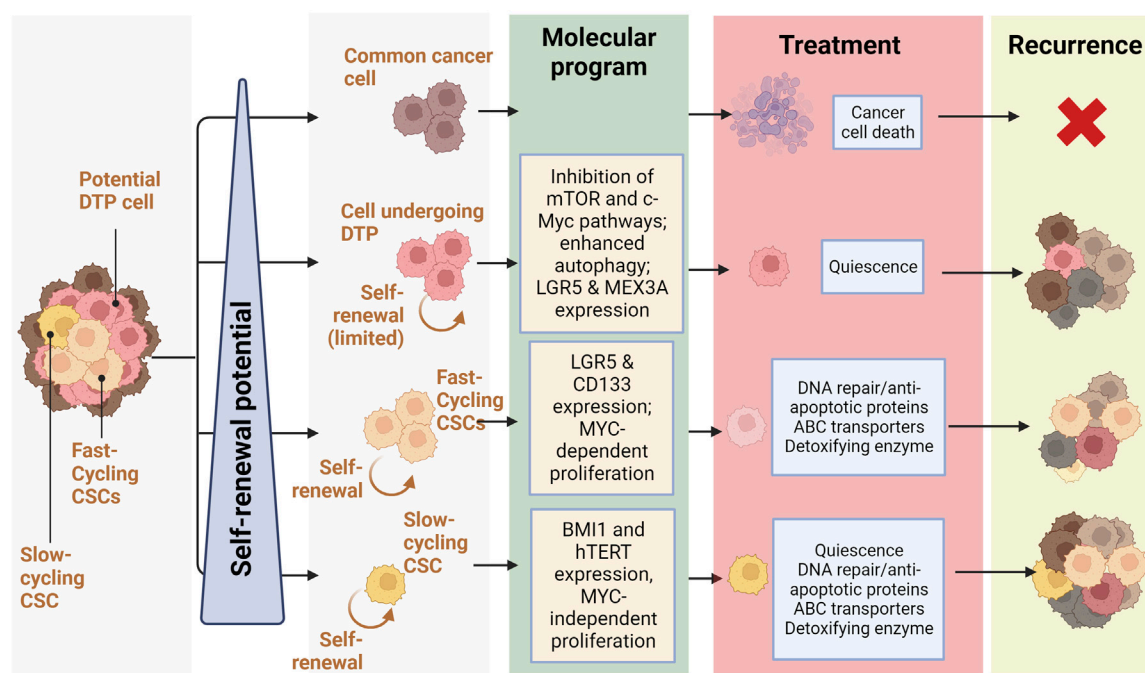


FIGURE 2

Relationship between self-renewal and recurrence in CRC: Schematic representation of treatment response and recurrence potential by different CRC cells depending on their self-renewal potential (slow-cycling CSCs and fast cycling CSCs and cells undergoing DTP) and the molecular programs they rely on (Quiescence, DNA repair/anti-apoptotic proteins, ABC transporters, Detoxifying enzyme) (Created with [BioRender.com](https://www.biorender.com)).

existence of various populations of cells capable of self-renewal in CRC, reflecting a level of inter-tumor heterogeneity.

In 2020 Fumagalli et al. investigated cells at the origin of CRC metastases using a mouse model of CRC and human tumor xenografts. Interestingly, they found that a majority of disseminated CRC cells do not express *Lgr5* but form distant metastases in which *Lgr5*-expressing CSCs progressively arise. This re-expression of *Lgr5* by non-CSCs seemed to occur independently of microenvironmental factors and was necessary for metastasis, but dispensable for primary tumor establishment, highlighting the key role of CSC-heterogeneity in important events like metastasis and how the dedifferentiation of non-CSC cells may contribute to this phenomenon (Fumagalli et al., 2020). These findings support the existence of a CSC phenotypic heterogeneity resulting in different populations of stem cells co-existing in colorectal neoplasms. They are also in accordance with the inter-tumoral clusters of stemness observed by (Liu et al., 2022), with high stemness clusters being based on inherent stemness of CSCs and low stemness clusters relying on dedifferentiation of non-CSCs cells. A balance seems to occur in tumors between two different CSC populations: *LGR5*⁺ crypt-base columnar stem cells (CBCs) and *LGR5*-negative regenerative stem cells (RSCs) (Gil Vazquez et al., 2022). CSCs exhibiting markers of CBC, RSC, or both, are capable of self-renewal whereas cells devoid of stem cell markers have very little clonogenic capacity. Despite similar self-renewal capacities, the predominance of APC and CTNNB1 mutations in CBC-enriched tumors seems to indicate that the CBC stem cell phenotype relies on mutations allowing a ligand-independent activation of Wnt signaling, whereas RSC-enriched tumors display enrichment of

KRAS, YAP, TGFβ, and inflammatory pathways (such as IFN-γ). Within neoplasms, an equilibrium between cell populations expressing CBC and RSC markers exists and plastic cells can adaptively shift between these stem cell phenotypes in response to microenvironmental pressures. This heterogeneous and dynamic stem cell population is suspected of being crucial for the adaptive response to selective pressures and to promote lesion outgrowth. Several other studies have demonstrated this apparent heterogeneity of CSC populations. For instance, CD133 expression may not be sufficient to identify the entire CSC population in mCRC, albeit metastatic CD133-expressing cells develop into aggressive tumors and express phenotypic markers of CSCs, notably CD44 (Shmelkov et al., 2008; James et al., 2015). Further evidence of CSC diversity and heterogeneity is the description by Srinivasan et al. of co-existing populations of fast- and slow-cycling colorectal CSCs, undergoing asymmetric division to generate each other (Srinivasan et al., 2016). Fast-cycling CSCs express stemness markers, like *LGR5* and *CD133*, and depend on *MYC* to proliferate, whereas slow-cycling CSCs are characterized by different markers, such as *BMI1* and *hTERT*, and are independent of *MYC* (Figure 2). We hypothesize that the inter-tumor heterogeneity characterised by Liu et al. could also result from different proportions of CSC subpopulations within tumors, the stemness-high cluster arising from tumors developed from slow-cycling CSCs and the stemness-low cluster corresponding to tumors originating from fast-cycling CSCs.

Several studies are now trying to combine stemness with other sources of intra-tumoral phenotypic heterogeneity and variability, such as those derived from the tumor microenvironment (Magee et al., 2012; Almendro et al., 2013; Kreso and Dick, 2014). This latter

plays a major role in the induction and maintenance of stem-like phenotypes in colorectal tumors. For instance, the specific localization of cells within a tumor was shown to be a key parameter in the pluripotency potential of CRC cells. Indeed, cellular clones responsible for tumor outgrowth in colon cancer predominantly originate from the outer tumor regions (Lenos et al., 2018). Tumor cells from these regions demonstrate higher clonogenic abilities independently of the markers they express. The increased contacts of cancer cells in these regions with components of the microenvironment, such as CAFs, would enhance clonogenic abilities. This is due to the release in the extracellular matrix (ECM) of stemness enhancing proteins such as osteopontin (Lenos et al., 2018) or Netrin-1 (Sung et al., 2019; Brisset et al., 2021). ECM has also been shown to influence stemness through biochemical and biophysical aspects (Nallanthighal et al., 2019). Collectively, these findings demonstrate that functional CSCs differ from cells expressing stemness markers and that the environment prevails over cell-autonomous features in determining stem cell functionality.

This impact of microenvironment further highlights stemness as a phenotypic state fueled not only by cell-intrinsic characteristics but also highly dependent on contextual conditions. Indeed, multiple environment-sensitive epigenetic parameters significantly influence stemness in colorectal CSCs, including DNA methylation, histone acetylation, histone methylation, ubiquitination, and microRNAs (Zeuner et al., 2014; Paschall and Liu, 2015).

Recent studies describe a subpopulation of CRC cells undergoing a phenotypic change following chemotherapy. Indeed, these cells described as “Drug Tolerant Persister” (or DTP) undergo a reprogramming similar to diapause, a reversible state defined by quiescence triggered by unfavorable environmental conditions, such as nutrient deprivation, during embryonic development (Rehman et al., 2021). In contrast to CSCs, all tumor cells have been shown to have an equipotent capacity to become DTPs, which cannot be determined based on genetic mutations but rather on epigenetic changes resulting in a decrease in mTOR pathway activity, alterations in chromatin modifications, hypo-transcription, and an increase in autophagy (Bulut-Karslioglu et al., 2016). Colorectal DTP cells express the genes LGR5 and MEX3A, represent a small subpopulation of the parental tumor (0.3%–5%), are chemoresistant and regenerate after therapeutic treatment (Álvarez-Varela et al., 2022) (Sharma et al., 2010; Liao et al., 2017) (Figure 2). In CRC mouse models, DTP cells displayed a marginal contribution to metastatic outgrowth; nevertheless, after treatment, DTP cells produced cell clones that regenerated the tumor. Conversely, LGR5+ MEX3A-cells were shown to differentiate towards a goblet cell-like phenotype and display no evidence of chemoresistance. It is believed that the DTP subpopulation, unlike CSCs, is not defined by genetical features but distinguished by unique transcriptional and epigenetic profiles, though they share several properties. For instance, DTP cells display fast-cycling and slow-cycling, reminiscent of the balance between CSC populations existing in CRC tumors.

Altogether, these studies put in evidence the plurality of pathways leading to self-renewal, tumor-induction and chemoresistance and explain the variability in expression of stemness markers, or phenotypic characteristics observed among colorectal CSCs. Moreover, the ability of non-CSCs to acquire stem-

like characteristics and become CSCs through the process of de-differentiation depending on genetic, epigenetic, or microenvironmental alterations, increase global phenotypic intra-tumoral heterogeneity and complexify the characterization of the different pools of self-renewing cells (Minami, 2015). Thus, dedifferentiation of non-CSCs driven by TGF-beta signaling was shown to enhance stemness and self-renewal abilities in CRC (Nakano et al., 2019). These dedifferentiated non-CSCs seemed able to acquire most phenotypic characteristics of CSCs, including expression of stemness-associated genes, such as LGR5 or CD44, and high tumorigenic potential, following priming by microenvironmental factors shaping CSCs niches (Borovski et al., 2011; Vermeulen et al., 2010) or induction of epithelial-to-mesenchymal transition (EMT) (Morrison and Spradling, 2008). The existence of inter- and intra-tumoral heterogeneity of CSCs in colorectal cancer is now known and represents another challenge for the effective treatment of CRC by chemotherapy. Previously mentioned CSC clusters exhibiting various phenotypic traits as well as differences in response to treatment results in wide range of cellular behavior, impeding prognosis and chemotherapies efficacy. A holistic understanding of the different CSC subpopulations and of signaling pathway alterations responsible for their resistance to chemotherapies is needed to enhance treatment efficacy and prevent tumor recurrence.

5 CSCS and chemoresistance

A growing body of evidence is emerging linking CSCs to tumor resistance to treatment and relapse. Indeed, therapies such as radiotherapies or chemotherapies are effective against differentiated and proliferative cancer cells but have an exceptionally low impact on CSCs (He et al., 2014). In colon carcinoma cells, apoptosis triggered upon chemotherapy is comparably lower in CSCs than in differentiated cancer cells (Colak et al., 2014). CSCs resist conventional chemotherapy by escaping DNA damage induced by therapies. Consequently, drugs inducing cell death by targeting DNA, such as cisplatin, oxaliplatin (DNA cross-linkers), methotrexate (DNA synthesis inhibitor), doxorubicin and daunorubicin (topoisomerase inhibitors) are inefficient against CSCs (Peitzsch et al., 2013). CSC resistance to DNA damage is modulated by several mechanisms, including alteration of cell cycle checkpoints (Solier et al., 2012), high expression of DNA damage repair proteins (such as p-ATM) (Khanna et al., 2001; Sánchez-Flores et al., 2015; Anuja et al., 2019), modified metabolism (Blondy et al., 2020), and an efficient scavenging of reactive oxygen species (ROS) induced by chemotherapy or radiotherapy (Diehn et al., 2009).

Increasing data suggest that quiescence contributes to CSCs chemoresistance, as some of the cytotoxic drugs mainly target highly proliferative cells (Is et al., 2015; Das et al., 2019; Das et al., 2020). After treatment, quiescent CSCs can resume their cell cycle and fuel tumor regrowth through the activation of signaling pathways fostering cell growth and proliferation (Francescangeli et al., 2020). The mechanisms underlying colorectal CSC quiescence are not yet fully understood, though the expression of transcription factors such as c-YES (Touil et al., 2014), ZEB-2 (Francescangeli et al., 2020) or HMGA-1 (Puca et al., 2014) were proven to be crucial

for the entry of CSCs into the reversible quiescent G0 cell-cycle phase.

CSCs exhibit an aberrant and flexible metabolism driving epigenetic and genetic changes necessary for tumor onset, development, intra-tumoral heterogeneity, and recurrence (Kaur and Bhattacharyya, 2021). The metabolic plasticity of CSCs allows them to switch between glycolysis and oxidative metabolism (OXPHOS) to adapt to microenvironmental stresses like nutrients deprivation and therapies (Kahlert et al., 2017). For instance, CSCs rely less on ATP and glucose-dependent lipid synthesis, suggesting that metabolic plasticity of CSCs could play a key role in their quiescence and consequently in their resistance to cytotoxic agents (Vincent et al., 2014). Moreover, 5-FU being an inhibitor of thymidine synthesis, it was reported to have a limited efficacy against CSCs due to their capacity to switch to high levels of oxidative phosphorylation, characterized by a high expression of pyruvate kinase M1 (PKM1) and repression of PKM2, and resulting in inhibition of the pentose phosphate pathway (Denise et al., 2015; Vellinga et al., 2015).

Aldehyde dehydrogenase (ALDH) is an enzyme catalyzing the oxidation of endogenous and exogenous aldehyde substrates, a key function of cellular detoxification (Tomita et al., 2016). Members of the ALDH1 family, such as ALDH1A1, ALDH1A3 or ALDH1B1, are strongly active in normal tissue stem cells and are considered to be markers of colorectal CSCs (Khorrami et al., 2015; Feng et al., 2018). The ability of ALDH to detoxify the active aldehydes formed by reactive oxygen species (ROS) protects CSCs from chemotherapy by increasing the level of ROS (Vishnubalaji et al., 2018).

ABC (ATP Binding Cassette) transporters is a family of large transmembrane proteins relying on ATP hydrolysis to reject metabolites, foreign bodies, and toxic substances from cells (Amawi et al., 2019). In colorectal CSCs, the common ABC transporters, ABCB1, ABCC1, and ABCG2, are highly expressed and actively excrete anti-tumoral drugs, decrease intracellular concentration of drugs, decrease the effect of chemotherapies, and result in multi drug resistance (MDR) (Kangwan et al., 2016; Butler et al., 2017).

Hence, resistance of colorectal CSCs to commonly used therapies is multi layered and relies on a high expression of DNA repair proteins, an acquired quiescence, an adapted metabolism, and expression of ALDH detoxifying enzyme and ABC transporters. These factors explain the resistance of colorectal CSCs against classical chemotherapy regimens like FOLFOX or FOLFIRI (Ilmer et al., 2016).

Furthermore, it is increasingly acknowledged that DTPs are key drivers of chemoresistance and tumor recurrence (Figure 2). As seen above, DTP cells exhibit gene expression patterns that mimic hormonally- and chemically-induced diapause upon chemotherapy. This diapause-like DTP state is characterized by the inhibition of mTOR and c-Myc pathways, as well as enhanced autophagy (Bulut-Karslioglu et al., 2016). By doing so, DTP cells display resistance to a wide variety of chemotherapeutic agents (De Angelis et al., 2019), radiotherapy (Zhao Y. et al., 2020), and targeted therapies (Cabanos and Hata, 2021). Although these DTP-cells revert to a chemo sensitive state after treatment withdrawal (Recasens and Munoz, 2019), tumors in a DTP state may act as a transient reservoir for the development of genetically resistant clones (Ramirez et al., 2016), demonstrating the importance of non-genetic heterogeneity in chemoresistance and recurrence.

As stated previously, intra-tumoral heterogeneity of CSCs in colorectal cancer has been established and contributes to chemoresistance and recurrence in CRC. The balance between the different subpopulation of CSCs and their direct interconversions undoubtedly allows a more diverse response to chemotherapeutic molecules and has been shown to come back to a steady-state post-treatment. For instance, despite the low frequency of slow-cycling colorectal CSCs, this long-lasting subpopulation might constitute a more chemoresistant reserve population needed for recurrence and tumoral repopulation after treatment (Srinivasan et al., 2016) (Figure 2). NOTCH1 signaling through HES1 and HES5 modulation was shown to be the key regulator of asymmetric division, modulating the balance between these two populations, thus directly impacting heterogeneity-based chemoresistance.

In conclusion, phenotypic heterogeneity of CSCs and of normal cancer cells is a driver of resistance to treatment and recurrence in CRC (Figure 2). This pluripotent cell-related heterogeneity fosters a highly adaptive response to chemotherapeutic agents, driving chemoresistance and the reconstitution of tumoral hierarchy after treatment, and is finely tuned by the modulation of molecular signaling, notably the Notch pathway (Ebrahimi et al., 2023).

6 Notch pathway general description

Notch signaling orchestrates several major aspects of tumor development by regulating differentiation, proliferation, and apoptosis.

There are four different Notch receptors (NOTCH1, -2, -3 and -4) in mammals. They undergo several post-translational modifications in the endoplasmic reticulum and Golgi apparatus such as cleavage by furins and glycosylation that are responsible for the different affinities of the Notch receptors for their ligands (Moloney et al., 2000; Vinson et al., 2016). The extracellular domains of Notch receptors contain a specific number of epidermal growth factor (EGF)-like repeats for each receptor: 36 for NOTCH1 and -2, 34 for NOTCH3 and 29 for NOTCH4. These EGF domains are essential to prevent ligand-independent activation of the receptors and allows homodimerization upon ligand interaction (Sakamoto et al., 2005). EGF motifs are followed by the Negative Regulatory Region (NRR), including a cysteine-rich LIN-12/Notch-related region (LNR) and two heterodimerization domains, named HD-N and HD-C (Fortini et al., 1993). Prior to being located at the membrane, NOTCH proteins are cleaved by furin-like convertases at site 1 (S1), converting the NOTCH protein into a NOTCH extracellular domain/transmembrane and intracellular domain heterodimer linked by non-covalent interactions (Ntziachristos et al., 2014). The NOTCH intracellular domain (ICD) is a transcriptional activator consisting of ankyrin repeats, a RPBj-Associated-Module (RAM) domain, a transactivation domain (TAD), a nuclear localization signal (NLS) and a PEST (proline-, glutamate-, serine- and threonine-rich region) domain modulating protein stability and proteolytic degradation. The structural differences of Notch receptors confer distinct functions, for instance, NOTCH1 ICD is a strong activator of the Hes1 promoter, while the NOTCH3 ICD appears to be a weaker activator and can even repress NOTCH1-dependent HES activation

in certain contexts (Beatus et al., 1999). Though, these distinct effects due to structural differences remain controversial. Indeed, studies by Liu et al., intending to decipher the impact of structural differences of Notch receptors on downstream signals failed to observe a significant structural-based difference between NOTCH1 and NOTCH2 receptors, as the switch in their ICD had no significant effect on carcinogenesis or on the development of organs in which either NOTCH1 or NOTCH2 have dominant roles (Liu et al., 2015).

Ligands of the NOTCH pathway are transmembrane proteins of the Delta/Serrate/LAG-2 family (DSL). In humans, five members of this family are described, three delta-like ligands DLL1, DLL3, and DLL4, and two jagged proteins JAG1 and JAG2 (Meuret et al., 2020). Interaction between the NOTCH receptor of the signal-receiving cell and ligands at the membrane of neighboring cells triggers the unfolding of the Notch regulatory region and unmasks the cleavage sites for ADAM10/17 and the γ -secretase, allowing the release of the NOTCH ICD (NICD) into the signal-receiving cell cytoplasm (Langridge and Struhl, 2017). In the nucleus, NICD binds to the CBF-1/Su(H)/LAG1 (CSL) transcription factor and recruits the transcriptional co-activator mastermind-like (MAML) (Kopan and Ilagan, 2009). The targets of this complex are the HES and HEY genes (also called HESR, CHF, HRT, HERP or gridlock), which encode for transcriptional regulators of the basic helix-loop-helix class which is mainly believed to act as repressors (Fischer and Gessler, 2007). HES and HEY proteins both dimerize to provide an intrinsic transcriptional repression activity. HES activation leads to the recruitment of the corepressor TLE/Groucho, whereas HEY confers marginal repression through the recruitment of the mSin3 complex containing histone deacetylase HDAC1 and the corepressor, N-CoR (Iso et al., 2001; Fischer and Gessler, 2007). The main difference between HES and HEY proteins is the lack of the WRPW tetrapeptide in the HEY proteins, preventing the binding of TLE corepressors.

Actors of Notch signaling, such as Notch receptors (NOTCH1, -2, -3 and -4), Notch ligands (JAG1 and -2, DLL1, -3 and 4), as well as Notch pathway targets (HES and HEY effector proteins) are aberrantly overexpressed in colorectal tumors. More precisely, at least 86% of colorectal cancers and 56% of adenomas display an overexpression of genes in the Notch pathway (Shaik et al., 2020). Moreover, the expression of NOTCH1, -3 and -4 receptors was reported to be significantly higher in colorectal cancers compared to normal and adenoma tissues (Zheng et al., 2015), and patients overexpressing NOTCH3 and NOTCH4 receptors, and the HEY1 transcriptional target, exhibit poorer overall survival (Rallis et al., 2019; Shaik et al., 2020). Finally, a functional crosstalk between Notch signaling the self-renewal regulating pathway Wnt has been well-documented in this cancer (Fre et al., 2009; Pannequin et al., 2009; Rodilla et al., 2009), suggesting a possible role for Notch pathway overactivation during colorectal tumorigenesis and possibly in the regulation of colorectal CSCs.

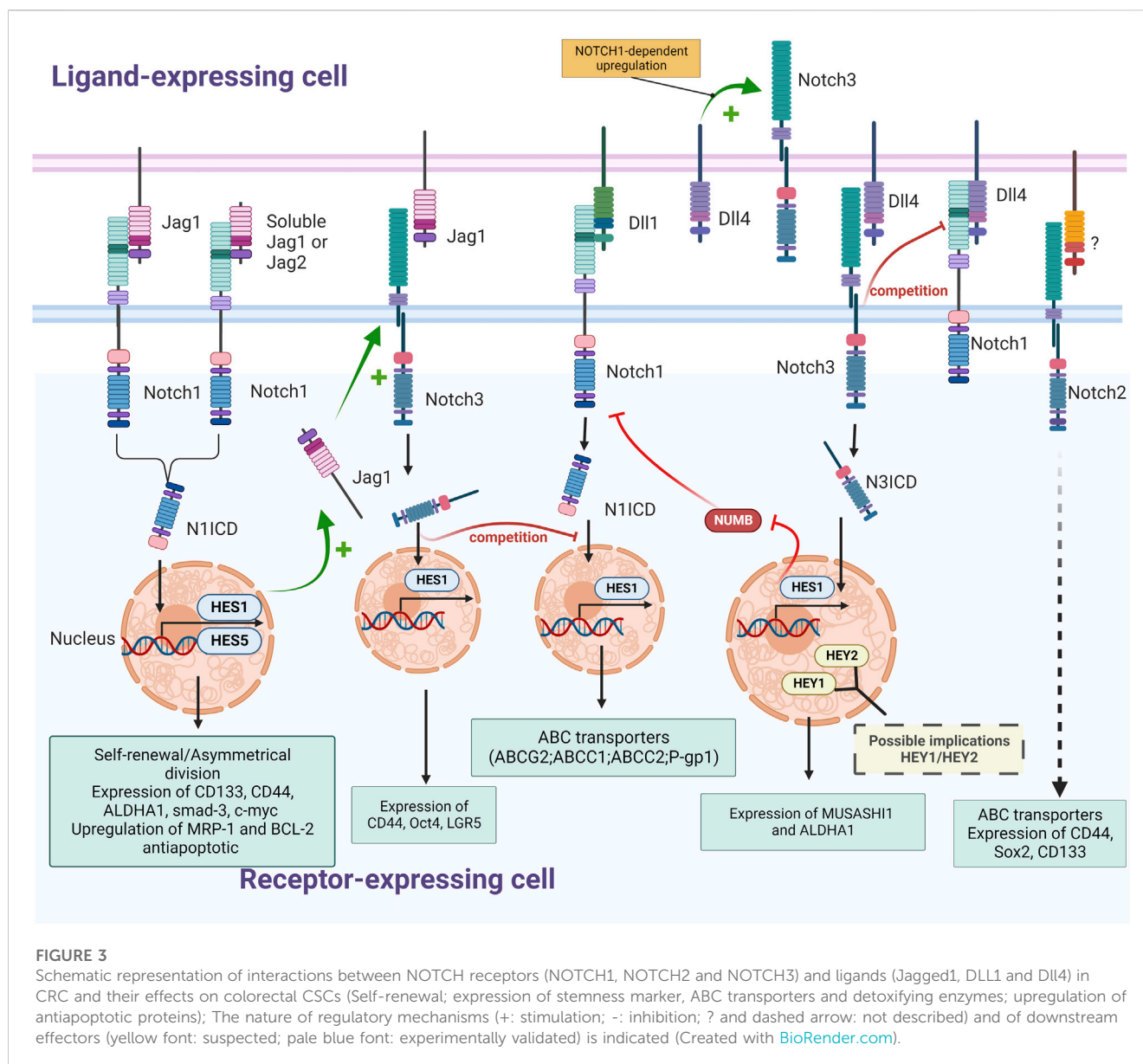
7 Notch pathway and colorectal cancer stem cells

Notch signaling is known to control cell fate decisions and stem-cell phenotypes. Alterations in *NOTCH* genes or in genes that regulate the specific signaling resulting in constitutive activation of the pathways, have been observed in CRC (Reedijk et al., 2008). Activation of Notch pathway was shown to be a key regulator of

stemness, pluripotency and self-renewal in a vast majority of solid cancers, as well as in patients with chemoresistance (Artavanis-Tsakonas et al., 1999; Espinoza et al., 2013; Yuan et al., 2015). Moreover, Notch ligand/receptor specificity was reported to influence tumor heterogeneity. For instance, in small cell lung cancer (SCLC), NOTCH1/DLL1 signaling clearly influence the balance between two populations of cells (Lim et al., 2017). Indeed, endogenous activation of Notch signaling regulates the switch between “non-neuroendocrine” SCLC cells displaying a high expression of HES1 effector, of Notch receptors, CD44 marker and of mesenchymal markers as well as sphere forming abilities, and “neuroendocrine” SCLC cells expressing high levels of DLL4 and the epithelial marker EpCam as well as a stochastic expression of Notch receptors. The non-neuroendocrine SCLC cells are quiescent and exhibit an enhanced chemoresistance, suggesting similarities with CSCs (Lim et al., 2017). The role of Notch in intra-tumoral heterogeneity in solid tumors is therefore known. Considering the importance of CSC subpopulation heterogeneity within a tumor in CRC resistance to treatment and post-treatment recurrence, we hypothesize that Notch signaling and the diversity of its pathways play a role into CSC diversity.

Notch signaling is well-known for its implications in stemness of intestinal crypt progenitors. For instance, the deletion of *NOTCH1* and *NOTCH2* or inhibition of global Notch activation by treatment with a γ -secretase inhibitor triggers colon columnar stem cells to differentiate into goblet secretory cells (van Es et al., 2005; Riccio et al., 2008). The aberrant activation of Notch receptors in CRC, as well as the well-known implications of these pathways in the induction and maintenance of pluripotency in intestinal crypt progenitors, indicate that activation of Notch signaling is involved in the modulation of stemness in CRC cells (Fre et al., 2005; Demitrack and Samuelson, 2016).

In a pathophysiological context, genes of canonical NOTCH signaling components, such as JAG1, JAG2, and NOTCH1 and the target genes of this pathway, notably HES1, HES4, and HES6, are all significantly higher in CSCs (Sikandar et al., 2010). For instance, JAG1/NOTCH1/HES1 signaling plays an important role in the maintenance and viability of CSCs through the inhibition of apoptosis and cell cycle arrest (Figure 3). Non-specific inhibition of Notch pathway via the use of gamma-secretase inhibitors (GSI) like DAPT ((N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) activates the intrinsic apoptotic pathway, causing cleavage of caspase-3 and increases levels of proteins responsible for cell cycle arrest, notably ATOH1, p27, and p57 (Sikandar et al., 2010). Notch is also critical for the intrinsic maintenance of colorectal CSC self-renewal and the repression of secretory lineage differentiation-related genes like *MUC2*, demonstrating its involvement in tumorigenicity of colorectal CSCs (Sikandar et al., 2010). Similarly, treatments with ADAM17 inhibitors such as MEDI3622 or TAPI-2, have shown similar negative effects on self-renewal of colorectal CSCs (Wang et al., 2015; Dosch et al., 2017; Li et al., 2018). Interestingly, a specific inhibition of the delta ligand DLL4 with a neutralizing antibody results in similar defects in colorectal CSCs (Fischer et al., 2011). Indeed, upon treatment with anti-DLL4, colorectal CSCs exhibit reduced self-renewal abilities and higher levels of differentiation promoting the expression of proteins like ATOH1 and CHGA through HES1 upregulation (Table 1).



The global activation of Notch pathway modulates stemness of colorectal CSCs through the repression of differentiation-related genes such as *MUC2* and *ATOH1* along with the inhibition of apoptosis triggered by cell-cycle arrest proteins like p27, fostering the maintenance of self-renewal. However, the effects of Notch signaling in colorectal CSCs go well beyond inhibition of apoptosis. The asymmetric division of slow-cycling and fast-cycling subpopulations of CSCs is NOTCH1-dependent and helps establish CSC heterogeneity by maintaining both slow-cycling, MYC-independent BMI1+ CSCs and fast-cycling, MYC-dependent LGR5+ CSCs (Srinivasan et al., 2016). The unbalanced distribution of NOTCH1 signaling promotes asymmetric BMI1/LGR5 daughter cell fates, which is dependent on the JAG1 ligand and on HES1 and HES5 transcriptional targets of the Notch pathway (Figure 3). This NOTCH1-dependent asymmetrical division has been shown to be directed by epigenetic events such as miRNA, in particular by miR34a which sequesters NOTCH1 mRNA to generate a bimodal

NOTCH1 signal which controls the choice between self-renewal versus differentiation (Bu et al., 2013), and by chromatin methylation of upstream Notch activators genes through H3K27me3 enrichment, which forms repressive chromatin domains upon STRAP silencing (Bu et al., 2013; Jin et al., 2017). Remarkably, treatment with the GSI DAPT results in reduced frequency of BMI1+/LGR5+ both *in vitro* and *in vivo*, demonstrating the importance of Notch pathway activation for the maintenance of heterogeneity and pluripotency in colorectal CSCs (Srinivasan et al., 2016).

The main effectors of the Notch pathway involved in CRC cell stemness seem to be the ligand/receptor couple JAG1/NOTCH1. The effect of JAG1 and NOTCH1 on pluripotency in colorectal cancer cells was observed *in vitro* where constitutive activation of Notch in colon tumor cell lines resulted in increased expression of EMT and stemness associated proteins. Transduction of constitutively active NOTCH1 or treatment with recombinant Jagged-1 led to expression of CD44, Slug, Smad-3, and induction of JAG1 expression (Fender et al., 2015)

TABLE 1 Effect of Notch pathway on CSCs and CSCs-related chemoresistance: Summary of previous studies addressing specific effects of NOTCH ligands and receptors, as well as NOTCH pathway effectors, on CSCs (stemness marker, anti-apoptotic protein and drug-efflux transporters expression), chemoresistance (resistance to drugs and expression of recurrence) and pluripotency (Self-renewal, tumorigenicity, and differentiation), as well as their interaction with other NOTCH pathway members.

Ligand/ Receptor (effector)	Effect on CSCs	Effect on other actors of the NOTCH pathway	Effect on treatment response	Effect on pluripotency
JAG1/NOTCH1	Expression of CD133, CD44, ALDH1, smad-3 (Fender et al. (2015) c-Myc, Gli1 Singh et al. (2022) and EPCAM Lu et al. (2013)	Upregulation of JAG1/Activation of NOTCH3 by JAG1 Fender et al. (2015)	Higher expression of <i>HES1</i> , associated with increased recurrence and poor prognosis after treatment with 5-FU Sun et al. (2017)	Maintenance of pluripotency and heterogeneity in colorectal CSCs through promotion of asymmetric BMI1/LGR5 daughter cell fates Srinivasan et al. (2016)
Soluble JAG1/ NOTCH1	Upregulation of MRP-1 and BCL-2 antiapoptotic proteins Fender et al. (2015), Singh et al. (2022)		Enhanced 5-FU resistance through MRP-1 and BCL-2 antiapoptotic proteins Liu et al. (2016)	Increase of sphere forming abilities Zhao et al. (2020b)
Soluble JAG2/ NOTCH1 (HES1/ HES5)	Modulation of differentiation through CD44, KLR5, SOX9 and NOX1 Rodilla et al. (2009)		Enhanced resistance to Oxaliplatin and SN38 Meng et al. (2009)	Differentiation blockade Rodilla et al. (2009)
DLL1/NOTCH1 (HES1)	Overexpression of drug efflux transporters ABCC1, ABCC2 and P-gp1 Xie et al. (2020)		ABCG2-mediated drug resistance to 5-FU Xie et al. (2020)	
JAG1/NOTCH3 (HES1)	Expression of CD44, OCT4, LGR5 Ying et al. (2015)	Interference with NOTCH1-mediated activation of HES1 through competition for RBP-Jk Beatus et al. (1999)	Enhanced 5-FU resistance Ying et al. (2015)	Increased sphere-forming abilities Ying et al. (2015)
DLL4/NOTCH3	Overexpression of <i>MUSASHI1</i> and <i>ALDH1</i> Okano et al. (2005), Pastò et al. (2014)	Upregulation of <i>NOTCH1</i> through NUMB inhibition Pastò et al. (2014), Choi et al. (2021)		Enhanced clonogenicity and tumorigenicity in colorectal CSCs Serafin et al. (2011)
(HES1/possibly HEY1 and HEY2 Serafin et al. (2011))		Reduction of NOTCH1 activation by DLL4 Serafin et al. (2011)		Maintenance of pluripotency Ying et al. (2015)
/NOTCH2	ABC transporters Jin et al. (2018), Wang et al. (2019)		-	Promotes sphere forming and metastases inducing abilities in colorectal cancer cells Huang et al. (2017), Jin et al. (2018), Wang et al. (2019)
	Expression of CD44, SOX2, CD133, CD26 Apostol et al. (2013), Huang et al. (2017), Jin et al. (2018), Wang et al. (2019)			Differentiation blockade Rodilla et al. (2009)
	Modulation of differentiation through CD44, KLR5, SOX9 and NOX1 (Rodilla et al., 2009)			
DLL4/NOTCH1		Upregulation of NOTCH3 receptor transcription and activation Serafin et al. (2011), Pastò et al. (2014)	-	
DLL4/NOTCH (HES1)	<i>ATOH1</i> and <i>CHGA</i> Upregulation Fischer et al. (2011)		Increased tumorigenicity and of recurrence after irinotecan treatment Hoey et al. (2009)	Promotion of self-renewal and sphere forming abilities Fischer et al. (2011)

(Figure 3). Interestingly, in this study, although NOTCH1 signaling was shown to activate the expression of CD44, Slug, and Smad-3, this was not via JAG1 but rather the consequence of a signaling cascade of other Notch receptors following the induction of JAG1 expression by NOTCH1. The most likely link between JAG1 and CD44 is the NOTCH3 receptor, since the expression of these three proteins are correlated. Moreover, both overexpression of *NOTCH1* and *NOTCH3* results in induction of CD44 and Slug expression (Figure 3). This complementarity between NOTCH1 and NOTCH3 implies that they

may both be needed to mediate stemness in colorectal CSCs with overlapping and distinct roles (Clark et al., 2023). Nevertheless, other studies found a similar effect of the activation of NOTCH1 on self-renewal and stemness markers, but with different markers, including CD44, c-Myc, ALDH1, and Gli1, finding at this occasion a direct link between NOTCH1 and CD44 (Figure 3) (Singh et al., 2022).

JAG1 appears to be the main ligand modulating the Notch-related stemness of colorectal CSCs, for instance in APC-deficient adenomas, *JAG1* deletion in LGR5+ CSCs disturbs stem cell niche

formation, suggesting that plasticity of these cells is highly dependent on JAG1 (Nakata et al., 2017). Though, the specific interactions of JAG1 resulting in enhanced stemness in CRC cells are not currently clear and may be influenced by the microenvironment. It was reported that JAG1 expression in the cytoplasm is correlated with the expression of NOTCH3 in tumor cells, suggesting an interaction between these two proteins (Serafin et al., 2011). Considering that the conversion of bone marrow-derived mesenchymal stem cells into CAFs is dictated by direct contact through JAG1/Notch pathway activation (Peng et al., 2014), it is plausible that Notch pathway modulation plays a key role in converting surrounding cells into colorectal CAFs, modelling the CSC niche. Moreover, other cells than CAFs, such as endothelial cells (ECs), colocalize with CRC cells in perivascular regions and positively influence stemness in CSCs through the excretion of a soluble form of C-terminally truncated JAG1, without any direct contact (Lu et al., 2013). This soluble form of JAG1 originates from a full-length protein cleaved by the protease ADAM17 and is secreted by ECs, increasing the tumorigenic potential of neighboring CRC cells, as well as their self-renewal. CRC cells in contact with this secreted JAG1 also exhibit an increase in the expression of CD133 and EPCAM markers along with an enhanced ALDH activity. Interestingly, following the cleavage of both JAG1 and JAG2 by ADAM17 in CSCs the truncated form of these ligands are released from the extracellular membrane where they promote CSC phenotype through NOTCH1 activation (Figure 3) (Wang et al., 2016). The crosstalk between the different effectors of the Notch pathway takes place within the CSC microenvironment and involves different receptors according to the cell receiving the signal.

However, JAG1 is not the only ligand of the Notch pathway enhancing the stemness phenotype. For instance, DLL4 was shown to enhance stemness through the activation of the NOTCH3 receptor, resulting in the overexpression of the stemness marker MUSASHI-1 (Okano et al., 2005; Bley et al., 2021) and inhibition of the NUMB protein (Pastò et al., 2014; Choi et al., 2021) in both colorectal cancer cell lines and primary cultures of colorectal cancer metastases (Figure 3). Inhibition of NOTCH3 in these cells via a neutralizing antibody or shRNA reduces sphere-forming abilities, whereas DLL4 stimulation enhances stemness features like ALDH1 expression, demonstrating an effect of NOTCH3 activation on stemness and self-renewal in CRC cells. In addition to JAG1 and DLL4, the JAG2/NOTCH pathway also exerts a cancer-promoting effect in colorectal cancer cells, as JAG2 knockdown in colorectal cancer cells inhibits their expression of CD133, decreasing in a similar manner their ability to form spheroids and to induce metastasis (Table 1) (Huang et al., 2017).

Indeed, NOTCH3 was shown to be overexpressed in spheroids derived from colorectal cancer cell lines (WiDr) compared to the parental cell line, confirming the implication of the NOTCH3 receptor in the maintenance of pluripotency in colorectal CSCs (Ying et al., 2015). These results are corroborated by the fact that *NOTCH3* silencing in CRC cells decreases clonogenic capacity *in vitro* and impairs tumorigenicity *in vivo* (Serafin et al., 2011). Interestingly, HES1 is not the only Notch effector suspected of being responsible for this effect since NOTCH3-induced activation or DLL4 stimulation results in a higher expression of HEY1 and HEY2 (Figure 3).

The HES genes cooperatively regulate maintenance and survival of intestinal stem and progenitor cells (Wu et al., 2017; Dzobo et al., 2020). In particular HES1, hypomethylation-linked activation of which leads to tumor cell proliferation and inhibition of differentiation of these tumor cells into intestinal epithelial cells (Kayahara et al., 2003). An *in vivo* study using a Rosa-NOTCH/Cre + mice (Artavanis-Tsakonas et al., 1999; El Marjou et al., 2004; Fre et al., 2005) demonstrated that activation of the NOTCH1 pathway limited the differentiation of intestinal progenitor cells through the activation of HES1 and repression of the mouse atonal homologue Math-1 and neurogenin-3, two proteins coded by essential genes for secretory and enteroendocrine cell lineage specification (Lee et al., 2001; Zine and de Ribaupierre, 2002). In contrast, HEYL negatively regulates metastasis of colorectal tumors in an *in vivo* model seemingly by inhibiting intravasation of metastasis-initiating cells. Interestingly though, HEYL overexpression results in a slight increase in liver metastases after intrasplenic xenotransplantation raising the possibility that HEYL is beneficial for stemness characteristics but detrimental for dissemination of metastatic cells (Weber et al., 2019).

Since Notch pathway mediates pluripotency and proliferation of CSCs, the targeting of Notch signaling might constitute an effective anti-cancer therapy. However, stem and progenitor cells in non-pathological intestinal regions, as well as in tumor cells, undergo differentiation into goblet cells following inhibition of NOTCH1/HES1 signaling, a non-specific targeting of these pathways could cause severe side effects (Fre et al., 2005). Therefore, a strategy to modulate Notch signaling specifically in cancerous cells without affecting healthy cells is needed, which undoubtedly relies on a better comprehension of the fine tuning of Notch pathway.

In conclusion, Notch signaling is a key regulator of stemness and self-renewal in colorectal CSCs. Activation of Notch receptors, notably NOTCH1 and NOTCH3 by their ligands JAG1 and DLL4 results in the repression of differentiation-inducing genes such as *MUC2* or *ATOH1* and inhibition of apoptosis triggered by cell cycle arrest. Members of the HES family, in particular HES1 and HES5, are the main effectors of Notch receptors activation on colorectal CSCs self-renewal, though the HEY effector family is suspected of being partly involved in this phenomenon. Moreover, Notch pathway plays a major role in asymmetrical division of colorectal CSCs and in the maintenance of several pools of pluripotent stem cell. The key implication of Notch signaling in the regulation and maintenance of CRC raises the possibility of its involvement in chemoresistance linked to CSCs and their heterogeneity.

8 Notch ligand-receptor specificity and colorectal cancer stem cells

Many studies have described the role of different Notch actors in the regulation of different stem cell properties. We may therefore wonder if Notch ligand-receptor specificity will affect specific stem cell subpopulations or regulate CSC heterogeneity. Indeed, the heterogeneity in Notch receptors, effectors, and pathways, could shape CSC heterogeneity.

Notch pathway is known to modulate stemness and the tumor initiating phenotype in CRC, though results are variable depending on the study, the model and on the actors of Notch pathway

observed. Notch signaling is often considered to be relatively simple since only a few proteins are involved. However, diverse signals output originates from the combinations of interactions between the different ligands and receptors. The role of Notch pathway in tumors is highly dependent on the spatial and temporal context of Notch activation, as well as on the status of other signaling pathways in the cells. This contextuality is probably the main reason for which studies observing the impact of different receptors on CRC stemness find a global augmentation of the CSC phenotype despite a non-redundancy or even antagonist effect of the different molecular actors involved.

For instance, a mutually exclusive relationship between NOTCH1 and NOTCH2 protein expression was observed in a CRC cohort of 1,003 patients. In this cohort, data supported the antagonistic roles of NOTCH1 and NOTCH2 in phenotypes of colorectal cancer cells, where NOTCH1 acted as an oncogenic enhancer whereas NOTCH2 played a tumor suppressor role (Chu et al., 2011). Despite this apparent antagonistic functions in primary colorectal cancer (Chu et al., 2011), in non-pathological intestinal epithelium, NOTCH1 and NOTCH2 receptors were shown to operate in a redundant manner for the maintenance of pluripotent progenitors in endothelial crypts (Riccio et al., 2008). Indeed, expression of both of these receptors leads to an increase in the transcriptional factor HES1, participating in cell cycling through transcriptional modulation of CDK inhibitors p27^{Kip1} and p57^{Kip2}. This analogous effect of NOTCH1 and -2 has also been found in tumors from Familial Adenomatous Polyposis (FAP) patients (Rodilla et al., 2009) and might be representative of the behavior of these receptors in the conventional adenoma-carcinoma pathway according to several *in vitro* studies (Guilmeau et al., 2010). In this type of colorectal tumors both receptors are activated by JAG1 and participate in blocking differentiation through HES1 but also through several other regulators such as CD44, KLR5, SOX9 and NOX1 (Rodilla et al., 2009).

Studies considering a more exhaustive observation of Notch pathway demonstrated differential expression and specific effects on CSC populations for each receptor. For instance a study revealed that NOTCH1 expression enhanced SOX2 and OCT4 expression, while reducing levels of CD44, whereas NOTCH2 positively influenced all of those factors as well as CD26 (Apostol et al., 2013). NOTCH3 and NOTCH4, may thus decrease CD44 expression, as seen by the upregulation of this marker upon *NOTCH3* and *NOTCH4* knockdown, but have a positive impact on expression of other transcription factors linked to stemness such as cMET, Setmar and CD26 in CRCs. NOTCH2 seems to be the receptor the most expressed in CSCs, followed by NOTCH1, NOTCH3 and lastly, NOTCH4. NOTCH2 expression positively regulates the transcription factors SOX2, OCT4, CD26, CD44 and c-MET, whereas the other receptors have mixed effects on the expression of these factors. Moreover, the link between actors of the Notch pathway can be different according to the presence and absence of partner proteins. For instance, it has been shown that the reduction of NOTCH1 levels inhibited the upregulation of the *NOTCH3* transcript by DLL4 (Serafin et al., 2011). This might be the effect of a direct regulation of NOTCH3 by NOTCH1 activation, as previously shown in leukemia (Palomero et al., 2006; Chadwick et al., 2009) or in some colorectal cancer models (Pastò et al., 2014). However, NOTCH3 ICD might interfere

with NOTCH1-mediated activation of HES1 by competing with the NOTCH1 ICD to access the RBP-J κ transcriptional factor modulated by the Notch pathway (Beatus et al., 1999), and by competing for a common activator, DLL4 (Serafin et al., 2011) (Figure 3). This inhibition of the HES1 activation by NOTCH3 might be circumstantial though, since several *in vitro* and *in vivo* studies demonstrated the activation of HES1 by NOTCH3 (Bellavia et al., 2000; Shimizu et al., 2002; Ye et al., 2022).

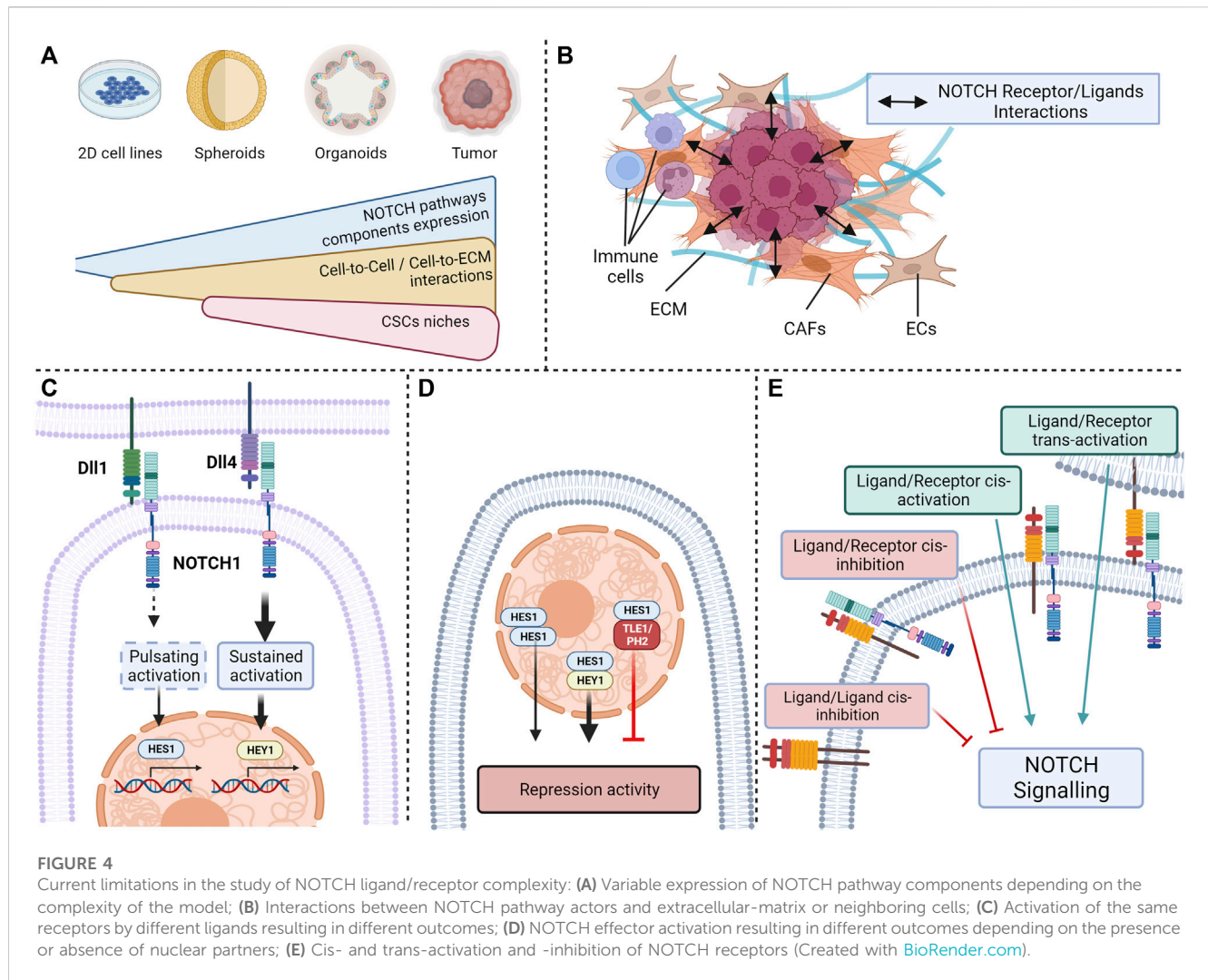
In colorectal cancer cell lines, as well as in primary cultures of colorectal metastases, a positive link between the activation of NOTCH3 and NOTCH1 receptor has been reported. Indeed, NOTCH3 activation via its binding to the DLL4 ligand triggers inhibition of the NUMB protein, known to inhibit NOTCH-dependent gene expression (Choi et al., 2021). Inhibition of the NUMB protein, in turn, enhances the activation of the NOTCH1 receptor which, via a positive feedback loop, increases NOTCH3 expression and activation (Pastò et al., 2014) (Figure 3).

Notch ligands, particularly JAG1 and DLL4, appear to both enhance stemness-related characteristics. Though, many studies on Notch pathway demonstrated in other pathophysiological contexts distinct or even opposite functions of the JAG1 and DLL4 ligands, especially in the modulation of angiogenesis. Indeed JAG1 and DLL4 ligands are both expressed in the embryonic aorta and both can activate the NOTCH1 receptor but leading respectively to the establishment of the hematopoietic and endothelial cell fates (Benedito et al., 2009; Gama-Norton et al., 2015). This ligand-specific lineage differentiation reinforces the existence of a ligand-receptor specificity in Notch signaling. Notch signaling specificity is impacted by Notch ligands crosstalk. An example of such a crosstalk between Notch ligands occurs in the intestinal epithelium, where inhibition of DLL4 or DLL1 does not impact intestinal stem cells, albeit their simultaneous inactivation inhibits pluripotency and results in loss of stem cells (Olfm4+, Lgr5+, and Ascl2+) (Pellegri-net et al., 2011).

HES1 is dominant during embryonic and neonatal stages, whereas the genes of HES-1, -3 and -5 cooperate to regulate adult intestinal homeostasis (Ueo et al., 2012). Though, the presence of LGR5⁺ cells in the intestinal tract of Hes-1, -3 and -5 cKO mice comparable to WT mice indicate that the HES transcription factors are not the only targets of Notch signaling involved in pluripotency maintenance.

Despite the numerous studies demonstrating the different effect of Notch pathway on colorectal CSCs, the crosstalk between the different actors of the Notch pathway and the specific effects they have render studies on the impact of these individual proteins on stemness inapplicable. Few studies focus on several Notch pathway proteins, which is needed to provide a more accurate insight into the impact of Notch pathway on stemness and self-renewal. These limitations and several other parameters need to be considered when exploring the relationship between Notch pathway, stemness phenotypes, CSCs heterogeneity and resistance to treatments.

One of the main limits encountered in studies investigating Notch pathway is the low endogenous activity of Notch signaling in many commonly used CRC cell lines in the absence of cytotoxic chemotherapy (Akiyoshi et al., 2008; Zhang et al., 2008; Meng et al., 2009). Although Notch signaling seems decisive in adenoma formation and CRC tumorigenesis, this



low level of expression does not accurately reflect the interactions and implications of the actors of Notch pathway in tumors (Marconi et al., 2021). Consequently, the impact of Notch pathway modulation was shown to be stronger in three-dimensional models like colorectal spheroids than in parental cell lines (Huang et al., 2015a). Notch signaling being dependent on cell-to-cell interactions or cell-to-ECM interactions, the use of immortalized two-dimensional cell lines is not optimal to observe the complex dynamics of Notch signaling proteins. For instance, a link between Notch signaling, more precisely on HEY2 expression and the tenascin-C, an hexameric glycoprotein from the ECM binding to fibronectin, periostin and integrins, was described (Oskarsson et al., 2011). Indeed, tenascin-C protects HEY2 transcriptional expression from inhibition STAT5, enhancing stemness phenotype and pluripotency of metastatic breast cancer cells. This positive effect of the ECM protein tenascin-c on Notch signaling and stemness has also been reported in glioma, this time on expression of JAG1, ADAMTS15, and activation of NOTCH1 and NOTCH2 receptors (Sarkar et al., 2017). Such a relationship between ECM components, Notch pathway and stemness necessitates models able to reproduce these

interactions, which is not the case of most 2D models. This statement about the use of two-dimensional cell lines is relevant for the studies of Notch signaling on stemness and self-renewal, given the major implications of the microenvironment and of the interactions between cells in these features (Le et al., 2008; Farahani et al., 2014; Avnet and Cortini, 2016; AlMusawi et al., 2021). A solution explored by several teams is the preferential use of three-dimensional models such as organoids or spheroids that are more suitable for studying complex interactions (Huang et al., 2015a; Höglström et al., 2018; Jackstadt et al., 2019). For instance, organoids generated from induced pluripotent stem cells can reproduce the transient expression patterns of HES1, reported in embryonic stem cells and deemed critical for physiological Notch signaling (Shimojo et al., 2008; Trujillo et al., 2019; Marconi et al., 2021). Such models are more amenable to reproducing the complex interactions between the molecular actors of these pathways occurring within the cell and with its microenvironment (Figure 4A).

Another parameter from the cell microenvironment that could explain the ligand/receptor specificity observed in Notch pathway is the crosstalk between tumor cells and surrounding

cells such as endothelial cells, CAFs or immune cells (Figure 4B). Such an example was given above with the effect of soluble JAG1 secreted from endothelial cells on CSCs (Lu et al., 2013). These interactions between CAFs and CRC cells could partially explain differences in Notch activities observed according to the localization of tumor cells. Indeed, in a similar manner to pluripotent cells, disparities of Notch activation have been observed between cells populating the edge of the tumor, with low signs of NOTCH activation, and cells originating from the center, with a high expression of NOTCH1 ICD (Schmidt et al., 2018). Regulation of Notch signaling by the tumor architecture, in this case the activation of *HEYL* transcription by JAG1 and JAG2 expression in neighboring cells, is also observed in other types of cancers, such as basal cell carcinoma (Eberl et al., 2018) and might be an important criterion in CRC. In addition, immune cells may drive Notch signaling at the edge of the tumor, as for breast cancers, where the stimulation of JAG1/NOTCH interactions by cytokine like IL-6 triggers a partial mesenchymal phenotype and an increase in stem-like characteristics (Bocci et al., 2019). Another example of this crosstalk between CSCs and immune cells is the effect of mammary stem cell-DLL1 on adjacent macrophages observed in the mammary gland, where NOTCH1, -3 and -4 activation in macrophages is triggered by this interaction results in the production of Wnt ligands stimulating pluripotency in mammary stem cells (Chakrabarti et al., 2018).

Furthermore, it was recently described that the different ligands of the Notch pathway can mediate distinct targets through their interactions with a common Notch receptor. For instance, Nandagopal et al. demonstrated by quantitative single cell imaging that DLL1 and DLL4 ligands have different effects on cell fate through their activation of the NOTCH1 receptor (Nandagopal et al., 2018). DLL1 triggered a pulsating activation of the NOTCH1 receptor, resulting in the activation of the HES1 transcriptional target, whereas DLL4 led to a sustained activation of the receptor causing HEY1 activation, these targets inducing specific cell fates. The dynamics of NICD production is a decisive parameter for signals triggered by a Notch receptor, but the intensity of the signal has also been shown to influence downstream pathways and to be dependent on the ligand (Figure 4C). A good illustration of this factor is the difference in the strength of signal triggered by Jag1 and Dll4 on hematopoietic stem cells (HSC) showed by Gama-Norton et al. with Notch1 activation trap mouse models with different sensitivity (Gama-Norton et al., 2015). Jag1 stimulates a low activation of Notch1 receptor, whereas Dll4 triggers a stronger Notch1 activation. This antagonistic relationship between jagged and delta ligand receptors based on the strength of Notch signaling has been observed in several physiological features (Benedito et al., 2009; Petrovic et al., 2015) and may be implicated in stemness.

The patterns of expression of effector proteins HEY and HES also seem to be crucial in outcomes triggered by Notch receptors. HES and HEY can be individually expressed in some cells, but they can also be co-expressed upon Notch receptors activation. HES and HEY homodimers and heterodimers bind similar DNA sequences, but with different repression activities levels (Leimeister et al., 2000; Iso et al., 2001; Van Wayenbergh et al., 2003; Kageyama et al., 2007).

In addition, other transcriptional regulators such as TLE1 or PHB2 can bind to HES1, thus repressing complexes (Rohena-Rivera et al., 2018). The complexity of these effectors results in different phenotypical outcomes triggered by Notch pathway (Figure 4D).

Finally, the plurality of interactions between the receptors and ligands is a confusion factor that may impact Notch pathway effect on stemness-related signaling. Indeed, different interactions between Notch receptors are possible depending on the cell expressing the ligand. Ligand/receptor trans-interaction, *i.e.*, between neighboring cells, results in classical trans-activation, whereas ligand/receptor cis-interaction, *i.e.*, within a cell, can also occur (LeBon et al., 2014). These particular types of interactions can either result in Notch receptor cis-activation (Formosa-Jordan and Ibañez, 2014; Nandagopal et al., 2019) or Notch cis-inhibition (del Álamo et al., 2011; Miller et al., 2009). Cis-activation has been observed through single molecule HCR-FISH detection of Notch targets (Nandagopal et al., 2019), whereas cis-inhibition, showed by Miller et al. using genetically modified *drosophila* models, decreases the capacity of a cell to receive signals from neighboring cells (Meir et al., 2002). In contrast, cis-activation affects the survival of neural stem cells, suggesting that self-renewing cells can sustain themselves, activating their own Notch signaling, in particular NOTCH1 and NOTCH2, in an autocrine manner (Nandagopal et al., 2019). In addition to these ligand/receptor interactions, ligand/ligand interactions have been observed through mutational analysis by Chen D et al., and highlight a type of cis-inhibition which is triggered by ligand dimers (Chen et al., 2023). Together, these finely balanced mechanisms dictate the strength, the flow, and the specificity of Notch signaling. This multitude of potential interactions complexifies the link between the expression of Notch receptors and ligands, and the resulting signaling on CSCs and has to be considered (Figure 4E).

Signaling resulting from the Notch pathway is a complex network of different receptors and ligands, the interactions of which coordinate distinct outcomes through activation of various effectors, underlying ligand and receptor specificity of the Notch pathway in colorectal CSCs. This complexity relies on interaction between CSCs, the microenvironment and neighboring cells, as well as the numerous types of interactions between the different actors of Notch pathway. This complex intricacy might be the reason, at least partially, contradictory results obtained by studies attempting to decipher the role of Notch pathway in colorectal CSCs. Though, another explanation is plausible, indeed, the multiple effects observed of the different Notch pathway actors on CSCs might be the reflection of Notch signaling impact on CSCs heterogeneity. As previously mentioned, CSC clusters exhibiting various phenotypic traits co-exist in CRC tumors, the regulation and tuning of these different clusters may be directly regulated through the ligand/receptor specificity of pathways such as Notch, as suggested by the asymmetrical division of slow- and fast-cycling CSCs directed by distribution of NOTCH1. Approaches such as genome-scale studies (Guruharsha et al., 2012), spatial transcriptomics (Cang et al., 2023), and computational modeling such as agent-based model (Reynolds et al., 2019) could be used to better determine the complexity of Notch pathway in adequation with CSCs heterogeneity.

9 Notch pathway ligand-receptor specificity and colorectal CSC: implications for post-treatment recurrence and chemoresistance

Similarities have been found between colorectal CSC-enriched cell lines via 3D-colonosphere culture and chemoresistant CRCs. Despite differences in these populations, these techniques have both been shown to enhance the proportion of CSCs (Vinogradov and Wei, 2012). Colon cancer spheroids and chemoresistant CRC cells both express the stemness markers, CD133 and CD44, and exhibited equivalent phenotypes. Notch signaling, notably the NOTCH1 pathway, was reported to play a major role in colorectal spheroids and in chemoresistant cell lines, suggesting that Notch activation is involved in maintaining a phenotypic characteristic common to spheroids and chemoresistant cells (Huang et al., 2015b). As presented above, cells able to self-renew such as CSCs, TICs and DTP exhibit an increased resistance to treatment and numerous studies present a direct link between Notch-dependent stemness in these cells and their resistance to treatments. The main reported associations between specific Notch ligand-receptor pairs, self-renewal and treatment response are summarized in Table 1.

For instance, HES1, often considered as the best marker of Notch pathway activation, is highly expressed in stage II CRC patients with higher recurrence rate and poor prognosis after treatment with 5-FU. These effects were reproduced *in vitro*, where cell lines overexpressing HES1 display higher 5-FU resistance (Sun et al., 2017). HES1 expression and the resulting chemoresistance is correlated with EMT signaling and increased expression of drug efflux transporters like ABCC1, ABCC2 and P-gp1 (Figure 3). This effect of HES1 on ABC transporters might be modulated by the DLL1-activation of NOTCH1 receptor. Indeed, the DLL1/NOTCH1 couple modulate the ABCG2-mediated drug resistance to 5-FU in colon cancer side population cells, which are a small subpopulation of cells exhibiting stemness characteristics and enhanced drug resistance (Figure 3) (Xie et al., 2020).

Indeed, NOTCH1 activity was shown to intensify resistance of CRC cells to 5-FU through the upregulation of MRP-1 and BCL-2 antiapoptotic proteins (Liu et al., 2016) (Figure 3). Moreover, the NOTCH1 pathway is linked to resistance to other drugs, as its activation, presumably by JAG1, is associated with resistance to Methotrexate, an inhibitor of dihydrofolate reductase used as a chemotherapeutic agent in colorectal cancer. This resistance is associated with stem-cell like characteristics such as an increased expression of markers CD166, CD26, CD44 and CXCR4, as well as enhanced sphere forming abilities (Zhao Q. et al., 2020). NOTCH1 activation is involved in resistance to other therapeutic agents such as oxaliplatin, 5-fluorouracil (5-FU), or SN-38 (the active agent of irinotecan treatment) (Meng et al., 2009). Indeed, these therapies all lead to an over-activation of NOTCH1 characterized by an increase in NICD and HES1 protein levels due to an increase in gamma-secretase activity.

Several studies reported a negative correlation between NOTCH2 expression in tumor and tumor stage, as well as differentiated carcinomas were shown to harbor a higher NOTCH2 expression compared to poorly differentiated ones (Chu et al., 2009), suggesting that NOTCH2 may be a marker of differentiation rather than of

stemness. However, NOTCH2 signaling has been shown to induce the expression of stemness-related genes such as CD133 and SOX2, to promote resistance to chemotherapies through the expression of drug transporters like ATP-binding cassette and enhance sphere forming abilities of CRC cells (Jin et al., 2018; Wang et al., 2019) (Figure 3). The implication of NOTCH2 in chemoresistance and pluripotency of CRC cells remains ambiguous and necessitates further research.

In the case of NOTCH3, its suppression *in vitro* leads to a decrease in sphere-forming abilities, in OCT4 and LGR5 expression, and an increased sensitivity to 5-FU treatment (Ying et al., 2015) (Figure 3). Interestingly, in this model, the inhibition of NOTCH1 had no effect on stemness characteristics, such as sphere forming properties, expression of stemness markers or resistance to treatment, suggesting that the implication of Notch receptors and actors of the Notch pathway in stemness-linked chemoresistance is highly contextual.

Although the precise implications of each protein of Notch signaling in chemoresistance remains elusive, evidence indicates that Notch pathway activation results in enhanced chemoresistance in CRC (Vinson et al., 2016). The use of GSI has a synergistic effect with most therapeutic agents used for the treatment of CRC including oxaliplatin, SN-38, and 5-FU (Meng et al., 2009), demonstrating that the overall activation of Notch pathway results in resistance to common chemotherapies used in CRC. In the specific context of stemness-linked chemoresistance, treatment of colorectal CSCs with GSI in combination with irinotecan results in a decrease in stemness marker, like ALDH, and a significant inhibition of tumor regrowth following treatment in a fraction of CRC tumors exhibiting high activation of Notch pathway (Arcaroli et al., 2012).

The use of GSI also enhanced mitotic arrest and apoptosis of colon cancer cells following taxane treatments like paclitaxel (Akiyoshi et al., 2008). However, this effect of gamma-secretase is believed to be independent of the Notch pathway since silencing of genes coding for NOTCH1, -2 and -3 receptors does not improve paclitaxel efficacy.

As mentioned above, DLL4 has a positive impact on stemness-related characteristic and its inhibition, for instance via a neutralizing antibody, results in the reduction of self-renewal and of tumor growth *in vivo* (Fischer et al., 2011). However, when combined with irinotecan, these effects are exacerbated and anti-DLL4 enhances cell death triggered by irinotecan by reducing levels of anti-apoptotic genes, such as HSPA6, and enhancing expression of proapoptotic genes like PDCD4. Blockade of DLL4 was reported to result *in vivo* in a decrease in tumorigenicity and of recurrence after treatment with irinotecan (Hoey et al., 2009).

Targeting the Notch pathway has emerged as a promising strategy for addressing two critical aspects of colorectal cancer treatment: chemoresistance and tumor recurrence. Chemoresistance poses a significant challenge in cancer therapy, leading to treatment failure and recurrence. In this context, the Notch pathway plays a pivotal role, particularly in colorectal cancer stem cells (CSCs), which contribute to treatment resistance and tumor relapse.

Studies have revealed that Notch signaling promotes the survival and maintenance of chemoresistant CSCs. It has been shown for instance that an elevated JAG1 expression was associated with poorer prognosis and chemoresistance in CRC patients (Kim et al., 2019). By inhibiting the Notch pathway, it is possible to restore sensitivity to chemotherapy agents, thereby overcoming

chemoresistance and improving treatment outcomes. Targeting Notch disrupts CSC self-renewal, enhances drug-induced cell death, and sensitizes CSCs to the cytotoxic effects of chemotherapy drugs (Takebe et al., 2015; Morel et al., 2017).

Preclinical studies have explored the combination of Notch pathway inhibitors with conventional chemotherapy agents to overcome chemoresistance and prevent recurrence. For instance, combining gamma-secretase inhibitors (GSIs) with chemotherapeutic agents, such as 5-fluorouracil (5-FU), has demonstrated synergistic effects in reducing tumor growth and overcoming resistance in colorectal cancer models (Meng et al., 2009). Inhibition of Notch signaling sensitizes CSCs to the cytotoxic effects of 5-FU, leading to enhanced cell death and improved treatment response. Simultaneously, it hampers the regrowth and self-renewal potential of residual cancer cells, minimizing the risk of recurrence (Strosberg et al., 2012).

Clinical trials investigating the combination of Notch pathway inhibitors with chemotherapy in chemoresistant colorectal cancer and its impact on recurrence prevention are underway (Takebe et al., 2014). Targeting the Notch pathway not only addresses chemoresistance but also holds potential in reducing the likelihood of tumor recurrence, offering a comprehensive therapeutic strategy for colorectal cancer patients (Suman et al., 2014).

In summary, Notch signaling generally leads to a higher expression of detoxifying enzymes such as ALDH, drug efflux transporters like ABCC1 and ABCC2, as well as an upregulation of anti-apoptotic genes and proteins like *PDCD4*, *HSPA6*, *BCL-2* and *MRP-1*. The protection of self-renewing cells following Notch pathway activation upon treatment leads to CRC recurrence and may be targeted to limit this event. The main contributors to this phenomenon are NOTCH1 and NOTCH3 receptors, the role of NOTCH2 being incompletely understood. Most of the Notch ligands studied seem to enhance chemoresistance, *DLL1*, *DLL4* and *JAG1* being the principal activators in this event. Moreover, the asymmetrical division of CSC subtypes orchestrated by Notch pathway contributes to the constitution of a slow-cycling CSC population, contributing to a pool of resistant stem cells.

Though, the studies exploring the implications of Notch pathway on chemoresistance and recurrence suffer from the same disparities as studies on CSCs. Indeed, as we have seen, Notch pathway components all have different effects on chemoresistance and CSCs related recurrence. This might be an indication of the regulation of CSCs different clusters through the ligand/receptor specificity of Notch pathway.

10 Conclusion

CSCs have been extensively studied in the context of chemoresistance and recurrence after treatment, these cells being considered as the main reasons for tumoral heterogeneity, and the ensuing resistance and recurrence. Indeed, these subpopulations exhibit features allowing multi drug resistance such as their proliferative quiescence, the high expression of DNA damage repair proteins, of detoxifying enzymes and of ABC transporters.

NOTCH pathway activation seems to be a key regulator of stemness as well as chemoresistance in CRC. Despite the lack of precise mechanisms linking NOTCH pathway activation to these features, an increasing amount of evidence supports the implication of NOTCH pathway proteins in stemness and chemoresistance via the modulation of asymmetric division, cell-cycle, and expression of anti-apoptotic proteins, ABC transporters and detoxifying enzyme. In addition to being an architect of intra-tumoral heterogeneity through its control of CSC asymmetrical division, the NOTCH pathway is a direct regulator of CSCs-related chemoresistance.

Though, considering the heterogeneity of CRC cells able to self-renew and the numerous molecular actors modulating NOTCH pathway, several parameters need to be taken into consideration to gain further insight into the implication of NOTCH pathway in these events and obtain reliable studies. Among these parameters, the microenvironment, and the general context in which NOTCH pathway proteins act (expression, interactions partners, and localization) are critical. A better characterization of these pathways, of interactions between these molecules, and of their implications in colorectal CSCs would allow the development of drugs targeting specific branches of the NOTCH pathway to be used in combination with commonly used chemotherapy or targeted therapies to prevent chemoresistance and recurrence in colorectal cancer.

Thus, to reconcile conflicting results between studies addressing the impact of NOTCH on stemness and recurrence in CRC, we argue that additional work should take into consideration NOTCH ligand/receptor specificity and its complex impact on CSC heterogeneity.

Author contributions

MB and FH wrote the manuscript drafts, PM and OM provided information about all aspects and reviewed/commented on draft manuscripts. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor JP declared a past co-authorship with the author FH.

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B. Notch3 in cancer

Despite sharing some structural similarities with other Notch receptors, Notch3 exhibits some differences notably a shorter intracellular domain. This difference may potentially be involved in the differential regulation of Notch3 intracellular domain cleavage. Notch3 is expressed in vascular smooth muscle cells [224], in the nervous system [225], and in regulatory T cells[226]. Notch3 has been recognized as a dependence receptor even though the mechanism of the apoptosis triggered by this receptor has not been identified yet[227].

The dysregulation of the Notch3 receptor has been extensively investigated from a pathological perspective, particularly in its relevance to CADASIL (Cerebral Autosomal Dominant Arteriopathy with Sub-Cortical Infarcts and Leukoencephalopathy), an inherited vasculature disease, and in tumorigenesis.

Notably, NOTCH3 is implicated in the stem cell phenotype of cancer cells. In ovarian cancer, the expression of NOTCH3 is associated with recurrence and poor clinical outcomes, directly influencing the stemness of cancer cells. The intracellular domain of NOTCH3 increases the expression of various genes, including Oct4, Nanog, Klf4, Rex1, Sall4, Rif1, NAC1, and the ATP-binding cassette ABCB1 [228]. Activation of the Notch3 pathway in cancer cells also contributes to the development of chemo-resistance, as observed in hepatocellular carcinoma (HCC) clinical specimens where the presence of CAFs and NOTCH3 expression correlate with lower patient survival. LSD1, a demethylase, is upregulated in CAFs and serves as an upstream driver of Notch3-mediated cancer stem cell self-renewal in HCC [229]. Moreover, cells expressing NOTCH3 exhibit characteristics of CSCs, such as higher ALDH activity and the ability to differentiate into cells that do not express NOTCH3. This may result from NOTCH3 inhibiting β -catenin, leading to increased Nanog expression and a higher CSC population in HCC cells [230].

The role of NOTCH3 in the EMT of cancer cells is not fully understood. In breast cancer cells, NOTCH3 inhibits EMT by activating GATA-3 or upregulating Kibra protein, activating the Hippo/YAP pathway in breast cancer epithelial cells. Conversely, NOTCH3 promotes EMT in ovarian cancer by enhancing ERK activation and in non-small cell lung

(NSCL) bone metastasis by upregulating Zeb-1 [230-232]. Interestingly, the effects of NOTCH3 pathway activation on EMT may vary depending on the type of cancer cell, acting as a tumour suppressor in small cell lung cancer (SCLC) and promoting EMT in NSCLC [233].

NOTCH3 is directly linked to its effects on tumoral angiogenesis. In human lung cancer, NOTCH3 is upregulated in tumour endothelial cells and limits tumour angiogenesis through its pro-apoptotic activity. The expression of Jag-1 by cancer cells during this process hinders apoptosis associated with the dependence receptor function of NOTCH3 [227]. However, in human tongue Oral Squamous Cell Carcinomas (OSCCs), approximately 30% of cases exhibit NOTCH3 receptor expression in CAFs, directly correlating with micro-vessel density in cancer stroma and tube formation [234]. Furthermore, NOTCH3 is essential for the endothelial-dependent differentiation of mural cells, vascular smooth muscle cells, and pericytes, playing a crucial role in vascular development and stability [235].

The NOTCH3 receptor, along with its downstream pathway PKC θ , cooperates with the NF κ B pathway to regulate FoxP3 transcription [236]. Additionally, NOTCH3 has an impact on macrophage polarization, with its inactivation during monocyte-to-macrophage differentiation leading to decreased transcription levels of inflammatory genes and reduced activation of AKT, MAPK, and NF κ B pathways [237]. In the context of tumours, there is a correlation between NOTCH3 and PD-L1 expression in breast cancer, particularly in breast cancer stem cells. NOTCH3 activation positively regulates PD-L1 expression through its downstream effects on the mTOR transcription factor [238].

Notch3 plays a significant role in cancer, particularly in influencing cancer stem cell properties, promoting, or inhibiting EMT, and impacting tumoral angiogenesis. Notably, its activation can either act as a tumour suppressor or contribute to tumorigenesis, depending on the specific type of cancer.

The dependence receptor characteristic of NOTCH3 is crucial, as it means that its activity can lead to either cell survival or apoptosis, depending on the presence or absence of its ligands. This dual role makes NOTCH3 an intriguing subject for study, as it can exert diverse effects on cancer progression and treatment response.

In the context of mCRC, studying NOTCH3 becomes particularly interesting due to its potential involvement in cancer stem cell regulation, EMT modulation, and angiogenesis. Understanding how NOTCH3 functions in CRC cells may provide insights into the mechanisms underlying metastasis, drug resistance, and overall disease progression. This knowledge could contribute to the development of targeted therapies and personalized treatment approaches for patients with mCRC.

In the context of cancer treatment, a deeper understanding of dependence receptors and their apoptotic signalling pathways could be therapeutically beneficial. These receptors, finely tuned to ligand presence or absence, have a dual influence over cellular processes, crucially impacting both survival and programmed cell death. Investigating these pathways holds potential for designing targeted therapies that exploit the apoptotic signals triggered by dependence receptors.

V. Dependence receptor-mediated apoptosis - a regulated destruction of the cell

A. General description of apoptosis

The term apoptosis was used for the first time by Kerr et al [239] to describe cell death resulting in a particular morphology: a rounding of the cell accompanied by pseudopods retraction, cytoplasm volume diminution, chromatin condensation (pyknosis), nucleus fragmentation (karyorrhexis), and plasma membrane sprouting leading to the formation of apoptotic bodies. From a biochemical point of view, apoptotic cells exhibit many changes, such as DNA fragmentation, as well as protein cleavage and cross-linking.

However, the plasma membrane retains its integrity steps in the process, and no major structural changes to the organelles are observed. The apoptotic bodies are quickly recognized and absorbed by the neighbouring phagocyte cells and then digested through their lysosomal activity [240]. The caspase family (Cystein ASPartyl protease) are cysteine proteases present in the cell constitutively in an inactive form (procaspase

or zymogene). During the apoptotic process, these caspases are cleaved and activated, and trigger an activation cascade of other caspase types, resulting in cell death. This post-transcriptional activation allows fine-tuning and rapid regulation of this enzyme and underlying mechanisms. From a structural point of view, the caspases are composed of a peptide with 3 domains: a pro-domain N-terminal, a large subunit p20 and a small subunit p10. After cleavage targeting an aspartic acid (Asp), procaspase is converted into an active form.

Once activated, the caspase can then cleave and activate another caspase in return. The caspase family is divided into three main groups, based on their functions and on the similarity of their sequences [241]: the inflammatory caspases (1, 4, 5 and 11), the effectors caspases (3, 6 and 7), and the initiators caspases (2, 8, 9, 10 and 12). Even though the caspase family is usually associated with proapoptotic and inflammatory phenomena, their involvement in numerous other processes such as stem cell differentiation [242]) or neuronal development has been demonstrated [243]. However, the processes by which these cells modulate a transient caspases activation remain still mostly unknown. At the level of the organism, the ability to trigger the death of cells composing the tissues in a specific manner is essential for shaping and maintaining physiological structure through the elimination of damaged or unnecessary cells. On the other hand, apoptosis is involved in the modulation of wound healing during major tissue remodelling as during the involution of the breast gland following lactation [244]. Apoptosis is also used as a detoxifying mechanism in the liver, where hepatocytes accumulating toxins are eliminated and release cytokines to stimulate the proliferation of new hepatocytes leading to liver regeneration [245].

The mechanisms of apoptosis are very complex and involve a cascade of energy-dependent molecular events. Currently, the scientific community tends to separate apoptosis into two main signalling pathways: the extrinsic pathway (death receptor pathway) and the intrinsic pathway (mitochondrial-dependent pathway). However, there is also evidence that these two signalling routes are linked and interact at several levels [246]. The extrinsic apoptotic pathway, mainly modulated by caspase 8, is induced by the activation of transmembrane receptors, following extreme disturbances of the

cell microenvironment [247]. Among the most commonly described of these receptors, we find the FAS receptor (also known as CD95 or APO1) [248], or the superfamily of TNF receptors, composed notably of the TNFR, TRAILR1 and TRAILR2 receptors [249]. Whereas the intrinsic pathway of apoptosis is mediated by intracellular signals triggered by various micro-environmental disturbances. These disturbances include DNA damage, endoplasmic reticulum stress, reactive oxygen species overexposure (ROS), growth factor deprivation, or replicative stress [240, 250, 251].

These disturbances cause the activation of pro-apoptotic proteins, the BH3-only proteins, effector of apoptosis. These pro-apoptotic proteins are modulated through direct interaction by anti-apoptotic proteins such as Bcl-2, Bcl-XL or Mcl-1, which prevent their pro-apoptotic action from acting as a competitive inhibitor. If these anti-apoptotic proteins reach saturation, BH3-only proteins can bind and activate BAX (BCL-2 associated X protein) and/or BAK (BCL-2 antagonist/killer). Once activated, BAX and BAK oligomerize and form pores triggering the permeabilization of the outer membrane of the mitochondria (or MOMP, for Mitochondria Outer Membrane Permeabilization). Once the outer membrane of the mitochondria is permeabilized, apoptotic proteins such as the Cytochrome C, SMAC (Second Mitochondrial-Derived Activator of Caspases) and protease serine OMI, are released in the cytoplasm leading to the formation of the apoptosome, a protein complex used as an activation platform for caspase 9. The activated caspase 9, in turn, cleaves the caspase effectors 3 and 7 to trigger the cell death process [252].

B. Apoptosis in cancer

The involvement of apoptosis as a mechanism of tumour prevention was discussed already in 1972 by Kerr et al [239], described as a mechanism operating the elimination of overly proliferative cells or developing in inappropriate sites. Since then, numerous observations have shown that escaping apoptosis is a key step for tumour escape and progression, and the ability to suppress apoptosis is considered a major hallmark of cancer [253].

Many checkpoints triggering cell death are present in the cell to detect stress related to hyper-proliferation, oncogenic activity or genomic instability, such as DNA damage or

reticulum stress endoplasmic. In addition, some transcriptional programs associated with proliferation, like the one orchestrated by MYC, also provide additional control by inducing a pro-apoptotic transcription [254].

Apoptosis is, therefore, an essential barrier, that requires the acquisition of selective advantages to progress toward tumoral phenotype. A good indicator of the importance of escaping cell death for tumour cells is the mutation of the tumour suppressor p53, the main apoptotic regulatory protein, which is found mutated in more than 50% of tumours [255]. Moreover, in numerous tumours, especially those resistant to therapies, there is an increase in antiapoptotic proteins of the Bcl-2 family in response to selective pressure provoked by apoptosis triggered by mutations and stress encountered by the tumour cell [256].

These observations highlight a major fact in cancer treatment. Although many stress sensing mechanisms such as p53 are inactivated in tumours, making them resistant to cytotoxic agents such as chemotherapy, the heart of the apoptotic machinery is still functional, which allows some innovative therapies through targeting this machinery.

C. Regulation of apoptosis: focus on the dependence receptors

In the realm of dependence receptors, the Trk family, encompassing TrkA, TrkB, and TrkC, stands out as tyrosine kinase receptors with dual roles. Despite primarily acting as neurotrophin receptors crucial for neuron survival, TrkA and TrkC have been identified as dependence receptors [257, 258]. The absence of nerve growth factor (NGF) or neurotrophic factor 3 (NT-3), respectively, leads to distinct apoptotic mechanisms. TrkA, when deprived of NGF, triggers ERK phosphorylation and caspase-7 activation, culminating in PARP cleavage and cell death [259, 260]. On the other hand, TrkC exhibits an unusual pathway; NT-3 withdrawal prompts internal cleavage, translocation of the intracellular fragment to the mitochondrial membrane, and activation of apoptosome, resulting in cell death [261].

Some representatives of tyrosine kinase receptors acting as dependence receptors, ALK and c-MET, have emerged [262, 263]. ALK, associated with anaplastic large cell

lymphoma, undergoes cleavage upon caspase 3 action, exposing a proapoptotic region and enhancing apoptosis, while ALK activation may decrease apoptotic signalling. The c-MET receptor, recently recognized as a dependence receptor, engages with hepatocyte growth factor (HGF) [264]. Withdrawal of HGF induces MET cleavage, yielding a 40 kD intracellular fragment, p40MET, which triggers calcium transfer to mitochondria, leading to mitochondrial permeability transition pore (mPTP) opening and subsequent cell apoptosis [265, 266].

Semaphorin receptors, crucial in nervous system development, have another representative of dependence receptors among them. Indeed, Sema3E, binding to PlexinD1, suppresses tumour cell death. Upon Sema3E withdrawal, PlexinD1 interacts with the nuclear receptor NR4A1, initiating a cascade that induces cell death [267, 268].

In recent years, additional receptors adopting the dependence receptor paradigm have been unveiled. NOTCH3, critical for vascular smooth muscle cell stability, operates as a dependence receptor, although its apoptotic induction mechanism remains unknown [227, 269]. Kremen1, a Wnt- β catenin pathway regulator, exhibits dependence receptor behaviour, with homodimerization inducing cell death [270, 271]. Finally, the KIT receptor, activated by Stem Cell Factor (SCF), undergoes caspase-induced cleavage in the absence of SCF, triggering a cascade of apoptosis in various cancer cell lines [272-274].

These receptors also show similarities in their way of triggering apoptosis. First, for most of the receptors, the monomerization of the latter in the absence of a ligand seems to be the starting point of the apoptotic cascades. Indeed, the monomerization of these receptors triggers the activation of different apoptotic protein partners. Those partners can be caspases, such as Caspase 2 for p75NTR [275], Caspase 7 for TrkA [260], or Caspase 8 and 9 for EphB3[276], or other apoptotic proteins such as the Death associated protein kinase DAPK1 for UNC5B[200] or Cobra-1(cofactor of BRAC1) for TrkC [261]. But it seems that there are some exceptions to this mechanism. For instance, it has recently been found that it's the multimerization of Kremen1, whether than its monomerization, that is triggered by the withdrawal of its ligand DKK1[272]. It's also possible that the apoptotic cascade does not include pro-apoptotic partners. For the c-

MET receptor, following its monomerization upon HGF withdrawal, c-MET is cleaved and yields a 40 kD intracellular fragment called p40MET[265]. This fragment localizes to the mitochondria-associated membrane region of the endoplasmic reticulum and triggers a calcium transfer from the endoplasmic reticulum towards the mitochondria and results in the release of mitochondrial components that trigger cell apoptosis[266]. However, since cleavage by caspase is required for most dependence receptors' apoptotic pathways activation, and most of these receptors activate caspases, it is plausible that this kind of receptor plays a major role in the amplification of caspase signalling. This could explain the regulation of several of these receptors, like UNC5B for example, by the tumour suppressor protein p53[277].

D. Netrin-1 receptors mediated cell death

Despite strong structural homology, the receptors of the UNC5 family do not share the same pro-apoptotic pathways. UNC5A, for example, induces cell death through its interaction with NRAGE (Neurotrophin Receptor-interacting MAGE homolog) via its ZU5 domain. In the presence of UNC5A, NRAGE is believed to be sequestered at the membrane. In the absence of its ligand, netrin-1, UNC5A undergoes cleavage, releasing the intracellular UNC5A/NRAGE complex into the cytoplasm [278]. NRAGE could then trigger apoptosis by interacting with and degrading the survival protein XIAP [279] or by activating JNK (c-Jun N-terminal kinase) and the caspase signalling pathway [280]. In the case of UNC5D, the absence of netrin-1 also leads to receptor cleavage by caspases, generating an intracellular fragment (UncICD) that translocates to the nucleus. This fragment then interacts with the transcription factor E2F1, which specifically activates pro-apoptotic genes [281].

As we have seen earlier, dependence receptors can play a role in the development and cellular homeostasis through their pro-apoptotic signalling. Specifically, UNC5B could partially regulate neuronal migration by inducing apoptosis in olivary neurons [282]. UNC5B could also play a crucial role in developmental angiogenesis. In this context, netrin-1 acts as a survival factor for endothelial cells expressing UNC5B, exerting a pro-angiogenic role by blocking apoptosis induced by UNC5B. The receptor would thus have a regulatory role in blood vessel formation [283].

From a mechanistic perspective, the known elements of the pro-apoptotic signalling pathway of UNC5B primarily involve direct interactions with the receptor at the membrane. In the presence of netrin, UNC5B is stabilized in a dimerized form and adopts a closed conformation where the ZU5 and DD domains interact [284]. In this conformation, it interacts on one hand with DAPK (serine/threonine Death-Associated Protein Kinase) and associates on the other hand with the phosphatase PP2A and its inhibitor CIP2A. When netrin-1 is absent, UNC5B exists in a monomeric form and adopts an open conformation, allowing the proteolytic cleavage of its intracellular domain and the loss of inhibition of CIP2A on PP2A [285]. The phosphatase can thus catalyse the dephosphorylation of DAPK, which then induces cell death through mechanisms that are still unknown [286].

E. MCL1: the link between Unc5B and apoptosis?

The apoptotic pathway of the Unc5B receptor is still elusive, though, Mcl-1 degradation is suspected to be a decisive factor. MCL1 belongs to the Bcl-2 family, itself divided into three groups, based on their function: the anti-apoptotic proteins (Bcl-2, Bcl-XL, Bcl-W, Mcl-1, BFL-1/A1), the pro-apoptotic proteins forming pores (BAX, BAK and BOK) and the BH3-only proteins (AfDB, BID, BIK, BIM, NOXA, PUMA, etc.) [256, 287, 288]. These anti-apoptotic proteins have a titrating effect on BH3-only proteins, preventing their action, constituting here a first level of inhibition. Activation of BAX and BAK by BH3-only proteins will therefore only be effective once sites BH3 of saturated Bcl-2 family proteins. This effect can be modulated by the intervention of BH3-only sensitizer proteins, unable to activate BAX and BAK, but able to saturate Bcl-2 anti-apoptotic proteins, and therefore influence cellular fate towards apoptosis.

The potential link between Unc5B and Mcl-1 is COMMD2 since its invalidation reverses Unc5B-mediated death in HCT116 cells (non-published data). This latter is a 199aa protein, belonging to the COMMD (copper metabolism MURR1 domain containing) family of proteins. It is a highly conserved family among eukaryotes, expressed ubiquitously, and involved in a wide variety of cellular processes [289]. All members of the COMMD family can associate with different Cullins, which are at the centre of Cullin-RING ligases (CRL) complexes, one of the biggest families of E3-ligases [290]. COMMD1,

by interacting with the CRL, would have the ability to move CAND1 (Cullin-associated Neddy8-dissociated Protein 1), a CRL inhibitor, thus causing the neddylation and activation of this complex. All these actors work together closely to regulate the intracellular levels of Mcl-1.

Transcription of the MCL1 gene is exacerbated following cellular stress such as hypoxia or reticulum stress endoplasmic. At the transcriptional level, Mcl-1 is regulated by different cytokines and growth factors acting on several transcriptional programs inducing Mcl-1 expression, such as the MAP kinases or PI3/Akt pathways. Conversely, the E2F1 transcription factor represses the promoter of MCL1 [291]. A second degree of regulation is the splicing of pre-mRNA allowing post-transcriptional control of MCL1 expression. MCL1 mRNA can be spliced into two alternative forms of Mcl-1 (Mcl-1S and Mcl-1ES). These two forms, unlike the untruncated form, would be able to induce mitochondrial-dependent cell death, binding to the total form of Mcl-1 [292]. These numerous points make the regulation half-life of Mcl-1 mRNA very short (2-3h). The intracellular level of Mcl-1 therefore depends on the various stresses targeting the cell, leading to eIF2 phosphorylation, and resulting in a general inhibition of translation [293].

Proteins of the Bcl-2 family promote tumour survival and are frequently overexpressed in tumours. Mcl-1, in particular, is one of the most amplified genes in human cancers, and its expression is often associated with chemoresistance and relapse due to its anti-apoptotic activities [294-297]. Good illustrations of it are the overexpression of Mcl-1 enhancing the development and maintenance of acute myeloid leukemia [298], and accelerating myc-induced lymphomagenesis [299].

The amplification of Mcl-1 can originate from the insertion of short specific sequences within its promoter, which correlates with increased Mcl-1 expression, and poor prognosis in the case of chronic lymphocytic leukemias for instance [300]. At the gene level, Mcl-1 dysregulations usually come from gene amplifications rather than from translocations of genes or point mutations [301, 302]. The mechanisms of phosphorylation and ubiquitination are another set of signalling pathways that the tumour cell can hijack to disrupt Mcl-1 expression levels. For example, an increased

expression of USP9X de-ubiquitin enzyme correlates with increased Mcl-1 levels in colorectal cancers or follicular lymphomas [303, 304].

Ways in which the cancer cell can increase levels of Mcl-1 intracellular are numerous, and tumours are often dependent on its expression for survival. Knowing the mechanisms leading to its expression is therefore a major challenge for predicting a tumour dependence on this protein. In addition, it would allow us to find sensitive signalling points to target this dependence and trigger the apoptosis of tumour cells. The UNC5B-dependent receptor, which was the focus of my study during my thesis, could be one of these regulatory tools.

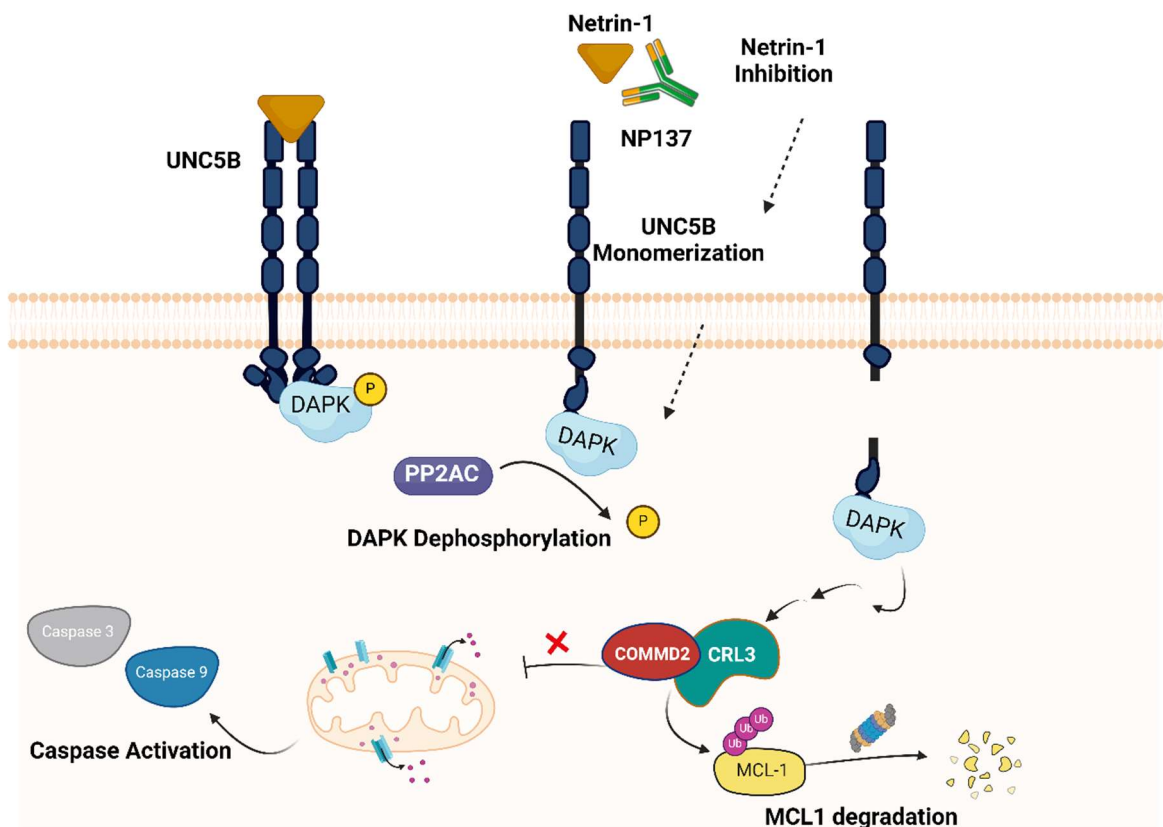


Figure 1.1 – Schematic representation of the actual primary hypothesis regarding UNC5B dependence receptor apoptotic pathway

VI. Gaps in knowledge

Targeting mCRC is crucial due to its significant impact on patient mortality and the limited efficacy of current treatments. Colorectal cancer is a leading cause of cancer-related deaths globally, and approximately 20% of patients are diagnosed with stage IV or metastatic disease, which is associated with a five-year survival rate of merely 13%. The metastatic spread of cancer cells, particularly to vital organs like the liver and lungs, poses a challenge in the CRC and mCRC treatment.

Current treatment modalities, such as chemotherapy and targeted therapies, have demonstrated limited effectiveness in achieving long-term remission for metastatic cases. Despite advances in medical interventions, the complexity of mCRC and its ability to develop resistance to standard treatments underline the urgent need for innovative therapeutic strategies. Discovering new ways to target mCRC is essential to enhance treatment outcomes, improve patient survival rates, and address the persistent challenges posed by mCRC.

The family of dependence receptors is expanding, growing with ongoing discoveries, as seen with recent findings on the activities of Kremen1 dependence receptors [305] and c-kit [273]. While functionally these receptors have been extensively characterized, the mechanistic aspects behind their function remain understudied. Despite UNC5B being one of the prototypical dependence receptors, many gaps persist in understanding its signalling, both in the presence and absence of its ligand, Netrin-1.

The challenges related to exploring the signalling pathways of UNC5B and NOTCH3 are numerous. From a fundamental research perspective, studying the receptor's signalling pathways would provide a better understanding of its role in a physiological context. On the other hand, in a pathological context, identifying UNC5B and Netrin-1's impact on CRC cells is crucial to considering them as potential targets for CRC treatment. Previous descriptions have highlighted dysregulation of the Netrin-1/UNC5B axis in the context of cancer, conferring selective advantages allowing tumours to evade UNC5B-induced tumour suppression [306-308]. Alongside these selective advantages, modifications in downstream signalling pathways of the receptor could further contribute to the tumorigenicity of colorectal cancer cells.

Despite considerable progress in understanding the role of Netrin-1 in cancer, the precise cellular targets responding to Netrin-1 signalling in the context of mCRC remain uncharacterized. Understanding the biological processes regulated by Netrin-1, such as cell migration, invasion, and angiogenesis, is essential for unravelling its complex role in cancer progression. Additionally, the consequences of blocking Netrin-1 signalling on these biological processes need further exploration. Elucidating these aspects will not only advance our comprehension of the molecular processes related to mCRC but also allow for the development of targeted therapeutic strategies aimed at disrupting Netrin-1-mediated pro-tumourigenic pathways. Addressing these knowledge gaps is pivotal for the design of more effective interventions in the management of metastatic colorectal cancer.

VII. Hypothesis and Aims

Studies discussed previously suggest that the UNC5B receptor plays a role in the regulation of cancer stem cells. These specialized cells within the tumour population are recognized for their self-renewal capacity and resistance to conventional therapies, contributing significantly to cancer progression and metastasis. It is postulated that UNC5B, as a dependence receptor, and its ligand Netrin-1, may orchestrate signalling pathways influencing the behaviour of CSCs in mCRC. Employing strategies to deprive the tumour microenvironment of Netrin-1, such as using NP137, and UNC5B-dependent signals may have targeted effects on CSCs. This approach could potentially interfere with the maintenance and expansion of the CSC population.

Hypothesis: Netrin-1 is involved in molecular pathways modulating chemoresistance and recurrence and represents a potential therapeutic target in mCRC

Aim 1: Investigate the role of Netrin-1 in the modulation of CRC cells

Given the dual role of Netrin-1 in cell survival and death, depending on ligand-receptor interactions, investigating Netrin-1 in the context of mCRC is crucial for uncovering novel mechanisms of cancer progression and identifying the downstream signalling pathways activated by Netrin-1 in mCRC cells. A comprehensive understanding of how Netrin-1 modulates mCRC cell behaviour should arise from this aim.

Aim 2: Investigate the therapeutic potential of targeting the Netrin-1 in mCRC

Using the NP137 anti-Netrin-1 antibody developed by our partner laboratory, we're planning on identifying key effects of UNC5B and Netrin-1 on CRC cells that would suggest a potential therapeutical effect of NP137 on mCRC.

Aim 3: Investigating the implications of other dependence receptors in these cellular processes

In the framework of this thesis project, our goal is to identify the implications of UNC5B, Netrin-1 and Notch3 in the modulations of important features of metastatic colorectal cancer, notably EMT and stemness as well as their relationship to chemoresistance and recurrence after treatment.

To achieve our aims, we evaluated the effect of Netrin-1 deprivation on stem cell features such as self-renewal with expression of stemness-related genes and chemoresistance and recovery after treatment, using techniques linked to phenotypical traits such as ELDAs (Extreme Limiting Dilution Assay), to genotypical traits using techniques such as single-cell RNA sequencing, and to apoptosis using techniques like AnnexinV assays. The aim is to apply the knowledge gained through this process to enhance the efficacy of existing therapeutic strategies in metastatic colorectal cancer patients.

Chapter 2: Material and Methods

I. Cell Lines and Tissue Culture Reagents

I.1. Adherent cell lines

The HEK293T cell line, originating from our lab cell bank, was used in the process of viral production. HCT116 and EMT6 cell lines, originating from our lab cell bank, were used for various *in vitro* experiments. These cell lines were grown in complete DMEM with the following composition: Dulbecco's Modified Eagle's Medium (DMEM) (From Lonza - #12 614F), Foetal Bovine Serum (FBS) at 10% concentration (From Bovogen Biologicals - #SBS-F), penicillin at 100 units/ml and Streptomycin at 100ug/ml (both from Life Technologies - #151540 122)

I.2. Patient-derived organoid cell lines

I.2.a Patient selection & ethics

Patients with CRC were identified prospectively from January 2016 to June 2016. Colorectal liver metastases and, when possible, matched healthy hepatic tissue and primary tumour were collected from patients undergoing treatment at Peter MacCallum Cancer Centre, The Royal Melbourne Hospital, St Vincent's Hospital, St Vincent's Private Hospital or Morwell Hospital by Dr Corina Behrenbruch. Tumour samples of approximately 0.25-1cm³ were collected directly from the operating theatre and used for the establishment of patient-derived organoids and FFPE blocks as well as RNA sequencing studies (performed by Sepideh Fouroutan prior to and during this PhD). The study leading to the generation of these organoid cell lines was approved by the Peter MacCallum Cancer Centre Human Research Ethics Committee (HREC/15/PMCC/112, project #15/169) and written consent was obtained from each patient according to National Health and Medical Research Council guidelines.

I.2.b. Organoid cell lines culture

Patient-derived organoids were established before this project by Dr Corina Behrenbruch. Organoids were typically grown in 48-well plates and passaged every 3 to 5 days at a split ratio of 1:2 - 1:3. Cells were grown inside a Growth-factor reduced Matrigel matrix (Corning, Cat #356231) forming a dome at the bottom of the well on-

which was added complete organoid growth medium (cf composition in Table 2.1 below) after completion of Matrigel polymerisation.

During splitting, media was removed, and each well was washed with ice-cold PBS to dissolve Matrigel. The contents of each well were then transferred to a 15ml polypropylene tube (Falcon, ref: CLS430791) filled with 10mL of ice-cold PBS. Following centrifugation at 220 g for 5 minutes, pelleted organoids were dissociated by mechanical trituration in fresh cold PBS such that large aggregates were broken into small fragments. Fragments were resuspended in the appropriate amount of Matrigel (as determined by split ratio) and plated by adding 40uL of cell suspension per well. When single-cell suspensions were required (for flow cytometry or ELDA assay seeding), pelleted organoids were resuspended in 1mL of pre-warmed TrypLE Express (ThermoFisher Scientific, Ref: 12604013) and incubated for up to 30 minutes at 37°C. TrypLE reaction was stopped by adding an equivalent volume of the complete organoid medium. Single cells from organoids were then filtered using Tubes with a Cell Strainer Cap (Corning™ 352235).

To correct for variation in protein concentration between Matrigel batches, all Matrigel was prediluted to a concentration of 4.35mg/ml in ice-cold basal DMEM/-F12. Matrigel domes were incubated at 37°C and allowed to solidify for 30-60 minutes. Following incubation, 500uL of complete organoid media was added to each well.

The composition of the complete organoid media is summarised in Table 2.1

Patient-derived organoids used in this study are derived from viably frozen early passages organoids. Organoid passages were limited to 30 to prevent genomic or epigenomic drifting.

Component	Concentration	Manufacturer
Advanced DMEM/F12		Thermo Fisher Scientific, Ref. 2634010
Penicillin	100U/ml	Life Technologies - Ref. 151540-122
Streptomycin	100U/ml	Life Technologies - Ref. 151540-122
Glutamax	5mM	Life Technologies - Ref. 35050-061
B27 supplement	2X	Life Technologies - Ref. 17504044
Leu [15] Gastrin I (human)	1µg/ml	Merck, Darmstadt - Ref. G9145
N-acetyl cysteine	1mM	Merck, Darmstadt - Ref. A9165
A83-01 (ALK inhibitor)	500nM	Merck, Darmstadt - Ref. SML0788
Recombinant Human Epidermal growth factor	50ng/ml	Miltenyi Biotec - Ref. 130-097-749
StemMACS™ SB202190 (p38/MAPK inhibitor)	10nM	Miltenyi Biotec - Ref. 130-106-275
YP-27632 (ROCK inhibitor)	10µM	Abcam - Ref. 120129

Table 2.1 - Composition of Complete Organoid Media (Concentration and Manufacturer)

I.2 Generation of CRISPR Cas9-modified organoids

I.2.a sgRNA design

For both UNC5B and NOTCH3, two sgRNA sequences (Table 2.2) were derived from the Brunello human CRISPR knockout pooled library (Doench et al., 2016). The reverse complements for these sequences were generated and adapters suitable to BsmB1 linearisation were added (TCCC-motif was added to forward guides and AAAC-motif was added to reverse guides).

Forward and reverse sgRNA oligos (100uM each) were annealed using T4 DNA Ligase buffer (New England Biolabs, Cat #B0202S) using the following thermocycler temperature settings: 85°C for 10 mins, 81°C for 2 mins, 77°C for 2 mins, 73°C for 2 mins, 69°C for 2 mins, 65°C for 2 mins, 61°C for 2 mins, 57°C for 2 mins, 53°C for 2 mins, 49°C for 2 mins, 45°C for 2 mins, 41°C for 2 mins, 37°C for 2 mins, 33°C for 2 mins, 29°C for 2 mins, 25°C for 2 mins, hold at 4°C.

Target Gene ID	Target Gene symbol	target transcript	genomic sequence	sgRNA	target sequence	PAM sequence	Exon Number
219699	UNC5B	NM_170744.4	NC_000010.11	antisense	Forward: TCCCAGGGTGGCGTACAGGCGATG Reverse: AAACCATCGCCTGTACGCCACCCT	CGG	3
219699	UNC5B	NM_170744.4	NC_000010.11	sense	Forward: TCCCATACCTAGCGATTTCGCCC Reverse: AAACGGGCGAAATCGCTAGGGTAT	GGG	10
4854	NOTCH3	NM_000435.2	NC_000019.10	sense	Forward: TCCCGACACCGATGTCTCAATGGG Reverse: AAACCCATTGACATCGGTGTC	GGG	5
4854	NOTCH3	NM_000435.2	NC_000019.10	antisense	Forward: TCCCGACAGGACAGTCTGACAGCG Reverse: AAACCGCTGTCAGACTGTCCTGTC	AGG	23

Table 2.2 - sgRNA guide sequences for UNC5B and NOTCH3 genes

1.2.b Construct digestion & ligation

Linearisation of the human FU-Cas9Cherry expression plasmid (Addgene, Ref. #70182) was performed using the Espel (BsmBI) restriction enzyme (Thermo Fisher Scientific, Ref. #ER0451), incubated in Tango buffer containing 20uM DTT at 37°C for 2 hours. 1µl DNA loading dye was added to 10µl of the digested plasmid and then run by electrophoresis on a 2% agarose gel containing RedSafe™ Nucleic Acid Staining Solution (1/20000 v/v; INtRON Biotechnology, Ref. #21141). Samples were loaded adjacent to a 100bp DNA ladder (New England Biolabs, Ref. #N3231S) to confirm the correct band size of the linearized plasmid. Electrophoresis was performed using the SafeBlue Electrophoresis System (Major Science, Ref. #MBE-150) at 130 V for 45 minutes and the resulting DNA fragment was visualised using a ChemiDoc XRS+ imaging system (Bio-rad; ChemiDoc XRS+, Ref. #1708265), enabling excision from the gel.

Purification from the agarose gel was achieved using the NucleoSpin® Gel and PCR Clean-up (Macherey Nagel, Ref. #740986.20) according to the manufacturer's instructions. Ligation of the FgH1t_UTG plasmid backbone (100ng) with the annealed sgRNA oligos (500pg) was performed by incubating with 1 x T4 ligase (New England Biolabs, Ref. #B0202) at room temperature for 30 minutes in T4 ligase Buffer. To confirm that cloning was successful, a sample of the resulting plasmid was sent for Sanger sequencing at the AGRF (Australian Genome Research Facility), and the resulting chromatograms were observed using Snap Gene Viewer software (GLE Biotech LLC, v.6.1.1) (Comparison of FgH1t plasmid chromatogram before and after ligation of UNC5B#2 SgRNA is given as an example in **Figure 2.1**)

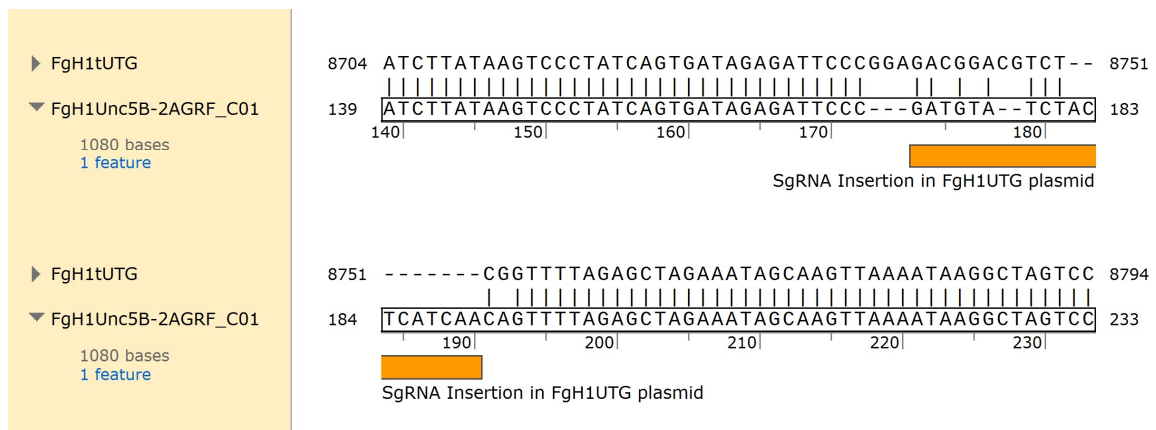


Figure 2.1 – Comparison of FgH1t plasmid chromatogram before and after ligation of UNC5B SgRNA

I.2.c Production of sgRNA and Cas9 viruses

Transformation of the produced plasmids into stable competent *E. coli* (Stbl3 strain) (New England Biolabs, Ref. C3040HVIAL) was performed using a standard heat shock protocol. The ligation product and Stbl3 cells were acclimatised on ice for 20 mins, heated to 42°C for 90 seconds, and put into ice for a further 2 minutes. 500uL of LB broth was then added and the mix was incubated at 37°C for 1 hour with agitation (130rpm) on an orbital shaker. Following centrifugation, the bacterial pellet was resuspended in 50uL of LB Broth and spread on an agar plate containing 75 µg/ml ampicillin and incubated overnight at 37°C. The following day, single colonies were selected and amplified by incubation overnight at 37°C with agitation (130rpm) in 4mL of LB broth containing 75µg/ml ampicillin. Plasmids were then purified from the bacteria using the AccuPrep® Plasmid Mini Extraction Kit (Bioneer, Ref. #K-3030) according to the manufacturer's instructions and quantified using the Qubit™ 4 Fluorometer (Thermo Fisher Scientific, Ref #Q33238).

I.2.e Viral medium production

To produce the medium containing the viral particles necessary for the transfection of organoids towards the CrispRCas9-mediated invalidation of target genes, the HEK-293T cells were transiently transfected using PEI (polyethyleneimine). HEK293T cells were seeded in complete DMEM in T25 flasks at 75% confluency and incubated overnight. The media was renewed 4 hours prior to transfection. A transfection mix including the

packaging vectors (pMDLg/pRRE - 700ng; Addgene #12251, pRSV-Rev - 350ng; Addgene #12253 and pMD2.G/VSVg - 420ng; Addgene #12259) and sgRNA-expressing DNA (1.4µg of the corresponding target-specific sgRNA or 1.4ug of sgScramble used as control) (the ratio sgRNA/plasmid components was equal to approximately 1:1 ratio) was combined with 1mg/ml PEI and incubated for 30 minutes at RT. The resulting cocktail was added to the media of HEK293T cells, and the latter were incubated overnight at 37°C and 5% CO₂. After incubation, DMEM media was refreshed, and lentivirus-containing supernatants were harvested 24 hours later. The viral supernatant was passed through a 0.4mm filter to eliminate cells and cellular debris and then used to transduce our organoid cell lines. To generate the Cas9-expressing virus, the above procedure was performed with the following amendments: HEK293T cells were seeded in 6 x T75 flasks at 75% confluency, Cas9 construct (14µg) was added to packaging vectors (3µg pMLDG, 1.5mg of RSV-REV and 1.8µg of VSVg) (the ratio Cas9 construct/plasmid components was approximately 2:1). The resulting media was then concentrated by centrifugation for 15 minutes at 3000g in Amicon® Ultra-15 centrifugal filters (Merck Millipore – Ref: UFC910024).

I.2.e CRISPR Cas9-modified organoids selection

To edit our organoid cell lines using CRISPR, cells were first transduced with Cas9 and with sgRNA viruses. In the Cas9 introduction, 4 wells of organoids were grown to confluency in 48 well-plates, then dissociated to single cells and resuspended in viral medium, 1µg of polybrene per mL of medium was added to enhance transduction efficiency. Cells in suspension were transferred to 2 wells of a 48-well ULA plate and spinoculated (both the viral vector and the cells are centrifuged together to aid viral binding to the target cells (600g for 90 minutes) to enhance viral replication, prior to overnight incubation at 37°C and 5% CO₂. After incubation, transduced cells were harvested by centrifugation and replated in Matrigel. Organoids were then amplified and passaged twice prior to flow cytometry-based sorting of the Cas9-mCh⁺ population (using mCherry as a marker of transduction of the Cas9-plasmid). For sgRNA transductions, an identical protocol was performed on the Cas9-mCh⁺ cells after 2 to 3 passages. After transduction, the Cas9-mCh⁺/sgRNA-GFP⁺ double-positive population was sorted by FACS and amplified. Doxycycline (1ug/mL) was added to the growth media

of organoid cell lines following the sorting of double-positive cells for 5 days to induce Cas9 cleavage. To maximise the homogeneity of knockdown efficiency, single organoids from the double-positive population were isolated (via manual selection using the tip of a p10 pipette) and amplified to produce monoclonal CRISPR Cas9 edited organoid cell lines. CRISPR editing in each monoclonal population was validated by TIDE analysis (section I.2.f.3) and western blot (section II.1).

I.2.f Validation of CRISPR Cas9 invalidation of target genes

I.2.f.1 Oligonucleotides primers design

cDNA sequences for UNC5B (RefSeq ID: NC_000010.11) and NOTCH3 (RefSeq ID: NC_000019.10) were downloaded from the National Centre for Online Biotechnology Information (NCBI) portal. Using the NCBI Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast>), primers were designed to generate amplicons of >500bp in length, with forward primers positioned approximately 250bp upstream of the PAM sequence for each guide (Table 2.3).

Construct	Direction	Primer Sequence (5' – 3')	Amplicon Size
UNC5B #1	Forward	TTGGGTGGGATTTTGCAGTG	576
	Reverse	TGTGAGATGGGATTAGGGCAG	
UNC5B #2	Forward	GCAATGTCAGAGACCCCTGT	503
	Reverse	CCAGCTTGTCAACCGCCTTT	
NOTCH3 #1	Forward	TGTCCAGCCATTGACACACA	523
	Reverse	TCAACACACCTGGCTCCTTC	
NOTCH3 #2	Forward	ACTCCCTTCCCTTCGATGTCT	509
	Reverse	CCTACACAATGGCACCTGCG	

Table 2.3 – Description of Primers used for Cas9 cut-site amplification

I.2.f.2 DNA Extraction & Polymerase Chain Reaction (PCR)

Genomic DNA (gDNA) isolated from CRISPR-edited PDOs was obtained using the DNeasy Blood and Tissue Kit (Qiagen, Cat #69504) following the manufacturer's instructions. The concentration and integrity of the gDNA were assessed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Cat #ND-2000).

For PCR, 100ng of gDNA was combined with forward and reverse sgRNA oligos (400nM each) and 2X MyTaq HA Mix (Bioline, Cat #BIO-25045). The amplification was carried out in a thermocycler under the following conditions: 1 denaturation cycle at 94°C (5 minutes); 40 amplification cycles comprising denaturation at 94°C (1 minute), annealing at 50°C (2 minutes), and elongation at 72°C (1 minute); and a final extension step at 72°C (7 minutes). Subsequent to PCR, 20µl of each PCR product was run through electrophoresis, and bands of interest were excised and purified from the agarose gel for further sequencing analysis.

II.2.f.3 Sequencing & TIDE analysis

For Sanger sequencing sample preparation, 20ng of the purified PCR product (or 1µg of plasmid, as detailed in section 2.3.2) was mixed with 1µL of DMSO and adjusted to a total volume of 12µL in ddH₂O. These samples were then submitted for sequencing at AGRF. For quality control, the resulting chromatograms were visually examined using SnapGene Viewer software (GLE Biotech LLC, v.6.1.1).

To assess the spectrum and frequency of small insertions and deletions (indels) in CRISPR-edited Patient-Derived Organoids, Tracking of Indels by Decomposition (TIDE) analysis [309] was carried out. This involved using the TIDE web tool (<http://shinyapps.datacurators.nl/tide/>), where Sanger sequencing trace files (.ab1) of edited PDOs were compared against their corresponding scramble (sgSCR) controls.

II. In vitro assays

II.1 Western blots

II.1.1 Whole Cell Protein Lysate Preparation

Tumour cell lines or Patient-Derived Organoids (PDOs) were harvested using trypsin or ice-cold PBS, respectively. Pellets were collected through centrifugation (220 g, 5 minutes) and washed once in ice-cold PBS to remove any residual FBS, Matrigel, and/or cellular debris. Whole-cell protein lysate was obtained by suspending cell pellets in Laemli lysis buffer (60 mM Tris-HCl, 10% v/v glycerol, 2% w/v SDS, 2% v/v β-mercaptoethanol) at room temperature for 10 minutes, followed by boiling the samples

at 90°C for 10 minutes. Protein concentrations were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific; 23227) following the manufacturer's instructions. The linear absorbance was measured at 562 nm using the EnSpire multimode plate reader (Perkin Elmer, Cat # 6055400). All protein lysates were stored at -80°C until used.

II.1.2 Western Blot and Protein Immunodetection

Protein lysates (20µg to 50 µg per lane) were subjected to Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for 75 minutes at 100V at room temperature using 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-rad, Cat #4561094). Pre-stained molecular weight standards (PageRuler Plus, Thermo Scientific, Rockford, IL, USA, Cat #26620) were included in each gel to determine the molecular weight of the identified proteins. Electroblothing was performed to transfer proteins to Immobilon-P PVDF membrane (Merck-Millipore, Bedford, MA, USA, Cat # IPVH00010) prior to activating with a 2-second methanol bath. The electroblotting was performed for 90 minutes at 90V at 4°C in a wet transfer apparatus containing Western transfer buffer (Tris-HCl 25mM [pH 6.8], glycine 192mM, 10% v/v methanol).

Following the transfer, PVDF membranes were blocked in BSA (5% w/v in Tris-buffered saline/0.5% Tween [TBS-T]) for 30 minutes at room temperature and then incubated overnight at 4°C with the appropriate primary antibody (**Table 2.5**) (in 5% w/v Milk in TBS-T). After primary antibody incubation, membranes were washed three times in TBS-T (5 minutes per wash) and subsequently incubated for at least 60 minutes at room temperature with the appropriate HRP-conjugated secondary antibody (in 5% w/v Milk in TBS-T).

Further washing with TBS-T (three times, 5 minutes per wash) removed excess secondary antibody before protein visualization using Clarity Western ECL Substrate-Clarity Peroxide Solution (Bio-rad; 10026546) for 5 minutes. Blot imaging was carried out using the ChemiDoc XRS+ imaging system (Bio-rad; ChemiDoc XRS+, Cat #1708265). To confirm equivalent protein loading between samples, all blots were re-stained with anti-β-actin or Ku80 antibodies.

Target	Clonality	Host species	Manufacturer	Dilution
Anti-UNC5B	Monoclonal	Rabbit	Cell Signalling (Ref 13851)	1:1000
Anti-NOTCH3	Monoclonal	Rabbit	Cell Signalling (Ref D11B8)	1:1000
Anti-βActin	Monoclonal	Mouse	Sigma Aldrich (Ref A4700)	1:5000
Anti-Vincullin	Monoclonal	Rabbit	Cell Signalling (Ref 13901)	1:1000
Anti-MCL1	Monoclonal	Rabbit	Cell Signalling (Ref 39224)	1:1000
Anti-JAG1	Monoclonal	Rabbit	Santa Cruz Biotechnology (Ref 390177)	1:1000
Anti-Ku80	Monoclonal	Rabbit	Cell Signalling (Ref 2180)	1:1000

Table 2.4 - Description and provenance of primary antibody used for Western Blot

II.2. Quantitative Polymerase-Chain-Reaction

Total RNAs were extracted utilizing the NucleoSpin® RNA Plus Kit (Macherey Nagel, Düren, Germany) by the manufacturer's protocol. RT-PCR reactions were conducted using the PrimeScript RT Reagent Kit (Takara Bio Europe, Saint-Germain-en-Laye, France). A total of 500 ng of total RNA underwent reverse transcription with the following program: 37°C for 15 minutes and 85°C for 5 seconds.

For expression studies, the target transcripts were amplified in a LightCycler® 2.0 apparatus (Roche Applied Science, Indianapolis, IN, USA) using the Premix Ex Taq probe qPCR Kit (Takara Bio Europe) following the manufacturer's instructions. The expression of target genes was normalized to TATA-binding protein (TBP) which served as the housekeeping gene. The quantity of target transcripts, normalized to the housekeeping gene, was determined using the comparative CT method: $CT = CT(a \text{ target gene}) - CT(a \text{ reference gene})$. $\Delta\Delta CT = \Delta CT(a \text{ target sample}) - \Delta CT(a \text{ reference sample}) = (CTD - CTB) - (CTC - CTA)$. Primer sequences :

NTN1 : Rev : 5'-GGC ATG CAG GTT GCA ; For: 5' GCA AGC CCT TCC ACT

TBP Rev: 5'-CAC ACG CCA AGA AAC ; For: 5'-CGG CTG TTT AAC TTC

UN5B Rev: 5'-CCA AGT ATC CGC CCA ; For: 5'-GAC ACG CCT GTA GCA

p21: Rev: 5'- CAT GGG TTC TGA CGG ACAT; For CGA AGT CAG TTC CTT GTG GAG

II.3 Flow cytometry

For all flow cytometry (FC) analysis and cell sorting, single-cell suspensions were resuspended in FACS Buffer (2% FBS, 0.2% EDTA in PBS) and passed through a 40 µm nylon mesh cell strainer to exclude large aggregates. Raw cytometry data was analysed using FlowJo software (Tree Star Incorporated, v 10.8.1).

II.3.1. Selection of cells during CrispRCas9

Cas9-mCh+/sgRNA-GFP+ double-positive population was analysed by flow cytometry. The background noise of fluorescence at ~510nm and ~622nm was determined using non-transduced PDOs prepared in the same way (dissociation/washing/nylon mesh stainer/ FCAS Buffer). The sorting was done by the Flow Cytometry facility of the Peter MacCallum Cancer Center by the flow cytometrists Fatma Panetta and Viki Milovac and was performed on a FACSAria™ Fusion cytometer.

II.3.2. Cell death assay in CD133+ cells

For AnnexinV and propidium iodide (PI) double staining, organoid cell lines were seeded onto 48-well plates at a density of 50% confluence. Cells were then incubated in media containing either recombinant Netrin-1 (100 ng/mL), NP001 isotypic control (10 µg/mL) or NP137 neutralizing antibody (10 µg/mL) for 72 h. Cells were then harvested and dissociated into single cells as previously described, washed with PBS and incubated for 10 minutes in Anti-CD133 (Prominin-1) Antibody (MAB4399-I, MilliporeSigma). Cells were then washed and resuspended in Annexin V buffer containing allophycocyanin (APC) conjugated to Annexin V and PI (BioLegend, San Diego, CA, USA). Annexin V-positive cells were then evaluated by fluorescence-activated cell sorting (FACS) on FACSCanto II (BD Biosciences, San Jose, CA, USA).

II.4 Extreme limiting dilution Assays (ELDAs)

The Extreme Limiting Dilution Assay is a statistical method commonly used in stem cell biology and cancer research. This assay helps estimate the frequency of functional cells with a particular activity within a heterogeneous cell population. It involves diluting the cells to such an extent that each well, on average, contains either one functional cell or

none. By observing the proportion of wells exhibiting the desired activity, we can calculate the frequency of the functional cells in the original population. This assay is particularly useful for studying rare cell populations, such as cancer stem cells, where traditional methods might be less sensitive.

II.4.1 ELDAs on PDOs

ELDA was employed to assess the self-renewal capacity of PDO cell lines. The protocol to perform an ELDA on our PDO cell lines was adapted from Hu, Y. et al article: “ELDA: Extreme limiting dilution analysis for comparing depleted and enriched populations in stem cell and other assays [310]”. In brief, organoid cell lines were seeded onto 48-well plates at a density of 50% confluence. Cells were then incubated in media containing either recombinant Netrin-1 (100 ng/mL), NP001 isotypic control (10 ug/mL) or NP137 neutralizing antibody (10 ug/mL) for 72 h. For ELDAs evaluating the effect of the caspase inhibitor Z-VAD(OH)-FMK on NP137 effect, Z-VAD(OH)-FMK (Ref: 161401-82-7, Selleck Chemicals) was supplemented to the organoid medium at 50ug/mL. For ELDAs evaluating the effect of NP137 on post-FOLFOX treatment self-renewal, FOLFOX was added to a final concentration of 1 μ M oxaliplatin + 1 μ M 5-Fluorouracil.

After 72h incubation, organoids were dissociated using TrypLe for 15 minutes at 37°C and mechanically pipetted until a single-cell suspension was achieved. The cells were then plated in 384-well Optical Imaging Flat Clear Bottom Black Polystyrene TC-treated plates (Corning Life Sciences, Tewksbury MA, USA, Catalogue #CLS3985) in 100 μ l of Matrigel diluted at 4.35mg/ml in ice-cold basal DMEM/-F12. Cells were seeded at the following densities: 250, 125, 25, 5 and 1 cell(s) per well, covering the dynamic range of the sphere-forming frequency of our organoid cell lines. Each of the four treatment groups had 10 to 16 replicates for every dilution, depending on the experiment. To prevent well drying and ensure a constant presence of the chemical tested, media change (with the appropriate treatment) occurred every 4 days in culture. It is to be noted that in experiments assessing the impact of FOLFOX, FOLFOX treatment was performed before seeding the ELDA but not maintained after single-cell seeding.

II.4.2 ELDAs on adherent cell lines

ELDA was employed to assess the self-renewal capacity of individual tumour cell lines, notably HCT116, following the methodology developed by Hu and Smyth (2009) [310]. Adherent cells were dissociated using Trypsin EDTA (Thermo Fisher Scientific, Ref: 15050065) for 5 minutes at 37°C, mechanically pipetted until a single-cell suspension was achieved) and passed through a 40µm nylon mesh cell strainer to exclude large aggregates. The cells were then plated in 96-well ultra-low attachment (ULA) plates (Corning Life Sciences, Tewksbury MA, USA, Catalogue #CLS3474) in 100µl Advanced DMEM/F-12 (Gibco, Cat #12634010) at cell densities of 1000, 333, 111, 37, 12, 4 and 1 cell(s) per well, covering the dynamic range of the sphere-forming frequency of adherent cell lines. Additional media supplements are detailed in **Table 2.5**. Each of the treatment groups had 12 replicates for every dilution. To prevent well drying, 50µl of media (with the appropriate treatment) was replenished after 5 days in culture.

Compound	Manufacturer	Final concentration
Advanced DMEM-F12	GIBCO (Ref: 12634-010)	
Insulin	Sigma Aldrich (Ref : I0908)	20µg/ml
N2 Complement	GIBCO (Ref: 17502-048)	1%
EGF	MACS (Ref : 130-097-749)	20ng/ml
bFGF	MACS (130-093-839)	10ng/ml
Glutamine	Sigma Aldrich (Ref : G7513)	20 mM
Penicillin	Life Technologies - Ref. 151540-122	100U/ml
Streptomycin	Life Technologies - Ref. 151540-122	100U/ml

Table 2.5 – Composition of Complete Spheroid Media (Concentration and Manufacturer)

II.4.3 ELDAs analysis

After 12 days in culture, the assessment of organoid or spheroid formation scoring in each well is based on the binary presence or absence of organoid(s) or spheroid(s). The fraction of responsive cells at each density was then subjected to analysis using the ELDA tool provided by the Bioinformatics Division of the Walter and Eliza Hall Institute of

Medical Research (<http://bioinf.wehi.edu.au/software/elda/>). This analysis aimed to calculate the stem cell frequency (SCF) along with corresponding 95% confidence intervals (CIs) for all tested treatment groups.

In individual experiments, pairwise differences in stem cell frequency among treatment groups were evaluated using a chi-square test. For aggregated data, statistical analysis was conducted using one-way, unpaired ANOVA, followed by the Bonferroni correction for multiple comparisons.

II.5 Cell viability

Organoids were dissociated using TrypLe for 15 minutes at 37°C and mechanically pipetted until a single-cell suspension was achieved. The cells were then plated in 384-well Optical Imaging Flat Clear Bottom Black Polystyrene TC-treated plates (Corning Life Sciences, Tewksbury MA, USA, Catalogue #CLS3985) in 100µl of Matrigel diluted at 4.35mg/ml in ice-cold basal DMEM/-F12. Cells were seeded at the 1000 cells per well density.

II.5.1 Growth assay

For experiments evaluating the effect of recombinant Netrin-1 on organoid growth, serial dilution was achieved to obtain the following concentration of recombinant Netrin-1 (200, 100, 50 and 25 ng/mL). PBS was used as a vehicle control. 50 uL of media was then dispensed into wells and cells were incubated at 37°C for 72 h.

II.5.2 Chemotherapy efficacy assay

For experiments evaluating the effect of Netrin-1, NP001 or NP137 on FOLFOX efficacy, 50 uL of media containing either recombinant Netrin-1 (100 ng/mL), NP001 isotypic control (10 ug/mL) or NP137 neutralizing antibody (10 ug/mL) was dispensed into wells. FOLFOX was then added in a volume of 5 µl per well to make a final concentration of 100, 33, 11, 3.3, 1.1. 0.3µM oxaliplatin and 100, 33, 11, 3.3, 1.1. 0.3µM 5-Fluorouracil, or with DMSO control (DMSO/PBS v/v of 1/3). Cells were then incubated at 37°C for 72 h. It is to be noted that FOLFOX treatment includes leucovorin in a clinical setting to

diminish the toxicity of 5-FU, leucovorin being non-relevant in an *in vitro* setting, we used 5-FU/Oxaliplatin treatment in vitro experiments (referred to as FOLFOX treatment).

II.5.3. Cell viability readout

Cell Titre Glo (CTG) 3DR from Promega was utilized for assessing viability. The CTG was allowed to reach room temperature, followed by the removal of plates from the incubator and a subsequent 15-minute equilibration at room temperature. CTG was then introduced at a 1:3 ratio (eg. 25 µl added to 50 µl media). The plate was shaken on a plate shaker at 100 rpm for 20 minutes, and the results were read on the plate reader (Perkin Elmer EnSpire). Each treatment and control well had 3 technical replicates in each experiment, and these experiments were independently repeated at least 3 times.

II.5 Single-cell RNA-seq

Organoid cell lines were seeded onto 48-well plates at a density of 50% confluence. Cells were then incubated in media containing either NP001 isotypic control (10 µg/mL) or NP137 neutralising antibody (10 µg/mL) for 72 h. Organoids were dissociated using TrypLe for 15 minutes at 37°C. Then, organoids were mechanically pipetted until a single-cell suspension was achieved) and passed through a 40 µm nylon mesh cell strainer to exclude large aggregates. The enumeration of viable cells was conducted using a Luna-FL Dual fluorescence cell counter (Logos Biosystems). Cells were loaded on Chip G and run on chromium iX to reach 7000 to 10,000 cells per sample, following the manufacturer's guidelines. Subsequently, single-cell RNA-seq libraries were generated utilizing the Chromium Single Cell 3' v.3.1 kit (10x Genomics, no. PN-1000121). These libraries were sequenced on the NovaSeq 6000 platform (Illumina) to achieve an average of approximately 50,000 reads per cell.

II.5.2 Bioinformatic analysis

Analysis was performed in the programming languages R (version 4.0.3) and RStudio (version 1.1.463). Data were loaded into R and processed using the Seurat package (version 3.2.3), a comprehensive framework for single-cell omics analysis developed by the Satija Lab. Cells with less than 2500 detected genes were removed, together with

cells with more than 15% mitochondrial reads. Filtering using ribosomal genes was not done since these genes did not have a significant effect on clustering (low differences of ribosomal protein-related genes among clusters)

Filtered data were normalized for sequencing depth using the Seurat NormalizeData function with the LogNormalize method and default settings. Cell cycle phase prediction was performed using the CellCycleScoring function in Seurat, which assigns two scores to each cell based on its expression of G2/M and S phase marker genes, followed by classification of each cell in either G2/M, S or G1 phase. The top 5000 highly variable genes across cells were identified with the Seurat FindVariableFeature function using a variance stabilizing transformation (VST) method. These variable genes were used for subsequent data scaling and dimensionality reduction steps. Differential gene expression between time points or between selected groups of cells was assessed using the Seurat FindAllMarkers function with the Wilcoxon Rank Sum test (logfc. threshold = 0.25, min.pct = 0.1, min.diff.pct = 0.2). Differences in gene between treatments Signature scores were calculated by the UCell method (v.2.2.0) and using different gene lists from MsigDb

(HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION, HALLMARK_P53_PATHWAY,
GOBP_MITOCHONDRIAL_RESPIRATORY_CHAIN_COMPLEX_ASSEMBLY,
GOBP_POSITIVE_REGULATION_OF_STEM_CELL_PROLIFERATION,
GOBP_POSITIVE_REGULATION_OF_STEM_CELL_POPULATION_MAINTENANCE,
GOBP_POSITIVE_REGULATION_OF_STEM_CELL_DIFFERENTIATION,
GOBP_NEGATIVE_REGULATION_OF_APOPTOTIC_SIGNALING_PATHWAY)

II.6 Immunohistochemistry (IHC)

Tumours from patients (see ethics and origin section I.2) were fixed in formalin-fixed, paraffin-embedded (FFPE) blocks and underwent sectioning into 3µm slices using a microtome. Embedded tumour slides were subjected to baking at 60°C for 1 hour and then dewaxed through immersion in sequential xylene and graded ethanol baths using the Jung XL AutoStainer by Leica. Heat-induced epitope retrieval (HIER) was executed in 10 mM citrate buffer (pH = 6) or 10 mM Tris-EDTA buffer (pH = 8) at 125°C for 15 minutes in a pressure cooker.

To inhibit endogenous peroxidase activity, slides were immersed in 0.3% H₂O₂ for 10 minutes and subsequently blocked in 2.5% w/v BSA in TBS-T for 30 minutes. Primary antibodies (refer to **Table 2.6**, in 2.5% w/v BSA in TBS-T) were incubated at room temperature for 1 hour in a humidified chamber. Before incubation with the HRP-conjugated secondary antibody (refer to **Table 2.7**; in 2.5% w/v BSA in TBS-T) for 30 minutes at room temperature, slides were washed three times (3 minutes each) in TBS-T. After an additional three washes (3 minutes each) in TBS-T, chromogenic detection was carried out using the DAB Peroxidase (HRP) Substrate Kit (Vector Laboratories, Burlingame, CA, USA, Ref: SK-4100) following the manufacturer's instructions.

Following counterstaining with hematoxylin, images were captured using VS120 Virtual Slide Microscope by Olympus. Visualization was accomplished and photographs were taken using the OlyVIA viewer software (v 2.9).

Target	Clonality	Host species	Manufacturer	Dilution
Anti-Netrin-1	Polyclonal	Goat	Abcam Antibody (Ref ab122903)	1:50
Anti-UNC5B	Polyclonal	Goat	R&D System (Ref AF1006)	1:20
Anti-NOTCH3	Monoclonal	Rabbit	Cell Signalling (Ref D11B8)	1:50

Table 2.6 - Description and provenance of primary antibody used for Immunohistochemistry

Target	Isotype	Conjugate	Manufacturer	Dilution
Anti-rabbit IgG	Secondary, polyclonal	HRP	Vector Laboratories (Ref MP-7401)	1:2
Anti-goat IgG	Secondary, polyclonal	HRP	Vector Laboratories (Ref MP-7405)	1:2

Table 2.7 – Description and provenance of HRP-conjugated secondary antibody

III. In Vivo Assays

Five-week-old (15-20 g body weight) female nude mice were obtained from the Charles River RMS Rhone Alpes Auvergne animal facility in France. The mice were housed in sterilised filter-topped cages and maintained in a pathogen-free animal facility.

ySAW190LT organoid cell line was implanted via subcutaneous injection into the right flank of the mice of a solution containing whole organoids in 100 μ L of growth factors reduced Matrigel (Corning) diluted in 100 μ L of DMEM/F12 medium. Each mouse received a quantity of organoids corresponding to 10 confluent wells in a 48-well plate. To do so, a total of 300 48-wells (for 30 mice) were split and left 72h to reach appropriate confluency, the total amount of organoids was then harvested, washed and resuspended in an appropriate amount of medium/Matrigel to ensure a steady concentration of organoids in each injections. Tumours were measured with a calliper and volume were calculated by the formula $V = 0.5 (\text{length} \times \text{width}^2)$. Randomization into groups was established when tumours reached an average of 70 mm³.

Netrin-1 antibody (NP137) or buffer solution used as a control was injected intraperitoneally into mice three times a week at 20 mg/kg once tumours reached 70 mm³. The FOLFOX regimen (solution combining 5-Fluoro Uracil (50mg/Kg IP), oxaliplatin (6mg/Kg IP) and disodium levofolinate (90mg/Kg IP)) was injected intraperitoneally, once daily for two days when tumours reached the 70 mm³ average. This protocol is routinely used and has been previously reported in the literature by Zoetemelk M et al [311] for the treatment of colorectal cancer liver metastases, where it was found to have minimal impact on mice welfare. Tumour volume was monitored at regular intervals (twice a week). After 10 weeks, mice were sacrificed.

IV. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad, v9.4.1) or R (R, v4.3.2). Data is expressed as the mean, plus or minus SD (Standard Deviation) unless stated otherwise. Data were pooled from a minimum of 3 independent replicated experiments. The threshold for rejecting the null hypothesis was $p < 0.05$; When statistical significance is integrated into a figure, it is denoted as: * for $p < 0.05$; ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p < 0.0001$. Paired t-tests were used in *in vitro* experiments with two conditions (ELDAs, Western Blot, mRNA expression (RNA sequencing, qPCR)), cell lines with a different number of passages being considered as a biological replicate. One-way ANOVA, Turkey's multiple comparison tests was used in *in*

vitro experiments with more than two conditions (ELDAs, Western Blot, mRNA expression (RNA sequencing, qPCR)). One-way ANOVA and Turkey's multiple comparison tests were used to determine the statistical significance difference among mice tumour weight. Two-way ANOVA and Dunnett's multiple comparison tests were used to determine statistical significance differences among mice tumour growth. The precise details on the type of test used, the number of replicates and the representation of the data are given in the figure's legends.

Chapter 3 – The Netrin-1/UNC5B couple in Colorectal Cancer: Expression Levels, pattern, and Context

I- Introduction

As introduced above, the development of metastatic cancer is a complex process involving numerous molecular players, whereas UNC5B and Netrin-1 have emerged as key regulators in several types of cancer.

Studies have demonstrated that Netrin-1, originally characterized for its role in axonal guidance, modulates the apoptosis of cancer cells through its interactions with the UNC5B receptor. In cancer, aberrant expression of UNC5B or Netrin-1 has been associated with enhanced tumour cell motility, invasion of local tissues, and metastasis to distant organs, highlighting the potential involvement of this pathway in processes important for metastatic tumour cells. The upregulation of Netrin-1 has notably been observed in colorectal, breast, and lung cancers, correlating with advanced disease stages and poorer prognosis [178, 312, 313]. Conversely, UNC5B, due to its dependence receptor characteristic, is subject to functional and regulatory alterations impairing its pro-apoptotic functions in several cancer types [314, 315]. However, its upregulation in another type of cancer such as breast or prostate cancer suggests a potential role for the UNC5B receptor in tumorigenesis and cancer cell modulation [165, 286, 308, 316, 317].

Multiple studies have found relationships between Netrin-1 and UNC5B in cancer cell phenotype associated with metastasis across different cancer types. For instance, elevated levels of Netrin-1 have been showed to be a biomarker of aggressiveness in bladder cancer, correlating with muscle invasion and poor prognosis in patients with brain metastasis [178, 208, 209]. This enhancement of metastatic phenotype by Netrin-1 seems to occur through different pathways. For example, mechanistically, Netrin-1 triggers EMT via activation of PI3K/AKT and ERK pathways in non-small cell lung cancer (NSCLC)[313] and hepato-carcinoma metastasis [211]. Whereas, in pancreatic ductal adenocarcinoma (PDAC), PDAC-secreted extracellular vesicles containing Netrin-1 stimulate hepatic stellate cells preparing the metastatic niche to facilitate the dissemination of migratory PDAC cells in the liver. Following activation by Netrin-1, HSCs

secrete retinoic acid absorbed by disseminated tumour cells, leading to the upregulation of Netrin-1 and Elf3 upregulation. Through autocrine signalling via UNC5B receptors, newly arrived disseminated cancer cells enhance their survival potential by secreting Netrin-1. Additionally, activated hepatic stellate cells deposit collagen into the microenvironment, facilitating the establishment of macro-metastases in the liver over time [318].

In addition, netrin-1 also emerges as a significant contributor to tumorigenesis and EMT in skin squamous cell carcinoma and human endometrial carcinomas. The efficacy of NP137, the netrin-1 neutralizing antibody, in reducing tumour progression and inhibiting EMT has been demonstrated in mouse models and supported by clinical trials in endometrial carcinoma patients [215, 216].

While emerging evidence suggests the involvement of UNC5B in the modulation of the Netrin-1 effect on metastatic cancer cells in several types of tumours, its exact role remains poorly described. For instance, knockdown or inhibition of UNC5B in ovarian cancer models has been associated with reduced tumour proliferation and a molecular signature indicative of EMT reversal, implicating UNC5B as a potential therapeutic target, but without any precise pathways put in evidence [212]. Indeed, despite the knowledge that NP137-mediated disruption of netrin-1–UNC5B signalling impairs EMT, little is understood about the pathways linking UNC5B to EMT [216].

Nevertheless, recently, studies found that in bladder cancer, the intracellular domain of UNC5B has been shown to play a crucial role in the promotion of cell proliferation and tumour formation, linking this effect to ribosomal protein [317]. This effect is achieved through the binding of the intracellular domain of UNC5B with numerous ribosomal proteins. Moreover, in the same study, they found that the overexpression of the intracellular domain enhances cellular migration, invasion, and metastasis by interacting with fibronectin, beta-catenin, and vimentin. This effect was independent of Netrin-1 since absent from the model, which interestingly wasn't subject to the apoptotic pathway of UNC5B. Indeed, in this study, the overexpression of the UNC5B receptor or its intracellular domain couldn't activate caspase-3 in bladder cancer cells. This study provides an insight into the potential mechanisms of modulation of EMT by UNC5B,

though, it also highlights the high importance of the context in studies about dependence receptors.

In colorectal cancer specifically, studies observing the expression of the Netrin-1 protein by immunohistochemistry using 60 primary colorectal cancer samples, 47 liver metastasis samples and 40 normal colorectal mucosa reveal that CRC patients with liver metastasis exhibited significantly higher positive expression of Netrin-1 compared to those without metastasis or normal colorectal epithelial mucosa [319]. However, we can note that this study is incomplete since it doesn't include the study of healthy liver tissue as a control for mCRC tumours, disregarding the possibility of an increased expression in liver tissue explaining the difference between primary and metastatic tumours.

Nevertheless, the fact that elevated levels of Netrin-1 in the serum of CRC patients correlate with an increased risk of CRC and liver metastasis suggests an existent role for Netrin-1 in promoting tumour growth, invasion, and modulation of mCRC cells and their microenvironment [319].

Given the key roles of Netrin-1 and UNC5B in metastasis and tumorigenesis across various cancer types, we first aimed to explore their expression patterns in CRC and mCRC. Understanding the expression profiles of UNC5B and NTN1 in CRC and mCRC is the first step towards having valuable insights into their contribution to mCRC cells' phenotype modulation and cellular processes specific to colorectal malignancies.

In this chapter, we performed an exploration of the expression patterns and levels of Netrin-1 and its receptor UNC5B in CRC, with a specific emphasis on mCRC. Using several techniques, our investigation explores multiple facets to provide a comprehensive understanding of these molecules in CRC progression. Initially, we explored large-scale data analyses, including TCGA cohort data, to discern potential trends in Netrin-1 and UNC5B expression across CRC patient populations. Then, we used RNA sequencing data derived from late-stage CRC tumours and organoids from hepatic metastatic CRC tumours, collected and sequenced by Dr Corina Behrenbruch and aligned to the human genome by Dr. Momenh (Sepideh) Foroutan before this project. These datasets allowed us to dissect Netrin-1 and UNC5B expression in the context of advanced CRC

stages, unravelling their potential roles in cellular processes directly linked to mCRC cell phenotype.

Complementing these computational analyses, we implemented immunohistochemical (IHC) staining techniques to detect the expression of Netrin-1 and UNC5B proteins in late-stage CRC tumour samples. In doing so, we aim to confirm the expression of Netrin-1 and UNC5B within the CRC tumour microenvironment. Furthermore, we took advantage of an innovative spatial transcriptomics study performed by Dr Tamara Vu in our laboratory using specifically spatial-visium 10X technology, to visualize gene expression patterns in intact tissue sections. This innovative approach enabled us to observe the spatial organization of Netrin-1 and UNC5B within CRC tumours, offering insights into their potential regulation by tumour-microenvironment interactions. Through this project, we aim to unravel the potential implications of Netrin-1 and its receptor UNC5B in CRC and mCRC tumours.

II- Results

a. Expression pattern of UNC5B and NTN1 in mCRC tumours

In our comprehensive exploration of expression profiles associated with Netrin-1 and its receptor UNC5B in CRC, we analysed both publicly available data from the Integrated TCGA Pan-Cancer Clinical Data and RNA sequencing data generated within our research team. The TCGA cohort, comprising 521 samples, provided a valuable source of information, revealing noteworthy patterns in NTN1 and UNC5B mRNA expression across different stages of colorectal cancer (**Figure 3.1-A & B**). We observed a higher expression of both NTN1 and UNC5B mRNA expression in the T4 stage of CRC compared to early T stages (T1, T2) of CRC ($p < 0.05$ and $p < 0.01$, respectively, One-way ANOVA, Tukey's multiple comparisons test). These findings suggest a potential involvement of Netrin-1 and its receptor UNC5B in disease progression, or molecular mechanisms modulating metastatic phenotype in CRC.

Simultaneously, our in-depth analysis of late-stage colorectal tumours (n=58) and non-pathogenic adjacent tissues (n=34), collected from our research cohort by Dr Cori

Behrenbruch and Dr Sepideh Foroutan, provided valuable data regarding the expression of NTN1 and UNC5B in the mCRC context. Notably, we observed a marked overexpression of UNC5B mRNA expression levels within tumour samples (mean = 0.2421 ± 1.255 logRPKM) compared to normal adjacent tissues (mean = -1.348 ± 1.086 logRPKM) ($p < 0.0001$, unpaired t-test) (**Figure 3.1-C**). This significant disparity highlights the potential involvement of UNC5B in driving cellular processes associated with mCRC modulation as well.

However, our investigation also revealed a noteworthy observation regarding the expression of NTN1 mRNA in late-stage CRC tumours compared to adjacent healthy hepatic tissue. We found a significantly lower expression of NTN1 mRNA in tumours (mean = -1.691 ± 1.396 logRPKM) compared to adjacent healthy hepatic tissue (mean = 0.7483 ± 1.399 logRPKM) ($p < 0.0001$, unpaired t-test). This finding might imply that the liver environment might provide an additional source of netrin-1 beyond the tumour itself, Netrin-1 being produced from both tumours and secreted by other cell types within the microenvironment.

To assess which receptor(s) may be responsible for a potential effect of Netrin-1 in CRC, particularly mCRC cells, we quantified the expression of Netrin-1 receptors mRNA, including DCC, UNC5A, UNC5B, UNC5C, and UNC5D, in our cohort of late-stage tumours (n=58) using RNA sequencing data (**Figure 3.1-D**). The analysis of this data revealed that among all these receptors, UNC5B displayed the highest expression levels ($p < 0.0001$, One-way ANOVA, Tukey's multiple comparisons test), with an expression of 0.2421 ± 1.255 logRPKM, whereas DCC, UNC5A, and UNC5D were found to be largely unexpressed in most mCRC tumours. It is to be noted that UNC5C displayed a level of expression higher than UNC5A, D and DCC (-1.839 ± 1.136 logRPKM), though lower than UNC5B expression (2-log-fold). These findings strongly suggest that UNC5B is the predominant receptor associated with any potential observed effects of Netrin-1 in CRC and mCRC tumours.

Considering these results, we focused our efforts on Netrin-1 and UNC5B in mCRC to understand their specific contributions to this disease.

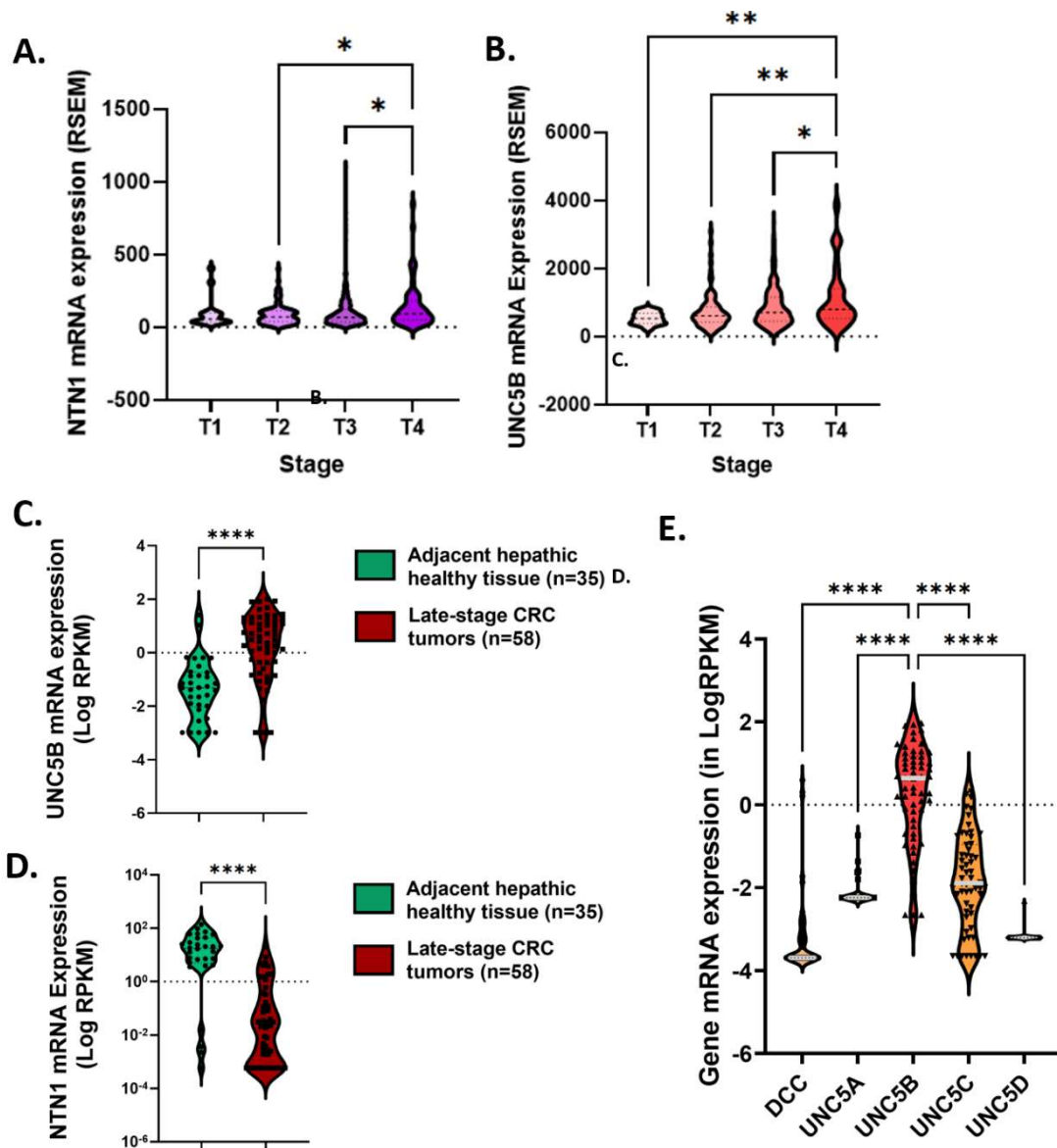


Figure 3.1 - Comparative Analysis of Netrin-1 (NTN1) and UNC5B mRNA (A) Expression Profiles of NTN1 mRNA in Colorectal Cancer TCGA according to T stage (B) Expression Profiles of UNC5B mRNA in Colorectal Cancer TCGA according to T stages ; (C) Comparison of mRNA Expression in Healthy adjacent liver and Late-stage colorectal tumour tissues for UNC5B (D) and NTN1; (TCGA cohort: n=521; late stage tumours cohort: n=58, Adjacent healthy tissue cohort: n=34); (E) Expression of Netrin-1 receptors (DCC, UNC5A-D) mRNA in late-stage CRC tumours (n=58) (bars indicate means); p Values: * = <0.05, ** = <0.01, **** = <0.0001; Statistical test: One-Way ANOVA - Tukey's multiple comparisons test for (A), (B) and (E), unpaired t-tests for (C) and (D)

b. Spatial distribution of Netrin-1 and UNC5B in colorectal cancer

To explore the expression levels of the Netrin-1 protein in colorectal tissues, we conducted an analysis using the Human Protein Atlas (HPA) database where we can find the profile of Netrin-1 protein expression detected in pathological tissue sections by immunohistochemical staining (using sigma-Aldrich antibody HPA056419) [320]. The investigation revealed that CRC stands as the fourth most prevalent cancer type among

patients exhibiting Netrin-1 protein expression, behind thyroid cancer, breast cancer, and melanoma cancer, though in comparable proportions (**Figure 3.2-A**). Specifically, this study indicated that Netrin-1 protein expression was detected at varying levels in 75% of the samples examined, with 66% showing a medium to high expression (**Figure 3.2-B**). This analysis not only demonstrates the relevance of studying the Netrin-1 protein in the context of CRC but also allows us to note that Netrin-1 expression is mainly localized at the cell membrane with a noticeable expression in the cytoplasm. The representative tissue staining results depicting medium expression levels in colorectal adenocarcinoma samples from an 86-year-old female patient, serves as an illustrative example of Netrin-1 expression in CRC tissues, with a combination of extracellular membrane and cytoplasmic staining (**Figure 3.2-C**). These results corroborate the prominent expression of Netrin-1 in the majority of colorectal cancer samples, warranting further investigation into its functional significance and clinical implications.

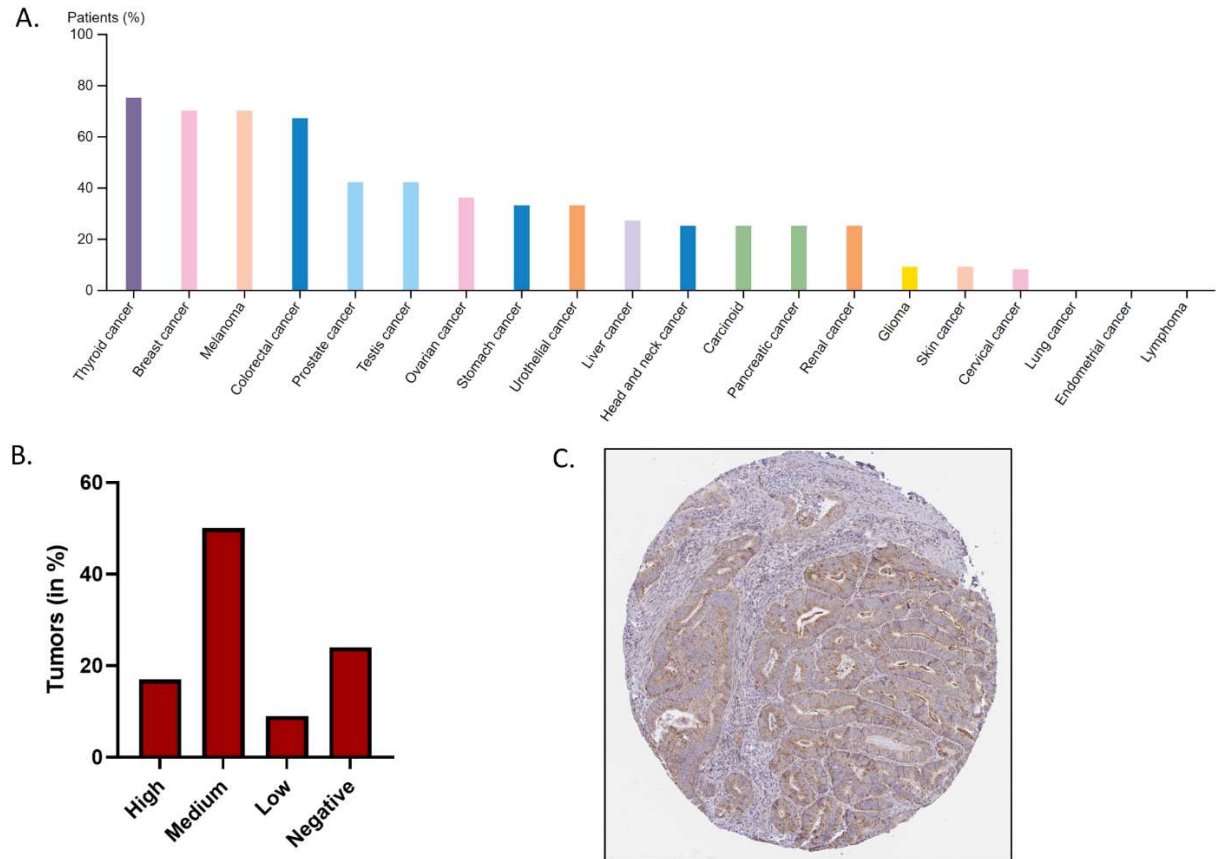


Figure 3.2 – NTN1 expression in colorectal cancer tissues (in The Human Protein Atlas (HPA) database) **(A)** NTN1 protein expression profiles in tumoral human tissues **(B)** NTN1 protein expression data in colorectal cancer patient tissues (in 12 patients) **(C)** Representative IHC image of NTN1 expression in normal colon cancer

To further confirm the expression of Netrin-1 protein in colorectal tumours, particularly in late-stage colorectal tumours, we employed a different antibody (Manufacturer: Abcam; Ref ab122903) to analysing the expression of Netrin-1 in 8 metastatic hepatic CRC tumours (VJR275LT, yPTM089LT, SJB486LT, PSH095LT, yPDW886LT, yPPM845LT, yPDC275LT, and yPPJ102LT) using IHC. These late-stage colorectal tumour samples (obtained by our laboratory under the supervision of Dr. Behrenbruch) enabled us to confirm the expression of Netrin-1 protein in liver metastasis samples (**Figure 3.3**). Through this approach, we successfully verified the presence of Netrin-1 at various levels within late-stage colorectal tumours. Notably, our immunohistochemical staining confirmed the predominant localization of Netrin-1 expression in both the cytoplasmic and membranous compartments of tumour cells. These findings are coherent with studies previously made and display various expressions of Netrin-1 among late-stage colorectal tumours.

In addition to exploring the expression of Netrin-1, we also investigated the expression pattern of the UNC5B receptor protein in 8 late-stage colorectal tumours (yPPM845LT, yPTM089LT, SJB486LT, PSH095LT, yPAM872LT, yPPJ102LT, yPDC275LT, and yPDW886LT) using IHC (**Figure 3.4**). Our findings revealed diverse expression levels of UNC5B across late-stage colorectal tumours. Notably, IHC staining unveiled a predominant membranous expression pattern of the UNC5B receptor protein, indicative of its localization within the cellular membrane. Additionally, we observed a minor cytoplasmic component of UNC5B expression within the tumour cells.

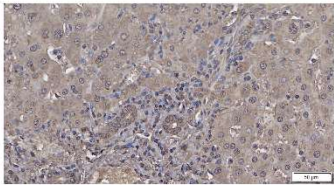
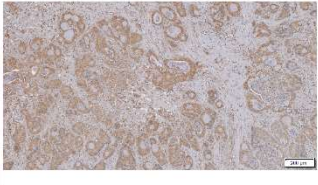
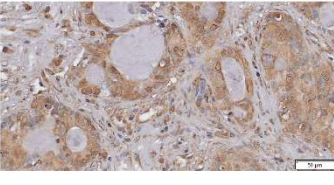
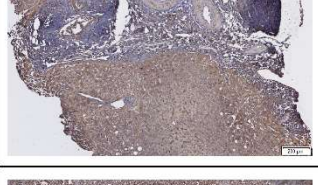
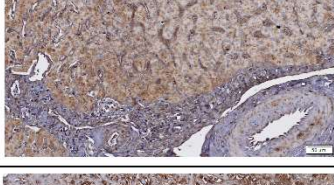
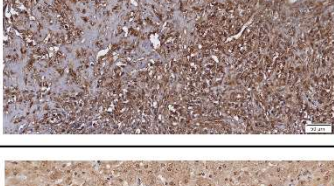
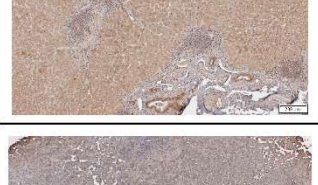
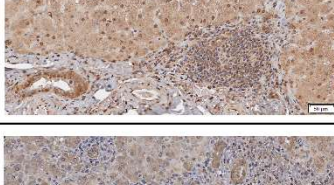
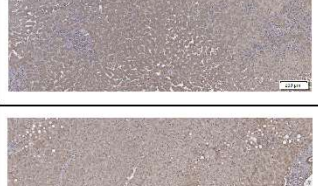
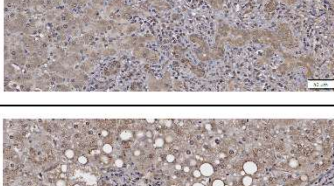
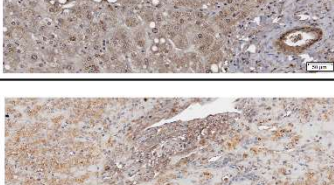
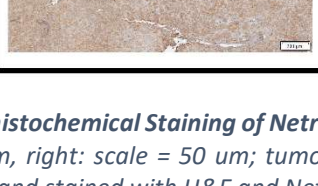
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Figure 3.3 Immunohistochemical Staining of Netrin-1 in a panel of Late-Stage Colorectal Tumour Sections (left: scale = 200 um, right: scale = 50 um; tumour tissues were fixated with formalin, embedded with paraffin, sectioned, and stained with H&E and Netrin-1Abcam Antibody (Ref ab122903))

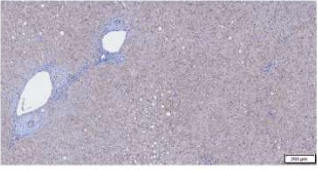
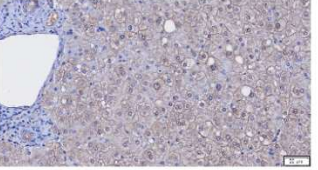
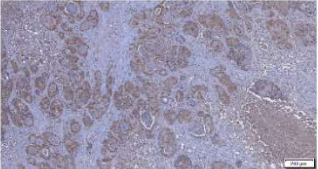
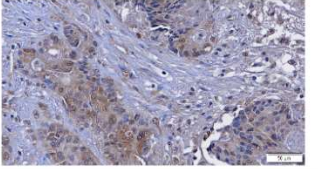
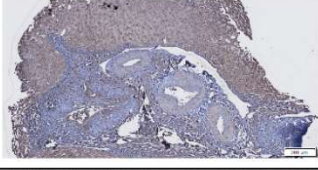
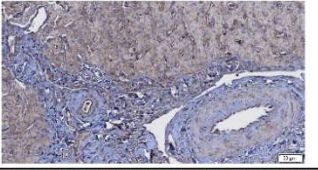
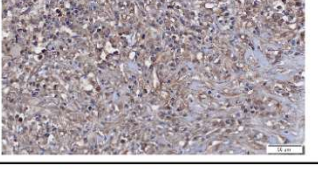
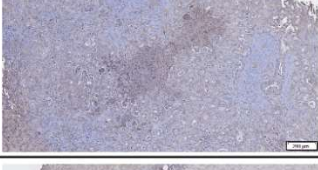
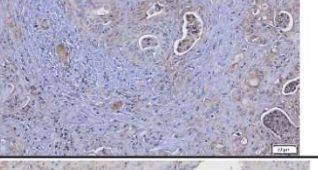
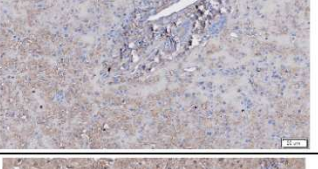
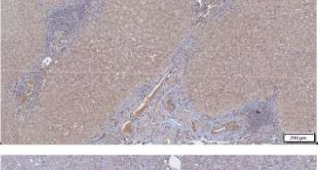
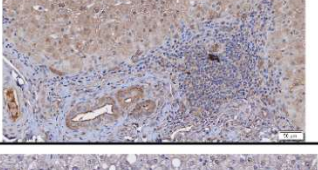
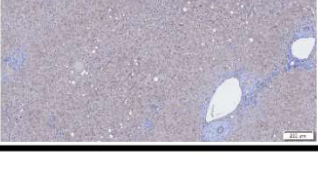
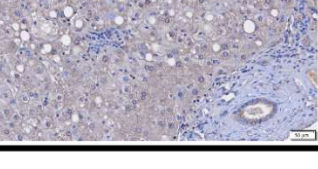
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yPDC275LT		

Figure 3.4 Immunohistochemical Staining of UNC5B protein in a panel of Late-Stage Colorectal Tumour Sections (left: scale = 200 um, right: scale = 50 um; tumour tissues were fixated with formalin, embedded with paraffin, sectioned, and stained with H&E and UNC5B antibody (R&D System; Ref AF1006))

Ongoing investigation (performed by Tamara Vu and Jessica Tang) regarding transcriptional landscape of late-stage CRC tumours using Visium 10X allowed us to have an insight into the expression patterns of NTN1 (Netrin-1) and UNC5B mRNA in mCRC tumours (**Data not shown**). Through this approach, we confirmed the expression of both UNC5B and Netrin-1 at the mRNA level in late-stage tumours but also uncovered intriguing findings regarding their spatial distribution. Data suggests that the cells expressing Netrin-1 and UNC5B may not overlap entirely within the tumour microenvironment. Indeed, analysis of the Visium 10X data revealed that the *UNC5B* gene expression pattern seems to differ from the *NTN1* expression one within the tumour. Suggesting that cells expressing UNC5B within the tumour could not be the ones expressing the most Netrin-1. Though, further investigations are needed to truly comprehend this relationship and describe more precisely the cellular types associated with the expression of these genes.

In conclusion, our investigation into the expression patterns of Netrin-1 and its receptor UNC5B in late-stage colorectal tumours has demonstrated their expression in CRC and mCRC, with diverse expression levels observed. Notably, both proteins exhibited predominantly membranous localization, relevant to their receptor/ligand functions within the tumour microenvironment. Furthermore, our exploration of mRNA expression patterns using Visium 10X spatial transcriptomics analysis not only corroborated the expression of NTN1 and UNC5B at the transcriptomic level but also revealed findings regarding their spatial distribution within the tumour, suggesting potential heterogeneity in the expression patterns of Netrin-1 and its receptor UNC5B, with distinct cells expressing these genes at the mRNA level.

After this exploration of Netrin-1 and UNC5B expression patterns in late-stage CRC tumours, our attention now turns to the investigation of molecular dynamics of this axis within our model of mCRC organoid cell lines. Based on the potential implication raised from tumour analyses, we aim to unravel the characteristics of Netrin-1 and UNC5B signalling pathways in a controlled laboratory setting, using our organoid model to elucidate their functional roles in mCRC cells.

c. Netrin-1 and UNC5B expression in organoid cell lines derived from hepatic colorectal cancer metastasis

In this section, we examined Netrin-1 and UNC5B expression levels within our organoid cell lines derived from metastatic colorectal tumours. Using RNA sequencing data collected by Dr. Fouroutan and Dr. Cori Behrenbruch.

Our investigation into Netrin-1 expression levels revealed interesting disparities between tumour tissues and organoid cell lines. Despite the presence of the *NTN1* gene expression in both settings, organoids exhibited significantly lower levels compared to tumour tissues. The mean expression level of *NTN1* in organoids was measured at -3.105 ± 0.395 logRPKM, indicating a relatively low expression. In contrast, tumour tissues demonstrated a mean expression level of -1.691 ± 1.396 logRPKM, significantly higher than that observed in organoids ($p < 0.0001$, unpaired t-test) (**Figure 3.5-A**). This substantial difference is coherent with the previous findings suggesting that Netrin-1 in late-stage tumours may be derived not only from cancer cells but mainly from surrounding non-cancer cells that populate the tumour microenvironment. Whereas *UNC5B* expression levels remained comparable between tumours tissues (mean = 0.2421 ± 1.255 logRPKM) and organoids (mean = 0.3140 ± 1.462 logRPKM, ($p < 0.0001$, unpaired t-test) (**Figure 3.5-B**). This similar expression between the expression of UNC5B in tumours and organoids suggests the relevance of our organoid model to studying the implications of this receptor in mCRC cells.

Analysis of Netrin-1 receptor expression in organoid cell lines confirmed that UNC5B should be the predominant Netrin-1 receptor in mCRC cells, at least at the mRNA level (**Figure 3.5-C**). The *UNC5B* gene exhibited robust expression across most organoid lines. In contrast, other Netrin-1 receptors, including DCC, UNC5A, and UNC5C, displayed minimal to no expression levels in organoids. This emphasizes the specificity of UNC5B as the primary receptor responsible for the potential effects of Netrin-1 in our mCRC organoid models.

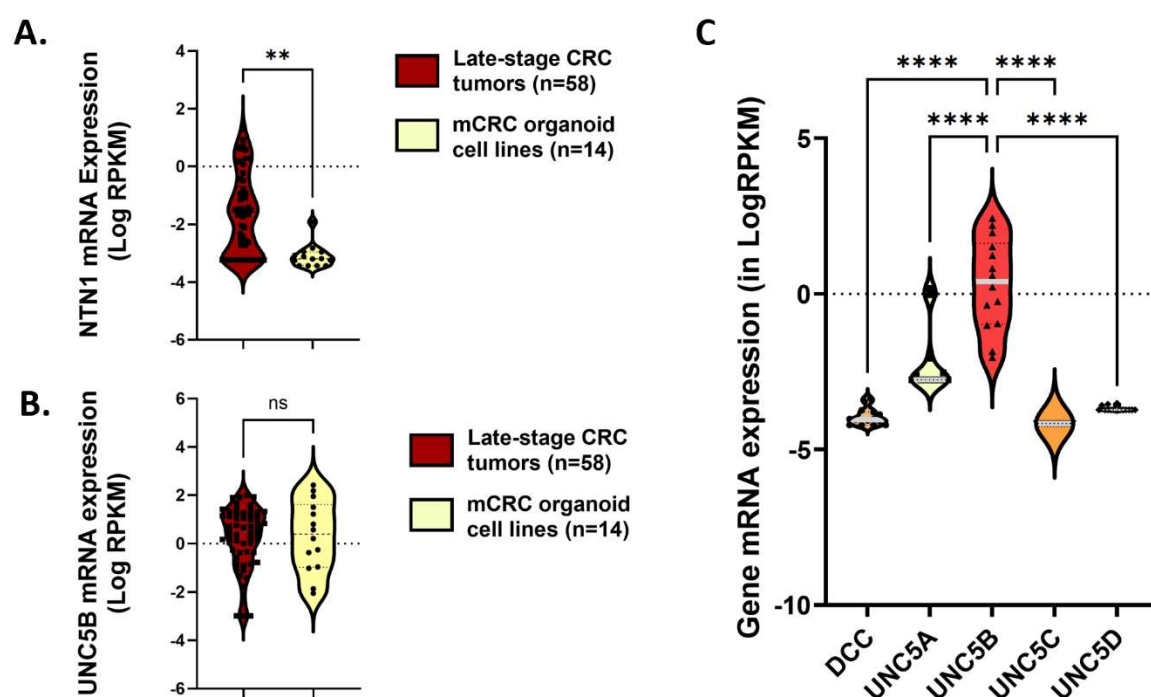


Figure 3.5 - Characterization of the expression of Netrin-1 and its receptors in mCRC organoids by RNA sequencing (A) Comparison of Netrin-1 (NTN1) expression levels in late-stage colorectal tumour tissues (n=58) and organoids derived from metastatic colorectal tumours (n=14); (B) Comparison of UNC5B expression levels in late-stage colorectal tumour tissues (n=58) and organoids derived from metastatic colorectal tumours (n=14); (C) Summary of mRNA expression levels of various Netrin-1 receptors in organoids derived from metastatic colorectal tumours (n=14). Normalized gene expression is expressed in logRPKM. Statistical significance was determined using unpaired t-tests for (A) and (B) and One-way Anova Tukey's multiple comparison tests for (C), ns = non-significant, ** = $p < 0.01$, **** = $p < 0.0001$.

To further investigate the expression of the UNC5B receptor in mCRC-derived organoid cell lines, we quantified the expression of UNC5B protein using Western blot across six different organoid cell lines derived from metastatic colorectal tumours: ySAW190LT, yPDB516LT, yPMK295LT, PIK315LT, VJR275LT, and PCC002LT (**Figure 3.6-B**). These cell lines were chosen to represent a spectrum of UNC5B expression levels in mCRC organoids based on the expression levels of UNC5B mRNA observed in our RNA sequencing data (**Figure 3.6-A**). Furthermore, these PDO cell lines were also selected to represent a range of responses to chemotherapy (from sensitive to resistant) in the provision of further analysis regarding the role of Netrin-1 and UNC5B in chemoresistance and recurrence of mCRC tumours.

Through Western blot analysis, we evaluated UNC5B protein expression levels in these organoid cell lines. Our findings revealed consistent results between mRNA and protein

expression. This alignment not only confirms the presence of UNC5B receptor expression in organoid cell lines but also highlights the reliability and relevance of characterizing mRNA levels to study UNC5B-mediated signalling pathways.

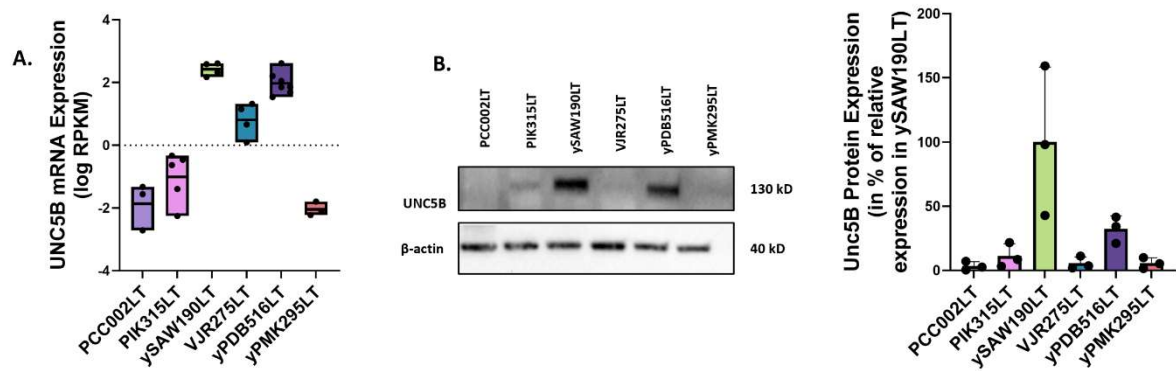


Figure 3.6 - Comparison of UNC5B Expression at mRNA and Protein Levels in Organoid Cell Lines (A) UNC5B mRNA expression in the 6 selected organoid cell lines (3 to 5 replicates per organoid cell lines; units in logRPKM) **(B)** Western Blot of the UNC5B protein in the 6 selected organoid cell lines (using β -actin as loading control; 3 replicates per organoid cell line; units in % of expression comparatively to ySAW190LT mean expression) Left: Western Blot, Right: representative histogram of UNC5B protein expression in organoid cell lines)

It is to be noted that UNC5B and NTN1 mRNA expression correlates positively in both TCGA cohort and in late-stage CRC tumours ($R=0.54$, $p = 7.46 \cdot 10^{-41}$ and $R=0.29$, $p = 0.025$ respectively) (**Figure 3.7**). This positive correlation between UNC5B and Netrin-1 expression in CRC suggests that tumour cells may upregulate both molecules to enhance survival and proliferation and is coherent with the dependence receptor model.

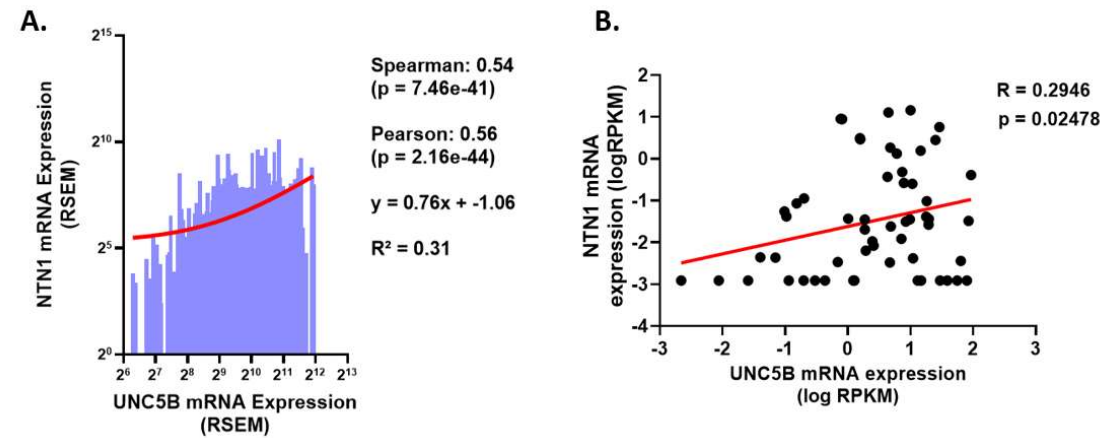


Figure 3.7 – Correlation between NTN1 and UNC5B mRNA expression (A) Correlation between UNC5B mRNA and NTN1 mRNA expression in TCGA cohort **(B)** Correlation between UNC5B mRNA and NTN1 mRNA expression in late-stage CRC tumours; (TCGA cohort: $n=521$, late-stage tumours

cohort: n=58); Statistical tests : (A) Spearman/Pearson, (B) Spearman's rank correlation coefficient (all values are displayed on graphs)

d. UNC5B and Netrin-1 expression correlate with that of genes related to epithelial-to-mesenchymal transition and stem-like phenotypes

Given the established involvement of Netrin-1 and UNC5B in promoting EMT and metastasis in other cancers, as evidenced in skin squamous cell carcinoma and human endometrial carcinomas, we sought to investigate the potential associations between *UNC5B* and *NTN1* mRNA expression and EMT-related genes, as well as genes related to metastasis initiation, using data from TCGA cohort (n=521), our library of late-stage CRC samples (n=58), and mCRC organoid cell lines (n=28). This exploration aims to assess the possible correlation between high expression of Netrin-1 and UNC5B and these processes. This is a first step towards determining if Netrin-1 plays a role in the regulation of EMT and metastasis initiation in CRC.

Our analysis reveals a strong positive correlation between the expression levels of *UNC5B* and *NTN1* genes and genes associated with EMT. Notably several transcription factor genes are pivotal in orchestrating EMT, including ZEB1, ZEB2, and SNAI2 (**Figure 3.8 - A-F**). ZEB1 and ZEB2 are members of the zinc finger E-box-binding homeobox (ZEB) family and are known to repress E-cadherin expression, promoting EMT and enhancing cancer cell invasion and metastasis. Similarly, SNAI2, also known as Slug, regulates EMT by suppressing E-cadherin expression and activating mesenchymal genes, thereby promoting tumour progression and metastasis. Moreover, our findings demonstrate significant correlations between *UNC5B* and *NTN1* expression and VIM mRNA expression (**Figure 3.8- G & H**). VIM being the gene coding for the Vimentin protein, an intermediate filament protein playing a role in the maintenance of cell shape, integrity, and signalling during EMT. These correlations suggest the existence of a link between *UNC5B* and *NTN1* expression and EMT processes in colorectal cancer.

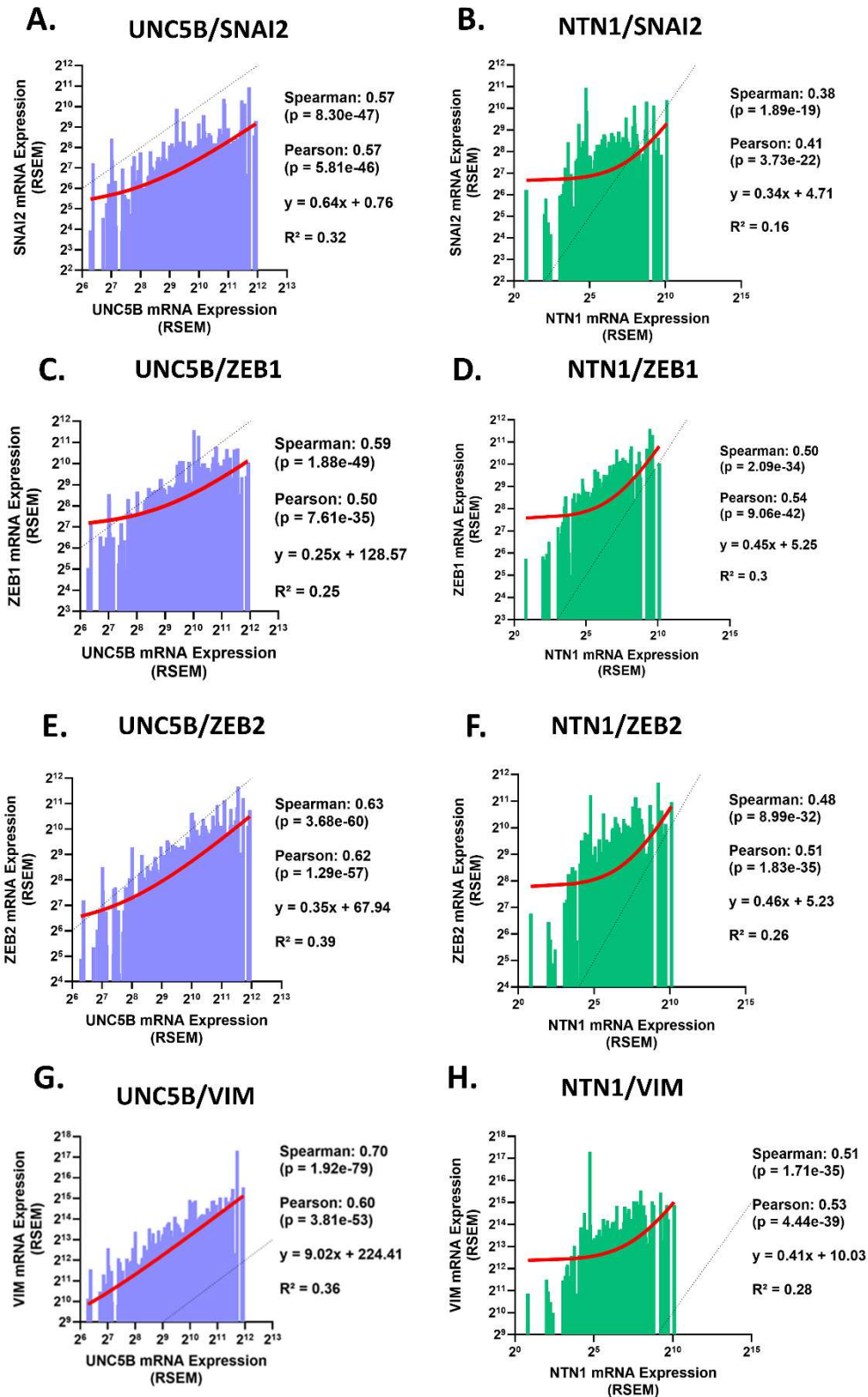


Figure 3-8 – Correlation between NTN1, UNC5B and Epithelial-to-Mesenchymal transition-related genes expression in the TCGA colorectal cancer cohort (A) Correlation between UNC5B mRNA and VIM mRNA expression (B) Correlation between NTN1mRNA and VIM mRNA expression (C) Correlation between UNC5B mRNA and ZEB1mRNA expression (D) Correlation between NTN1mRNA and ZEB1 mRNA expression (E) Correlation between UNC5B mRNA and ZEB2 mRNA expression (F) Correlation between NTN1mRNA and ZEB2 mRNA expression (G) Correlation between UNC5B mRNA and SNAI2 mRNA expression (H) Correlation

between NTN1mRNA and SNAI2 mRNA expression; (TCGA cohort: n=521); Statistical tests: Spearman/Pearson (all values are displayed on graphs)

To investigate the correlation between the EMT-associated phenotype and *UNC5B* mRNA expression in late-stage CRC, we employed the "song score" method, a robust tool that quantifies and summarizes the relative expression levels of signature genes from individual samples by incorporating stably expressed genes [321]. Utilizing this method, we applied an EMT score to each of the tumour samples using the JP. Thiery EMT signature, a widely recognized and validated set of genes indicative of EMT across various cancer types [322]. This approach allowed us to establish an EMT score across the late-stage CRC tumours in our cohort. Notably, our analysis revealed a significant and positive correlation between *UNC5B* mRNA expression and EMT-related gene signature in late-stage CRC (**Figure 3.9-A**), confirming the strong association observed in the TCGA cohort. Specifically, we found a Pearson correlation coefficient (R) of 0.58 and a p-value of 1.6×10^{-6} , indicating a robust relationship between *UNC5B* expression and the EMT process in late-stage CRC. Moreover, using the same method, we found a significant correlation between the expression of *NTN1* and the same EMT signature (**Figure 3.9-B**) with a correlation coefficient of 0.27 and a p-value of 0.037.

Following our observations in stage 4 CRC tumours, we further investigated the potential involvement of *UNC5B* in EMT in our model mCRC organoid cell lines and examined the relationship between *UNC5B* expression and EMT-related genes. Our analysis revealed a negative correlation between *UNC5B* expression and *CDH1*, which encodes E-cadherin (**Figure 3.9-B**). E-cadherin is a critical cell adhesion molecule that plays a pivotal role in maintaining epithelial integrity and tissue architecture. Downregulation of E-cadherin is a hallmark event of EMT and leads to the loss of cell-cell adhesion and acquisition of a mesenchymal phenotype.

In our investigation of *UNC5B* and Netrin-1 expression with stemness and EMT, we explored their correlation with Nanog and SOX4 transcription factors (**Figure 3.9 - D-F**). Nanog is a key regulator of stem cell pluripotency, playing a crucial role in maintaining self-renewal and inhibiting differentiation [323]. Its expression has been linked to the acquisition of stem cell-like properties in cancer cells, contributing to tumour initiation,

progression, and therapy resistance. On the other hand, SOX4 is a transcription factor involved in various cellular processes, including embryonic development, cell fate determination, and tumorigenesis. In cancer, SOX4 has been associated with promoting EMT, driving metastasis, and enhancing cancer stem cell properties [324, 325]. Our findings revealed significant correlations between *UNC5B* mRNA expression and Nanog, indicating a potential role for *UNC5B* in regulating stemness in CRC. Additionally, we observed correlations between *NTN1* and *NANOG*, as well as *SOX4*, suggesting a possible involvement of Netrin-1 in stem cell maintenance processes in CRC. These correlations raise the possibility of an interplay between *UNC5B*, Netrin-1, stemness, and EMT in CRC and mCRC.

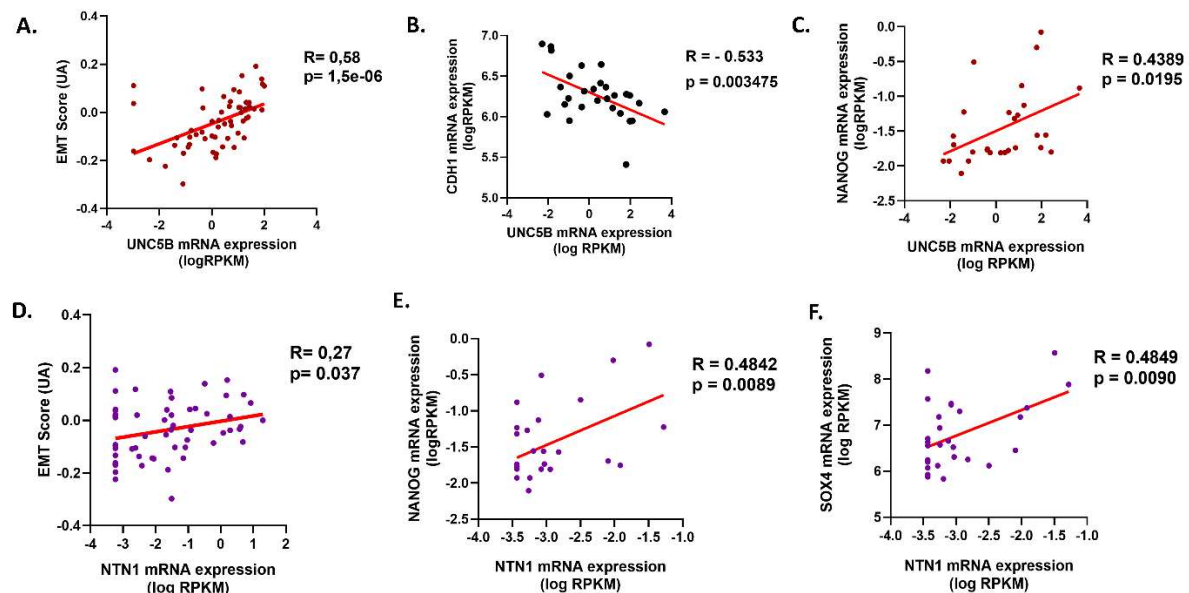


Figure 3.9 - *UNC5B* and *NTN1* mRNA expression correlates with EMT and stemness related genes in late-stage CRC tumours and mCRC organoids (A) Correlation between *UNC5B* mRNA and EMT-signature Score (JP.Thiery EMT signature) in late-stage CRC tumours (B) Negative correlation between *UNC5B* mRNA and *CDH1* mRNA expression (C) Correlation between *UNC5B* mRNA and *NANOG* mRNA expression (D) Correlation between *NTN1* mRNA and EMT-signature Score (JP.Thiery EMT signature) in late-stage CRC tumours; (E) Correlation between *NTN1* mRNA and *NANOG* mRNA expression (F) Correlation between *NTN1* mRNA and *SOX4* mRNA expression; (late stage tumours cohort: $n=58$, mCRC organoids: $n=28$); Statistical tests: Spearman's rank correlation coefficient (All values are displayed)

e. Regulation of *UNC5B* expression in chemotherapy-treated mCRC

We aim to characterize the role of the UNC5B/Netrin-1 pathway in mCRC cells to explore the therapeutic potential of targeting this pathway to enhance the efficacy of the current treatment. Therefore, to confirm that the targeting of Netrin-1 is still relevant during chemotherapeutic treatment, we had to assess that the UNC5B receptor was still expressed upon chemotherapy. To do so, we investigated the expression of the UNC5B receptor using RNA sequencing upon treatment with FOLFOX, the most frequent chemotherapy regimen used in patients with mCRC, comprising folinic acid, fluorouracil, and oxaliplatin. It is to be noted that FOLFOX treatment includes leucovorin in a clinical setting to diminish the toxicity of 5-FU, leucovorin being non-relevant in an *in vitro* setting, we used 5-FU/Oxaliplatin treatment in vitro experiments (referred to as FOLFOX treatment). Using our organoid model to recapitulate *in vitro* the tumour responses to chemotherapy, we compared the expression of *UNC5B* in vehicle-treated organoids with those treated with FOLFOX (**Figure 3.10-A**). The first observation was that UNC5B is still expressed in mCRC organoids at the end of FOLFOX exposure (n=14), supporting the potential interest of prospective investigations regarding the potential of a combination treatment between commonly used chemotherapy and Netrin-1 inhibition using the NP137 antibody. Moreover, we observed a noticeable upregulation of the UNC5B mRNA expression upon FOLFOX treatment in four of our mCRC organoid cell lines: ySAW190LT, PSH095CoT, PSH095LT, and SSF997CoT, following treatment with FOLFOX chemotherapy (25 μ M; for 72 hours) (**Figure 3.10-B - E**).

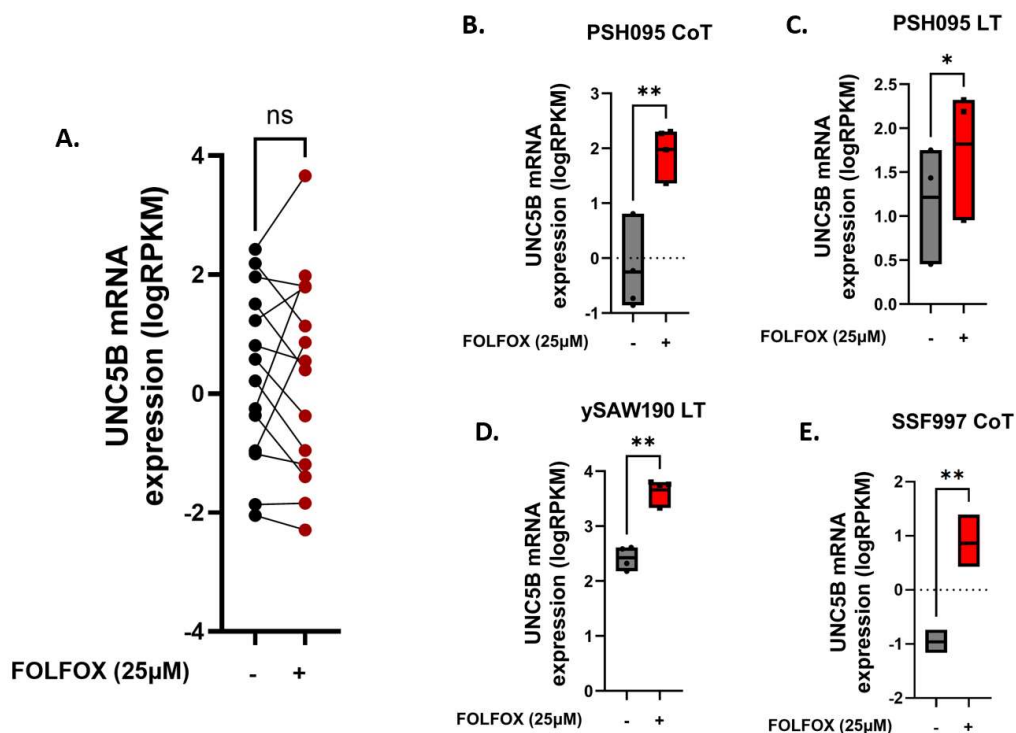


Figure 3.10 – Upregulation of UNC5B mRNA expression upon FOLFOX treatment in mCRC PDOs (A) UNC5B mRNA expression in mCRC PDOs (n=14) after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; **(B)** UNC5B mRNA expression in **PSH095CoT** PDO after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; **(C)** UNC5B mRNA expression in **PSH095LT** PDO after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; **(D)** UNC5B mRNA expression in **ySAW190LT** PDO after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; **(E)** UNC5B mRNA expression in **SSF997CoT** PDO after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; Statistical significance was determined using paired t-tests, * = p < 0.05, ** = p < 0.01.

We further investigated the common traits between these organoid cell lines that might explain this overexpression of the UNC5B receptor upon treatment with chemotherapy. During our investigations, we noticed that these four organoid cell lines (ySAW190LT, PSH095CoT, PSH095LT, SSF997CoT) do not carry a mutation of the *TP53* gene (results from C. Behrenbruch and S. Foroutan) and they all expressed a high level of *TP53* mRNA (**Table 3-1**). This suggested that the observed UNC5B overexpression upon FOLFOX treatment may be attributed to the activation of the P53 pathway following chemotherapy treatment. Previous studies by Cho et al. in 2020 and Tomicic et al. in 2019 have observed the activation of P53 by 5-FU or Oxaliplatin, components of the FOLFOX regimen [326, 327]. Additionally, the regulation of the Netrin-1 pathway by P53 has also been documented [165]. To investigate this possibility, we examined the

expression of CDKN1A mRNA, which codes for the p21 protein—a well-known downstream target of P53, in mCRC PDOs upon FOLFOX treatment (**Figure 3.11-A**). Doing so, we observed a significant increase in the expression of CDKN1A mRNA, confirming the activation of P53 in our mCRC organoid model following FOLFOX treatment. Based on this information, we quantified the expression of UNC5B mRNA upon FOLFOX treatment in TP53^{high} organoids (expression superior to mCRC organoids mean expression: 1.726 logRPKM) and confirmed the significant increase of UNC5B mRNA in this particular sub-group (**Figure 3.11-B**). It is to be noted that the increase of expression of UNC5B was not significant in PCC002LT (-1.865 ± 0.741 logRPKM vs -1.845 ± 1.233 logRPKM) despite the high expression of TP53 in this organoid cell line. However, the PC002LT cell line exhibits a missense mutation of the TP53 gene which explains the absence of UNC5B upregulation in this cell line. Consequently, we excluded it from the TP53-high expressing sub-group.

Organoid Cell Line	TP53 status	Number of replicates	Minimum	Maximum	Range	Mean	Std. Deviation
PCC002LT	Mut	3	4.117	4.45	0.3332	4.287	0.1667
PSH095CoT	WT	4	3.856	4.497	0.641	4.18	0.2818
ySAW190LT	WT	4	3.801	4.807	1.007	4.156	0.4525
PSH095LT	WT	3	3.567	4.018	0.4507	3.782	0.226
SSF997CoT	WT	3	3.051	3.445	0.3946	3.276	0.2032
yPJF520LT2	WT	4	0.5549	1.345	0.7904	0.9931	0.3516
yPJF520LT1	WT	3	0.5744	1.091	0.5168	0.8771	0.2696
yPMK295LT	WT	3	0.3849	1.341	0.9561	0.827	0.4821
yPDB516LT2	Mut	7	0.1137	1.399	1.285	0.794	0.4463
VJR275 CoT1	Mut	4	0.1799	0.9995	0.8196	0.5822	0.3483
yPDB516LT1	Mut	5	0.1179	0.796	0.6781	0.5266	0.2556
PIK315LT	Mut	5	-0.3397	0.604	0.9437	0.02915	0.3704
VJR275 CoT2	Mut	3	-0.02626	0.03451	0.06077	0.01121	0.03277
VJR275 LT	Mut	4	-0.804	0.5367	1.341	-0.1691	0.5514

Table 3-3 Recapitulative table of TP53 mRNA expression in mCRC organoid cell lines – Expression determined by RNA sequencing expressed in logRPKM – Organoid cell lines are ranked according to their TP53 expression (top: high expression, down low expression) – organoid cell lines exhibiting an increased expression of UNC5B mRNA upon FOLFOX (25uM, 72h) has been highlighted in red.

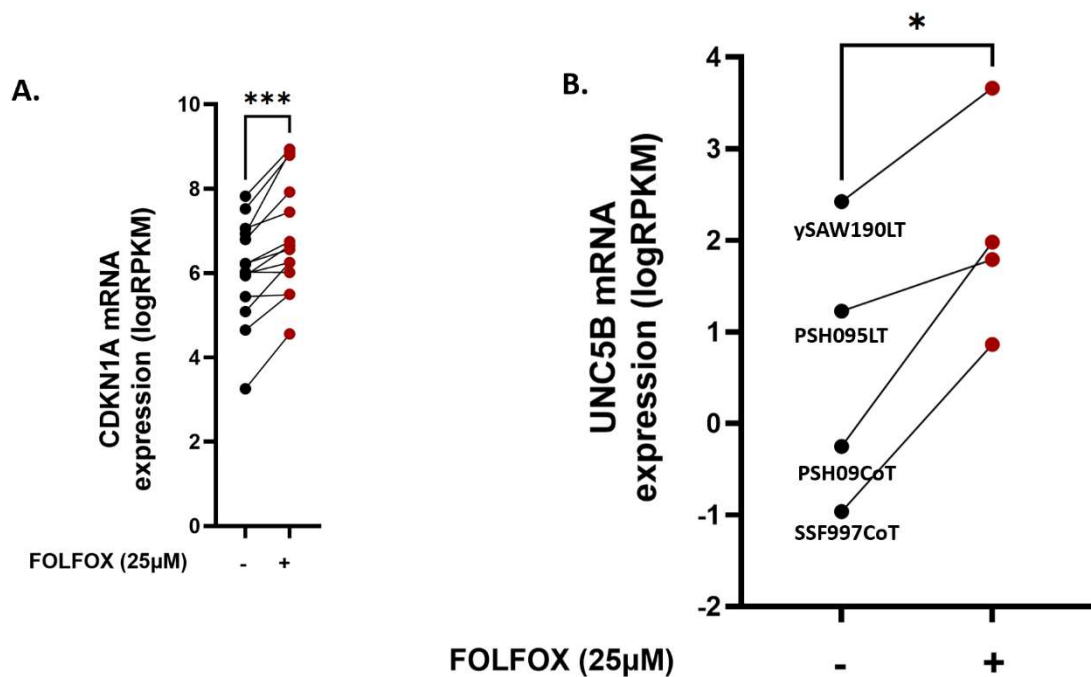


Figure 3-11 - Upregulation of P53 activation and UNC5B expression upon FOLFOX treatment in mCRC PDOs (A) CDKN1A mRNA expression in mCRC PDOs after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; **(B)** UNC5B mRNA expression in TP53^{high} PDOs after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; Statistical significance was determined using paired t-tests, * = $p < 0.05$, *** = $p < 0.001$.

To further explore the role of P53 in the regulation of *UNC5B* and *NTN1* gene transcription, we investigated the effect of an activator of P53, Nutlin 3A, on their expression levels. Nutlin 3A is a small molecule that inhibits the interaction between P53 and its negative regulator MDM2, leading to the stabilization and activation of P53. By using Nutlin 3A, we aimed to mimic the activation of P53 observed during chemotherapy treatment and assess its impact on the transcription of *UNC5B* and *NTN1* genes. As a control for P53 activation, we monitored the expression of the *CDKN1A* gene, which is known to be upregulated upon P53 activation. We performed quantitative PCR (qPCR) to measure the mRNA levels of *UNC5B*, *NTN1*, and *CDKN1A* in our experimental conditions. Upon treatment with 5 or 10 μM of Nutlin3A, we observed a significant increase in both *UNC5B* mRNA (approximately 6-fold change, $p < 0.05$, paired t-test) and *NTN1* mRNA (approximately 4-fold change, $p < 0.05$, paired t-test) compared to the non-treated condition (**Figure 3.12-B and C**). Additionally, we confirmed the activation of p53 by Nutlin 3A treatment through the significant upregulation of *CDKN1A* mRNA

expression (approximately 20-fold change) (**Figure 3.12-A**). These findings suggest that P53 activation indeed modulates the UNC5B and Netrin-1 pathways in mCRC, both in the presence and absence of chemotherapy. This might suggest that patients with a non-mutated form of the TP53 gene could benefit to a greater extent from a combination of therapy composed of FOLFOX and a molecule targeting UNC5B, although further investigation is warranted to fully understand the implications of these interactions between the p53 pathway and the UNC5B/Netrin-1 axis.

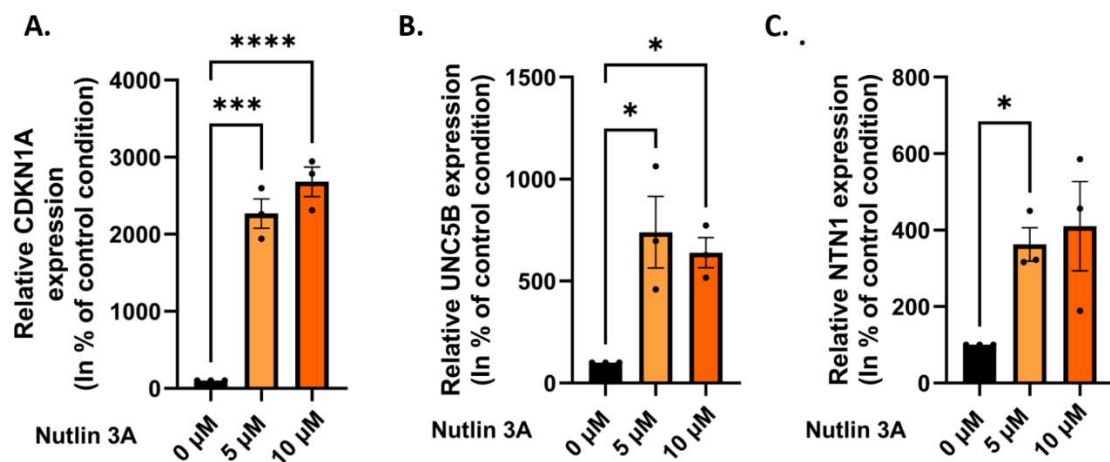


Figure 3-12 – Crosstalk Netrin-1 pathway and P53 pathway during chemotherapy treatment (A) Quantification of relative *CDKN1A* (p21) mRNA expression level in ySAW190LT PDO after 24h of treatment with either the vehicle or Nutlin 3A (P53 activator) (5 or 10 μM) by real-time qPCR; **(B)** Quantification of relative *UNC5B* mRNA expression level in ySAW190LT PDO after 24h of treatment with either the vehicle or Nutlin 3A (P53 activator) (5 or 10 μM) by real-time qPCR; **(C)** Quantification of relative *NTN1* mRNA expression level in ySAW190LT PDO after 24h of treatment with either the vehicle or Nutlin 3A (P53 activator) (5 or 10 μM) by real-time qPCR; Statistical significance was determined using paired t-tests; Error bar indicate SD ; * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

III- Conclusion and Discussion

Our investigation into the expression profiles of Netrin-1 and its receptor UNC5B in colorectal cancer aimed at unravelling the potential implications of this pathway in mCRC cells phenotype modulation. Using a combination of publicly available data from the Integrated TCGA Pan-Cancer Clinical Data and RNA sequencing data obtained from our research, we analysed patterns of expression of Netrin-1 and UNC5B across different stages of CRC. The TCGA cohort, consisting of a substantial 521 samples, provided a rich source of information, allowing us to discern significant upregulation of both NTN1 and

UNC5B mRNA levels in later stages of CRC, suggesting their potential involvement in the modulation of CRC metastatic cells.

Moreover, our investigation extended beyond mRNA expression analysis to explore the protein-level expression of Netrin-1 and UNC5B in late-stage CRC tumours. Through immunohistochemical analysis, we confirmed the presence of Netrin-1 and UNC5B proteins in tumour tissues, unveiling a diverse expression pattern across patients. Notably, both proteins exhibited predominant membrane localization, coherent with their functional role, and suggesting their involvement in mediating cell-cell communication.

We also have observed various levels of expression for both Netrin-1 and UNC5B mRNA and protein in mCRC organoids, suggesting their involvement in mCRC cells may not apply to all CRC tumours. Moreover, the higher expression of Netrin-1 in healthy liver tissues compared to mCRC tumours and organoids suggests a potential role for non-cancerous cells in secreting Netrin-1 within the tumour microenvironment, a phenomenon previously documented in colorectal CAFs by Sung et al. in 2019 [164]. This observation aligns with our Visium data, which suggests distinct expression patterns between *UNC5B*-expressing and *NTN1*-expressing cells within the tumour landscape.

In contrast, our study demonstrates comparable UNC5B expression levels between late-stage tumours and mCRC organoids, supporting the use of this model to investigate the implications of the UNC5B receptor in metastatic colorectal tumours.

These findings raise intriguing questions about the specific cellular pathways modulated by UNC5B and Netrin-1 in late-stage colorectal tumours, particularly in the context of tumorigenesis and metastasis. Moving forward, it is imperative to investigate the functional implications of UNC5B and Netrin-1 expression in mCRC. By elucidating the precise cellular pathways and mechanisms influenced by these molecular actors, we can gain a better understanding of their roles in CRC and mCRC cell modulation.

Alongside UNC5B, Netrin-1 can theoretically also interact with other receptors, including Deleted in Colorectal Cancer (DCC), and the UNC5H receptor family (UNC5A, UNC5C, and UNC5D) [165, 179, 282]. DCC is a well-known receptor for Netrin-1 but is frequently deleted or downregulated in colorectal adenocarcinoma [171, 328]. UNC5A, UNC5C, and

UNC5D are also implicated in various cellular functions, including apoptosis and axon guidance[180, 278, 281, 282]. However, their specific roles in CRC cell biology remain less understood. Despite their potential involvement in Netrin-1 signalling, our analysis demonstrated UNC5B is the predominant receptor expressed in mCRC organoid cell lines, suggesting a very limited role for other Netrin-1 receptors signalling in driving the effects of Netrin-1 in CRC and mCRC cells.

We further investigated the potential associations between Netrin-1/UNC5B expression and crucial molecular processes in mCRC, including EMT and cancer stemness. Correlation analysis revealed robust positive associations between NTN1 and UNC5B expression levels and genes linked to EMT, such as Vimentin and transcription factors ZEB1, ZEB2, and SNAI2. These findings strongly suggest the potential regulatory roles of Netrin-1 and UNC5B in driving EMT-related processes in CRC and mCRC cells.

The correlations between *UNC5B* and *NTN1* mRNA expression and stemness-related genes, such as Nanog and SOX4, transcription factors that play critical roles in regulating stemness, particularly in the context of cancer [128, 323-325], suggest a role for the UNC5B/Netrin-1 couple in the regulation of CSCs in CRC.

Moreover, we characterised the behaviour of the Netrin-1/UNC5B pathway in mCRC upon treatment with chemotherapy. RNA sequencing data from organoid cell lines treated with FOLFOX chemotherapy revealed a sustained or increased expression of UNC5B mRNA upon treatment, suggesting a similar sustained expression of the receptor in late-stage CRC tumours during chemotherapy treatment. Further experiments confirmed the upregulation of UNC5B and Netrin-1 genes during and after FOLFOX treatment, potentially mediated by P53 activation. The activation of P53 with Nutlin 3A leads to increased expression of UNC5B and Netrin-1 genes, indicating the involvement of P53 in the modulation of the UNC5B/Netrin-1 pathway in mCRC.

In summary, through integrated analyses of mRNA and protein expression, and spatial transcriptomics, we have unravelled indirect evidence of an association between Netrin-1/UNC5B signalling pathways and key molecular processes modulating mCRC pathogenesis. Indeed, UNC5B and Netrin-1 may contribute to the maintenance and expansion of cancer stem cell populations within the tumour microenvironment,

thereby influencing tumour heterogeneity, metastasis, and therapy response. By elucidating the impact of UNC5B and Netrin-1 on CSCs, our study would provide valuable information about the molecular mechanisms responsible for CRC cell modulation. Targeting UNC5B and Netrin-1 signalling pathways may offer promising therapeutic strategies for inhibiting tumour progression, metastasis, or treatment-related issues (such as chemoresistance and recurrence of the tumour after treatment) by disrupting EMT or targeting CSCs. However further investigations are needed to fully understand the role of UNC5B and Netrin-1 in CRC stemness and its potential as a therapeutic target, as we will discuss in the next chapter.

Chapter 4 – Netrin-1 modulates stemness and **apoptosis in mCRC**

I- Introduction

The experiments and analysis discussed last chapter suggest the involvement of the UNC5B receptor and its ligand Netrin-1 in the modulation of colorectal cancer stem cells (CSCs). Notably, we observed correlations between the expression levels of UNC5B and Netrin-1 mRNA and the regulation of key stemness-associated factors such as SOX4 and NANOG mRNA, alongside a decrease in E-cadherin expression indicative of EMT. Given these findings, the primary objective of this chapter will be to investigate the potential role of the UNC5B/Netrin-1 axis in modulating stemness in mCRC cells and to use the knowledge from these investigations to evaluate the therapeutic impact of Netrin-1 targeting, particularly in CSCs-mediated resistance and recurrence.

Since resistance and recurrence of CRC appear to be determined by specific subpopulations of cells closely related to CSCs [329], finding if modulating the UNC5B/Netrin-1 pathway could impact CSCs could be therapeutically relevant. Indeed, therapies such as radiotherapies or chemotherapies are effective against differentiated and proliferative cancer cells but have exceptionally low effectiveness on CSCs [133].

Chemoresistance in colorectal CSCs involves several mechanisms discussed previously that collectively allow these cells to evade chemotherapy effects [330]. One of these mechanisms is quiescence, where CSC enter a dormant state, making them less sensitive to drugs targeting actively dividing cells [145]. Additionally, CSCs often upregulate the expression of anti-apoptotic proteins, inhibiting programmed cell death induced by chemotherapy. Furthermore, CSCs may overexpress ATP-binding cassette (ABC) transporters, facilitating the efflux of cytotoxic drugs out of the cells [157, 158]. Another contributing factor is the heightened expression of detoxifying enzymes, particularly aldehyde dehydrogenase (ALDH), which metabolize chemotherapeutic agents and decrease their efficacy [153, 154]. Moreover, CSCs frequently exhibit enhanced DNA repair mechanisms, enabling efficient repair of chemotherapy-induced DNA damage and promoting resistance to treatment [135]. These various mechanisms collectively confer chemoresistance to colorectal CSCs, representing a significant challenge for effective cancer therapy.

These mechanisms make CSCs key players in the resistance to chemotherapies of CRC and mCRC tumours [331-333]. Moreover, this subpopulation of cancer cells is responsible for post-treatment relapse due to their self-renewal capabilities, limitless replication, differentiation potential and tumour induction abilities [122]. Self-renewal, one of the main phenotypical traits of CSCs, refers to the ability of these cells to perform asymmetrical division. Indeed, When CSCs divide at least one of the daughter cells maintains the same stem cell characteristics. The second daughter cell is either a cancer stem cell as well (symmetric division) or a cell with differentiating potential (asymmetric division). This self-renewal capability plays a crucial role in driving the malignant behaviours of colorectal CSCs. Although the breadth of molecular mechanisms modulating self-renewal in these cells remains incompletely understood, it has been demonstrated that the abnormal activation of signalling pathways such as Wnt, Notch, and TGF- β (transforming growth factor- β)/BMP (bone morphogenetic protein) as well as the specific functions mediated by microenvironmental factors, are implicated in orchestrating this self-renewal regulation [334].

To correctly analyse the effect of the Netrin-1/UNC5B pathway in CSCs, we should be attentive to the methodology applied to their study. Colorectal CSCs can be identified through the analysis of the expression of specific membrane receptors [335]. These markers include CD44, a receptor for cell adhesion and migration; CD133 (prominin-1), associated with stemness and tumour initiation; EpCAM (epithelial cell adhesion molecule), crucial for cell-cell adhesion and signalling; SMOC2 (SPARC Related Modular Calcium Binding 2), a matricellular protein highly expressed during embryogenesis and wound healing, and LGR5 (leucine-rich repeat-containing G-protein coupled receptor 5), a marker of intestinal stem cells and a key player in Wnt signalling. Moreover, transcription factors like SOX2 (SRY (sex-determining region Y)-box 2), OCT4 (octamer-binding transcription factor 4), KLF4 (Kruppel-like factor 4), and NANOG (Nanog homeobox) are known as key regulators of stemness. They enhance stem cell properties by activating genes vital for self-renewal and pluripotency, while simultaneously inhibiting the expression of genes associated with differentiation. Expression of these markers and transduction factors is often associated with aberrant activation of several proliferative pathways such as Notch, Hedgehog and Wnt in CSCs [123, 124].

To characterize the effect of Netrin-1 on the CSC population in CRC, we will use two distinct yet complementary methods: assessing the expression of stem cell markers such as CD133 through fluorescence-activated cell sorting (FACS), and the observation of a phenotypical characteristic, their self-renewal capabilities, using extreme limiting dilution assays (ELDA). ELDA is a valuable tool used to observe self-renewal capability in a sample of heterogeneous cells, a capacity associated with CSC populations, operating on the principle that only CSCs possess the capacity for asymmetric division to regenerate the heterogeneous tumour. This assay involves serial dilution of a single cell sample over a range of concentrations, allowing us to evaluate each culture's individual capability to form complex structures such as organoids and spheroids (**Figure 4.1**). We then performed statistical analysis of the results using the ELDA web tool, which is a limiting dilution analysis software providing meaningful confidence intervals for all limiting dilution assay datasets.

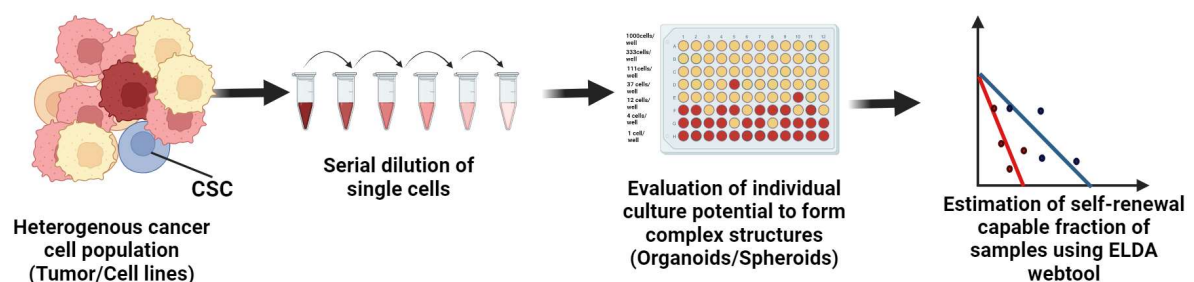


Figure 4.2 - Schematic representation of In Vitro ELDA (Extreme Limiting Dilution Assay)

To mimic either the presence or absence of Netrin-1, we will use either recombinant Netrin-1 (Adipogen, AG-40B-0040) or the neutralizing antibody NP137 developed and supplied by our collaborators (Team Apoptosis, cancer and development at Universite de Claude Bernard Lyon 1, France).

To investigate the influence of both UNC5B and Netrin-1 in the HCT116 human colon adenocarcinoma cell line initially to observe the impact of this pathway on self-renewal capabilities in a simple colorectal cancer cell model. Afterwards, to examine the implications of UNC5B and Netrin-1 on CRC cell phenotype in a model that better reflects human pathology, we translated and pursued our investigations into our model of patient-derived organoid (PDO) cell lines. These complementary approaches provide

valuable insights into the role of UNC5B in regulating stem cell phenotypes in CRC cells across different experimental contexts.

Given the implications of the UNC5B receptor and its ligand Netrin-1 in apoptosis, related to the dependence receptor characteristics of UNC5B and the other Netrin-1 receptors, we also analysed the impact of Netrin-1 on mCRC cell apoptosis. To achieve this, we used PI/AnnexinV assay to quantify apoptosis and used the pan-caspase inhibitor z-VAD-FMK [336] in settings where inhibition of apoptosis was needed.

In our comprehensive investigation, we used the strength of our organoid model, the faithful reproduction of phenotypical responses of tumours to various chemotherapeutic regimens. Through this model, we investigated the dynamics of the UNC5B/Netrin-1 pathway both upon and after exposure to chemotherapy in our model of mCRC organoid. We aimed to gain an understanding of how the UNC5B/Netrin-1 pathway modulates the response to chemotherapy and influences the development of resistance. Furthermore, to open a potential pathway towards the use of NP137 in a clinical setting, we transitioned our investigations into *in vivo* studies. By engrafting patient-derived organoid cell lines into a mouse model, we could closely monitor the real-time response of tumours to therapeutic interventions and validate the therapeutic potential of targeting the UNC5B/Netrin-1 axis in decreasing colorectal cancer progression.

II- Results

a. Netrin-1 & UNC5B modulate stemness in HCT116 cells

To investigate the involvement of Netrin-1 in the self-renewal characteristic of CRC cells, we first tested the effect of its inhibition in a simple and well-characterized colon cancer model. To accomplish this, we evaluated the impact of the neutralizing antibody NP137 on the sphere-forming capabilities of HCT116 cells, a commonly used human colorectal carcinoma cell line, using ELDA.

Previous studies investigating the effects of Netrin-1 on various biological processes, including neurogenesis, angiogenesis, and the modulation of dormancy of hematopoietic stem cells, typically observe an effect of the molecule within a

concentration range of 50 to 200 ng/mL [337-340]. Therefore, for our study on colorectal CSC modulation, we chose to utilize a concentration of 100 ng/mL (equivalent to 1.3 nM) of recombinant Netrin-1. This concentration was selected based on its reported effects in relevant cellular contexts. Additionally, following the recommendations of Netris Pharma, the manufacturer of NP137, we employed this neutralizing antibody at a concentration of 10 µg/mL (66 nM). This concentration ensures that the antibody is present in excess, allowing it to effectively neutralize both exogenous recombinant Netrin-1 and endogenous Netrin-1.

Our observations revealed that HCT116 cells treated with NP137 (10 µg/mL) exhibited a significant decrease in self-renewal capacity compared to both untreated cells and cells treated with the isotypic control antibody NP001 at an equivalent concentration (10 µg/mL) (**Figure 4.1-A**). This significant reduction in self-renewal underscores the potential role of Netrin-1 in driving the self-renewal of colon cancer cells, at least in the HCT116 cell line.

To investigate the implication of UNC5B in this process, we used HCT116 cells transduced with a doxycycline-inducible plasmid driving UNC5B overexpression. To guarantee the specificity of our experiments, cells transduced with the UNC5B-inducing plasmid were sorted by fluorescence-activated cell sorting (FACS) using a GFP marker also expressed by the plasmid, ensuring that only properly transduced cells were used in subsequent analyses. Using Western blotting, we confirmed the successful robust overexpression of UNC5B protein upon doxycycline treatment (2 µM) in UNC5B-transduced cells, compared to WT cells or non-treated cells (**Figure 4.1-B**). We then observed the effect of this UNC5B overexpression on the self-renewal potentials of HCT116 cells using ELDA. Our findings revealed a significant increase in the fraction of self-renewal-capable cells ($65.18 \% \pm 3.276$) in UNC5B-transduced cells treated with doxycycline compared to both untreated cells and wild-type cells treated with doxycycline, which exhibited self-renewal rates ranging from 28% to 36% (WT-CTR: $28.35 \% \pm 3.468$; WT-Dox: $36.62 \% \pm 7.460$; UNC5B+-CTR: 29.03 ± 11.15) ($p < 0.01$, One-way ANOVA - Tukey's multiple comparisons test) (**Figure 4.1-C**). This observation suggests that with an endogenous level of Netrin-1, the UNC5B receptor enhanced the self-renewal abilities of CRC cells. It is to be noted that the baseline level of self-renewal

observed in the HCT116 is relatively high and not representative of the tendency observed in other colorectal cell lines, though it's a known feature of these cells that has previously been noticed in other studies [341].

To investigate whether UNC5B overexpression retains its impact on self-renewal in the absence of Netrin-1, we examined the effect of a control (NP001, 10 µg/mL) or Netrin-1 specific (NP137, 10 µg/mL) antibody on self-renewal in cells expressing UNC5B. As illustrated in **Figure 4.1-D**, in the presence of NP001, there was still a notable and significant increase in the fraction of self-renewal-capable cells among those expressing UNC5B and treated with doxycycline (2 µM), compared to untreated cells ($19.03 \% \pm 7.484$ vs $28.83 \% \pm 5.140$; $p < 0.05$ paired t-test). Conversely, in cells treated with NP137, this increase was not significant, suggesting the role of Netrin-1 inhibition in mitigating the effects of UNC5B overexpression on self-renewal (**Figure 4.1-E**). However, intriguingly, even in the presence of NP137, there was a discernible but statistically non-significant trend towards increased self-renewal in UNC5B-overexpressing cells ($10.39 \% \pm 3.375$ vs $16.72 \% \pm 0.875$), hinting at the possibility that UNC5B may exert an effect independently of Netrin-1 expression, or that NP137 concentration is insufficient to neutralize the entirety of Netrin-1 present in UNC5B-overexpressing HCT116 medium. However, we can observe a similar trend in HCT116 WT treated with doxycycline, suggesting that this could also be the result of doxycycline in UNC5B-overexpressing HCT116 cells.

These observations suggest a role for Netrin-1 and its receptor UNC5B in the positive regulation of self-renewal in CRC cells. However, it's essential to acknowledge that the HCT116 cell line may not be the most ideal model for studying CRC stem cells. Firstly, the lack of heterogeneity inherent to 2D cell lines may not fully recapitulate the complexity of CRC tumours, which are characterized by diverse cell populations. Secondly, the high rates of sphere-forming abilities observed in HCT116 cells may not accurately reflect the behaviour of CRC tumours in vivo. Additionally, we observed a certain variability in the frequency of sphere-forming abilities of this cell line across experiments, highlighting the importance of focusing our observations on values within the same experimental context to ensure the reliability of our findings. We hypothesized that this discrepancy might be due to a difference in the number of passages of the

HCT116 cells between the experiments (15 to 17 passages for the experiment with NP137 whereas passages of cells used for further experiments underwent between 25 and 30). Moving forward, employing more representative and heterogeneous models of CRC stem cells will be crucial for elucidating the intricate mechanisms underlying Netrin-1 and UNC5B implications in self-renewal regulation in CRC.

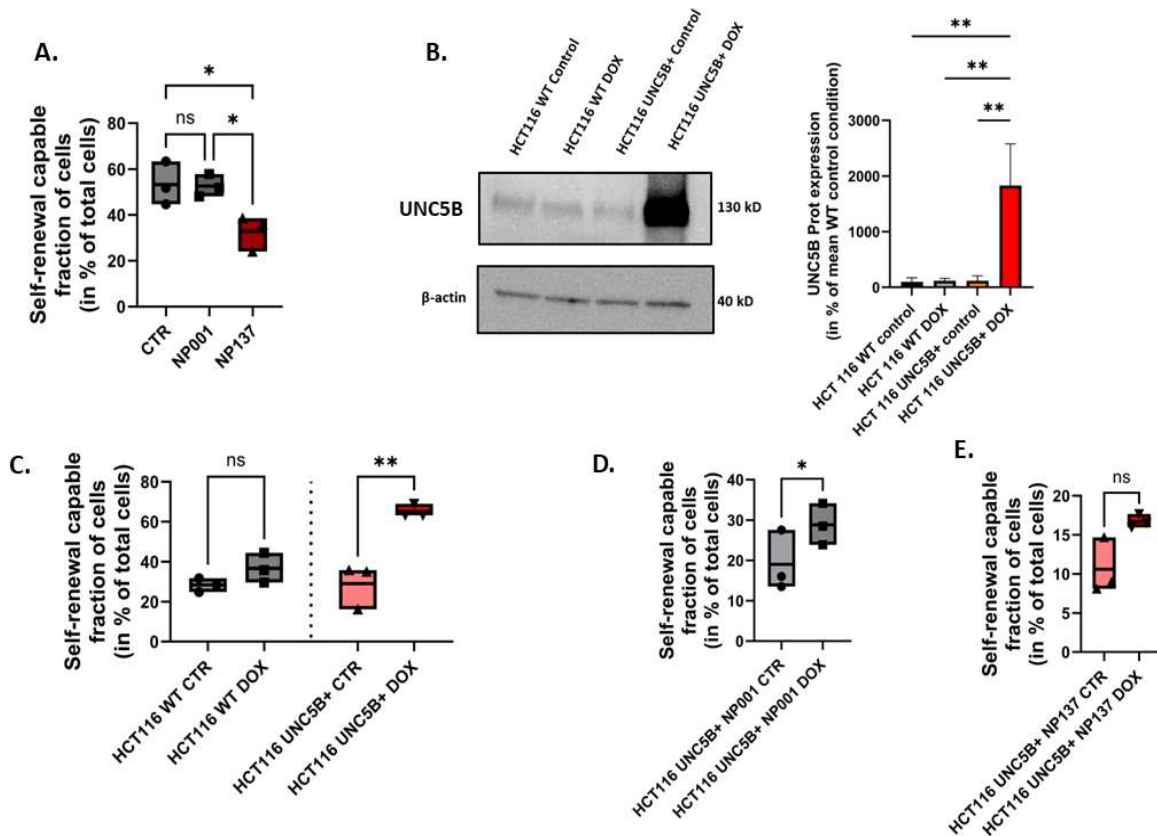


Figure 4.2 : Netrin-1 and UNC5B are enhancing self-renewal in HCT116 cell line (A) Self-renewal capable fraction of HCT116 cells treated with either nothing (Control), NP001 (10 μ g/mL) or NP137 (10 μ g/mL) observed by ELDA; **(B)** Expression of UNC5B protein in HCT116 cell lines (WT or UNC5B-plasmid transduced) treated with nothing (Control) or 2 μ M of Doxycycline (Left) Western Blot, (Right) Histogram representation of UNC5B protein expression (Error bars indicate SD); **(C)** Self-renewal capable fraction of HCT116 cell lines (WT or UNC5B-plasmid transduced) treated with nothing (Control) or 2 μ M of Doxycycline; **(D)** Self-renewal capable fraction of HCT116 cell lines (UNC5B-plasmid transduced) treated with NP001 (10 μ g/mL) and either nothing (Control) or 2 μ M of Doxycycline observed by ELDA ; **(E)** Self-renewal capable fraction of HCT116 cell lines (UNC5B-plasmid transduced) treated with NP137 (10 μ g/mL) and either nothing (Control) or 2 μ M of Doxycycline observed by ELDA; Statistical significance was determined using One-way Anova for (A), (B) and (C) unpaired t-tests for (D) and (E), ns = non-significant, * = $p < 0.05$, ** = $p < 0.01$; N=3

b. Netrin-1 modulates stemness in patient-derived mCRC organoids

To extend our observations into a more representative and heterogeneous model of CRC, we employed ELDA to assess the proportion of cells with self-renewal abilities in our mCRC organoid cell lines. Doing so, we observed a robust correlation ($R = 0.95$, $p = 0.01$) between the expression levels of UNC5B receptor protein and the proportion of cells exhibiting self-renewal abilities in our mCRC organoid cell lines, quantified using ELDA (**Figure 4.2-A & B**). This correlation again suggests that the effect of the couple Netrin-1/UNC5B ligand/receptor on the modulation of self-renewal observed in HCT116 cells is relevant in our mCRC organoid cell lines.

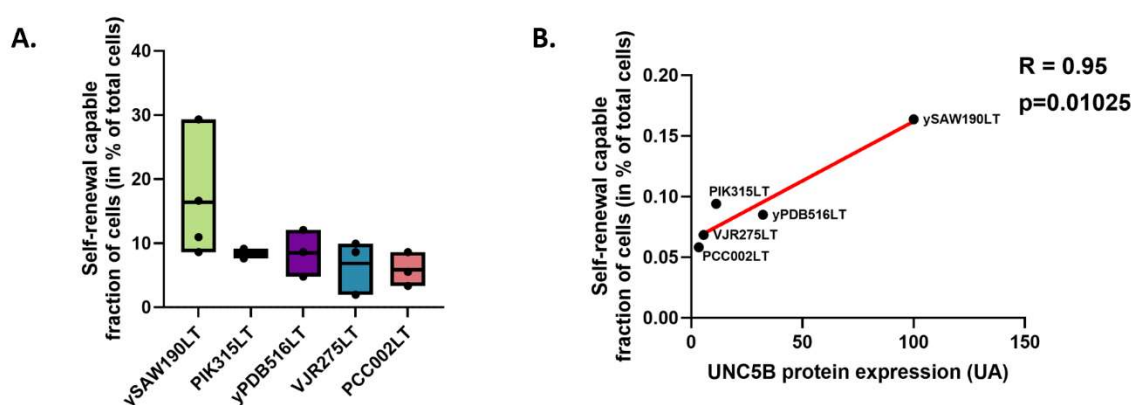


Figure 4.3 - UNC5B protein expression correlates with self-renewal in mCRC organoids (A) Histogram representing self-renewal capabilities of mCRC organoid cell lines observed using ELDA (error bars indicate SD; $N=3$ ($N=4$ for ySAW190LT cell line)) (B) Correlation between expression of UNC5B protein and self-renewal capable fractions of mCRC organoids; Statistical test used: Pearson correlation test

To further explore if the effect of Netrin-1 found on HCT116 cell line self-renewal was still occurring in our patient-derived mCRC organoid cell lines, we investigated the impact of supplementing our organoid cell lines with either recombinant Netrin-1 (Adipogen, AG-40B-0040), the neutralizing antibody NP137, or a combination of both, on self-renewal properties. To gauge self-renewal in our organoid cell lines, we adapted the ELDA protocol outlined by Hu et al. in 2009 for culturing our organoid cell line in a Matrigel matrix.

We focused on two organoid cell lines: ySAW190LT, which exhibits high levels of UNC5B mRNA and protein expression, and PIK315LT, displaying medium to low levels of UNC5B mRNA and protein (**Figure 3-7.A and B**) but higher levels of NTN1 than ySAW190LT. Upon treatment with NP137 (10 $\mu\text{g}/\text{mL}$) we observed a significant decrease in self-

renewal capabilities in both organoid cell lines compared to non-treated cells and cells treated with NP001 (10 µg/mL) as control (ySAW190LT: control = 12.33% ± 3.851, NP001: 12.47% ± 1.541, NP137 3.042 % ± 1.081 p<0.05%; PIK315LT: control = 10.48% ± 0.302, NP001: 9.871 % ± 1.007, NP137 4.09 % ± 2.205, p<0.01%, One-way ANOVA – Turkey's multiple comparison test) (**Figure 4.3-A & B**). These results indicate that inhibition of Netrin-1 leads to an important reduction of self-renewal in our mCRC organoid model at endogenous levels of Netrin-1.

Furthermore, to validate our findings, we conducted experiments using recombinant Netrin-1. Upon supplementing the culture medium with Netrin-1 at a concentration of 100 µg/mL, we observed a trend towards enhanced self-renewal in both organoids (ySAW190LT: Control = 11.33% ± 0.908, Net-1: 17.75% ± 8.631, Net-1 + NP001: 19.83% ± 6.709, Net-1 + NP137: 3.279 % ± 1.245; PIK315LT: Control = 8.38% ± 0.757, Net-1: 9.87% ± 0.878, Net-1 + NP001: 10.06% ± 0.908, Net-1 + NP137: 5.3 % ± 1.742). Accordingly, treatment with NP137 (10 µg/mL) effectively inhibited the effects of recombinant Netrin-1 and resulted in a decrease in self-renewal to levels similar to those observed in the absence of recombinant Netrin-1 (**Figure 4.3-C & D**). These results confirm the significant impact of Netrin-1 on self-renewal ability in mCRC organoids, suggesting a critical role of the Netrin-1 pathway in regulating colorectal CSC behaviour.

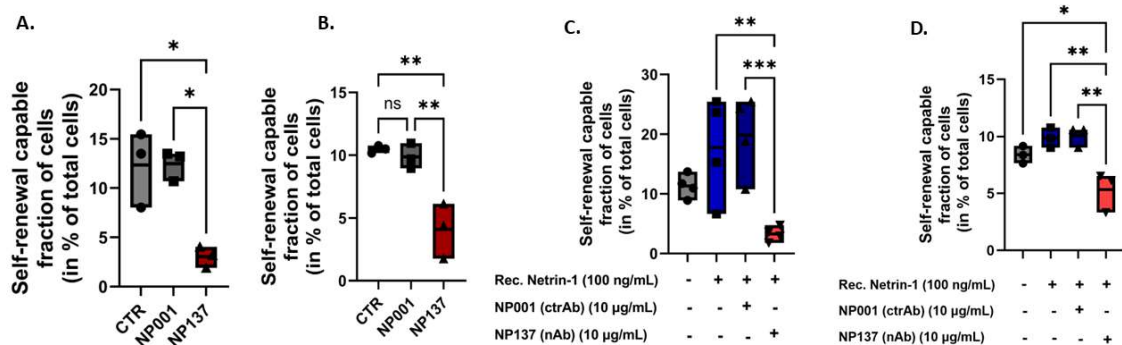


Figure 4.4 Netrin-1 enhance self-renewal in mCRC organoids (A-B) Self-renewal capable fraction of ySAW190LT (A) and PIK315LT (B) mCRC organoid lines, either left untreated (Control), or treated with NP001 (10µg/mL) or NP137 (10µg/mL) antibodies, and measured using ELDA; (C) Self-renewal capable fraction of ySAW190LT (C) and PIK315LT (D) organoids in the presence or absence of recombinant Netrin-1 (100 ng/mL), NP001 (10µg/mL) and/or NP137 (10µg/mL) and measured using ELDA; Statistical significance was determined using One-way Anova – Turkey's multiple comparison test, ns = non-significant, * = p <0.05, ** = p <0.01, *** = p <0.001. N=3 for (A), (B) and (D); N=4 for (C)

To further corroborate the potential role of Netrin-1 in the modulation of colorectal CSCs, we examined its effect, as well as the one of NP137, on the CD133-positive cell

population within our ySAW190LT organoid using flow cytometry (**Figure 4.4**). Although not completely restricted to CSCs, the CD133+ population is known to include this subpopulation of cancer cells [342-344]. Our findings revealed a significant increase in the proportion of CD133-positive cells in mCRC organoid cells treated with recombinant Netrin-1 (100 ng/mL), both with and without NP001, compared to non-treated cells (Control: 13.78 % $\pm$ 0.263; Net-1: 18.7 % $\pm$ 3.358; Net-1 + NP001: 17.4 % $\pm$ 1.944; $p < 0.01$ and $p < 0.05$ respectively, One-way ANOVA – Turkey’s multiple comparison test). Importantly, this effect of recombinant Netrin-1 on the CD133-positive population was effectively reversed by the addition of NP137 lowering the concentration to a level observed in non-treated cells. These results provide compelling evidence of the role of Netrin-1 in modulating the CD133-positive CSC population within mCRC organoids, emphasizing its potential significance in CRC tumours.

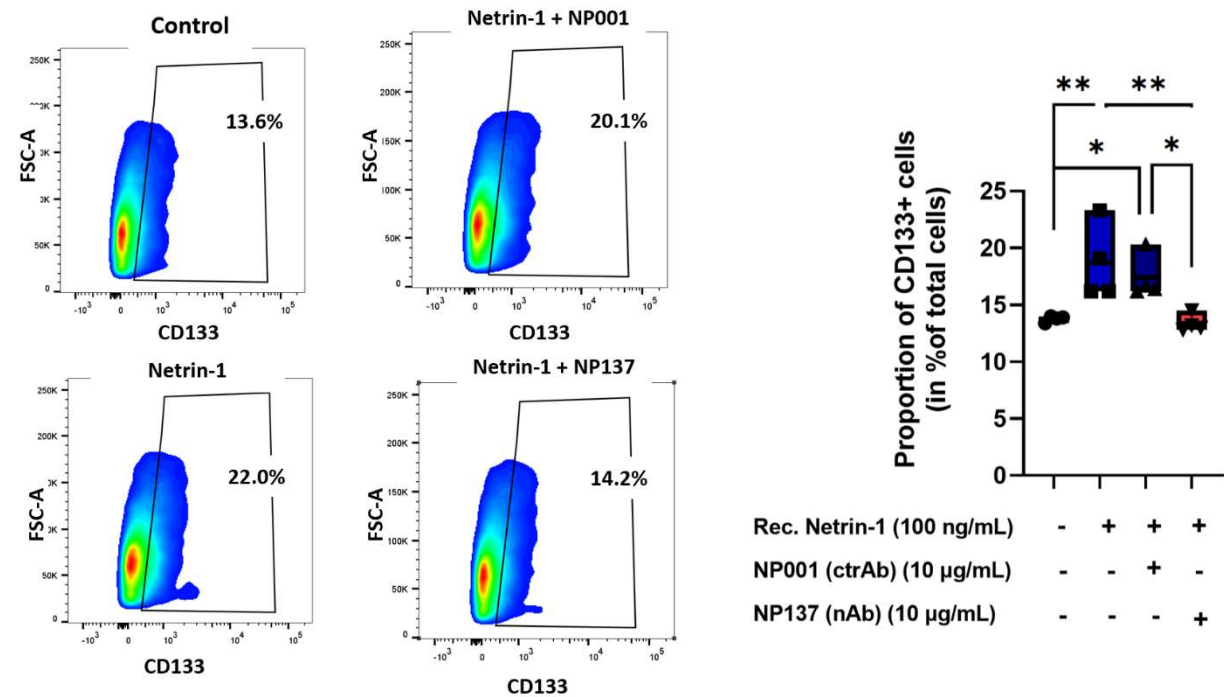


Figure 4.5 - Netrin-1 increases CSC proportion in mCRC organoids (Left) Representative flow cytometry analysis (Gate was determined using a non-marked control and left unchanged for all samples) and (Right) bar graph summarising proportions of CD133-positives cells in ySAW190LT organoid after 72h of treatment with recombinant Netrin-1 (100 ng/mL), NP001 (10 μ g/mL) and/or NP137 (10 μ g/mL); Statistical significance was determined using One-way Anova – Turkey’s multiple comparison test, ns = non-significant, * = $p < 0.05$, ** = $p < 0.01$. N=4

c. Netrin-1 modulates apoptosis in mCRC cells

Given that Netrin-1 is the ligand for several dependence receptors, which are known to induce apoptosis in the absence of their ligands and considering that we showed that its inhibition is associated with decreased self-renewal and CSC numbers, we investigated whether apoptosis plays a role in the effect of Netrin-1 in mCRC organoids. To explore this possibility, we evaluated late apoptosis in organoids treated with recombinant Netrin-1, either alone or in combination with NP137 or NP001, using a staining technique employing propidium iodide (PI) and Annexin V coupled with CD133 marking by FACS. This method relies on the principle that Annexin V binds to phosphatidylserine, a cell membrane phospholipid exposed at the cellular membrane during early apoptosis, while PI stains the nuclei of cells with compromised membrane integrity, indicative of late apoptosis or necrosis [345]. By employing this PI/Annexin V staining approach, we were able to assess late-stage apoptosis in our mCRC organoid cells under various netrin-1 concentration conditions using flow cytometry. Our results revealed a decrease in late apoptosis in CD133-positive cells treated for 72 hours with recombinant Netrin-1, with or without NP001, compared to untreated organoids. In addition, the decreased percentage of apoptotic CD133-positive cells induced by Netrin-1 was reversed after treatment with NP137 (12.35 % $\pm$ 1.27 for non-treated and 12.28 % $\pm$ 1.786 for NP137 treated cells vs. 9.45% $\pm$ 1.1 for Netrin-1 treated cells) (**Figure 4.5-A**). Importantly, this effect appeared to be specific to the CD133-positive population, as the impact of Netrin-1 on the CD133-negative cells was not significant despite an apparent trend (6.53 % $\pm$ 1.63 for non-treated and 5.89 % $\pm$ 1.786 for NP137 treated cells vs. 5.635% $\pm$ 1.93 for Netrin-1 treated cells) (**Figure 4.5-B**). These findings suggest a potential role for Netrin-1 in modulating apoptosis specifically within the CD133-positive CSC population.

To confirm whether the influence of Netrin-1 on self-renewal was dependent on apoptosis, we investigated its effects in an experimental setting where apoptosis was inhibited. For this purpose, we employed z-Vad-FMK, a pan-caspase inhibitor known for its ability to block apoptosis by targeting a wide range of caspases. By incorporating z-Vad-FMK (50 μ g/mL) into the organoid medium as well as recombinant Netrin-1 and NP001 or NP137 72h before and during the ELDA, we effectively prevented the negative

impact of NP137 on self-renewal, restoring self-renewal rates to levels comparable to those seen in cells treated with Netrin-1 and NP001 (**Figure 4.5-C**). This finding demonstrates that the effects of Netrin-1 on colorectal CSCs self-renewal are closely intertwined with the inhibition of apoptosis. Unravelling this mechanistic link put in evidence the relationship between Netrin-1 signalling and apoptosis in CRC.

As discussed in the introduction, my PhD research group at the Lyon Cancer Research Centre is currently investigating apoptotic pathways associated with Netrin-1 withdrawal, and their unpublished data suggests that apoptosis induced by NP137 treatment may involve active degradation of the antiapoptotic protein MCL1, particularly in the context of UNC5B-dependent apoptosis. To explore this possibility, we examined the effect of Netrin-1 inhibition on MCL1 expression in ySAW190LT organoids. Corroborating this hypothesis, our observations revealed a significant decrease in MCL1 protein expression after 18 hours of treatment with NP137 (10 µg/mL) (**Figure 4.5-D**), strongly suggesting that the link between Netrin-1 inhibition and apoptosis in mCRC cells is indeed mediated through MCL1.

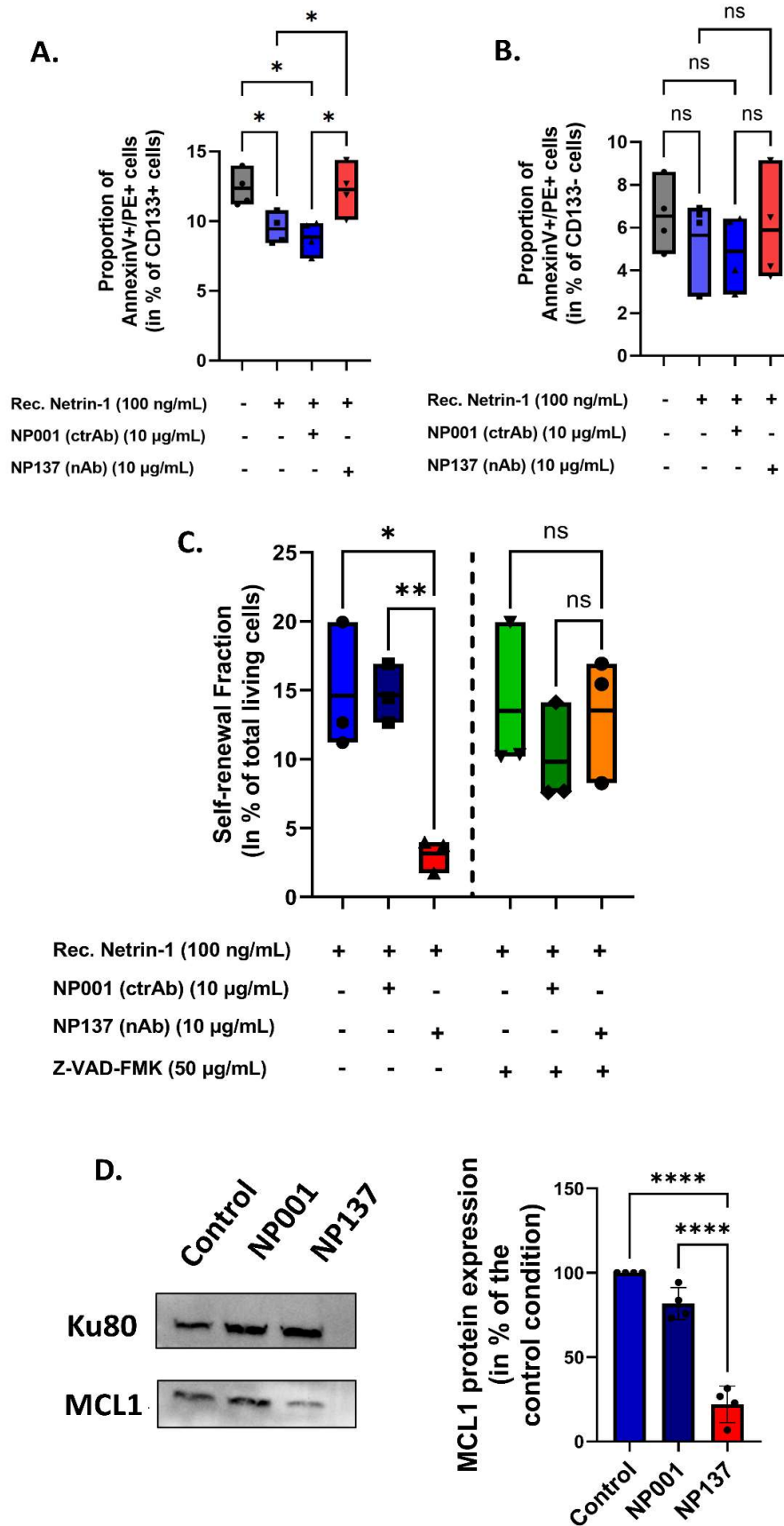


Figure 4.6 - Netrin-1 modulates self-renewal in mCRC through CSCs apoptosis (A) FACS analysis late apoptosis observed by AnnexinV/PI staining in CD133-positive cells in ySAW190LT organoid after 72h of treatment with recombinant Netrin-1 (100 ng/mL), NP001 (10µg/mL) and/or NP137 (10µg/mL) ; **(B)** FACS

analysis late apoptosis observed by AnnexinV/PI staining in ySAW190LT organoid cells after 72h of treatment with recombinant Netrin-1 (100 ng/mL), NP001 (10µg/mL) and/or NP137 (10µg/mL) ; **(C)** Self-renewal capable fraction of ySAW190LT organoid cell line in presence or absence of recombinant Netrin-1 (100 ng/mL), NP001 (10µg/mL) and/or NP137 (10µg/mL) observed by ELDA **(D)** Expression of MCL1 anti-apoptotic protein expression in ySAW190LT organoid after 18h of treatment with either nothing (Control), NP001 (10µg/mL) or NP137 (10µg/mL) observed by Western blot using Ku80 as a load protein control, (Left) Western Blot, (Right) Histogram representation of MCL1 protein expression ; Statistical significance was determined using One-way Anova – Turkey’s multiple comparison test, Error bars indicate SD; ns = non-significant, * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$. N=4 for (A), (B) and (D), N=3 for (C)

d. Netrin-1 & UNC5B modulate self-renewal in mCRC cells

Given the observed modulation of apoptosis and self-renewal in mCRC organoids by Netrin-1, along with our findings indicating that the UNC5B receptor influences self-renewal in HCT116 cells and that its expression is closely correlated with the self-renewal-capable fraction in our organoids, we supposed that the effects of Netrin-1 on mCRC cells are mediated through UNC5B receptor activities. Moreover, this assumption is supported by our RNA sequencing data where we observed that the UNC5B receptor is roughly the only Netrin-1 receptor expressed in late-stage CRC tumours as well as in our organoid model (**Figure 3.1-E** and **3.6-C**). To investigate this hypothesis, we utilized CRISPR Cas9 technology to knock out the *UNC5B* gene in PIK315LT organoids. To ensure the validity of our observations and to confirm that the effects observed are indeed due to the loss of the *UNC5B* gene and not to a specific mutation or off-target effect, we independently employed two different sgRNAs in separate organoids. Each sgRNA targeted a distinct region of the *UNC5B* gene, resulting in the generation of two distinct polyclonal organoid lines with *UNC5B* gene knockout, enabling the independent validation of *UNC5B* knockout consequences in each organoid cell line. Analysis of Sanger sequencing traces comparing the parental organoid with the scramble control (sgRNA) or sgUNC5B using Tracking of Indels by Decomposition analysis (TIDE) [346] confirmed that genome editing occurred at the predicted Cas9 cut site and was specific to the sgUNC5B conditions (**Figure 4.6-A**). We further confirmed the UNC5B protein knockout by Western blot (**Figure 4.6-B**).

It is to be noted that we attempted to invalidate the *UNC5B* gene in the ySAW190LT organoid cell line. This organoid exhibits the highest expression of UNC5B among all organoids, we thought that observing the effect of this gene invalidation in this organoid

would be informative. However, despite a similar protocol and several attempts, the invalidation was unsuccessful and led to incomplete (11.3 %) gene invalidation (**Supplementary Figure 4-1.A**). In our investigation to understand this observation, we analyzed by flow cytometry the ySAW190LT organoid cell line transduced by the Cas9 and SgRNA two weeks before the experiment (**Supplementary Figure 4-1.B**). We can observe that despite a stringent selection of cells expressing both GFP (SgRNA) and mCherry (Cas9), the rates of double-positive cells drop to 35.4 %. This considerable decrease could be linked to either a mechanism triggering the rejection of plasmids expressing the Cas9 and the SgRNA in this organoid cell line, or by the fact that *UNC5B* invalidation would represent a disadvantage for survival or growth of the cells, the few cells expressing *UNC5B* after sorting would grow faster or survive in higher rates than successfully *UNC5B*-invalidated cells, leading to a high proportion of *UNC5B*-valid cells after certain time. This possibility would suggest a high dependence of the ySAW190LT organoid cell line on the *UNC5B* receptor.

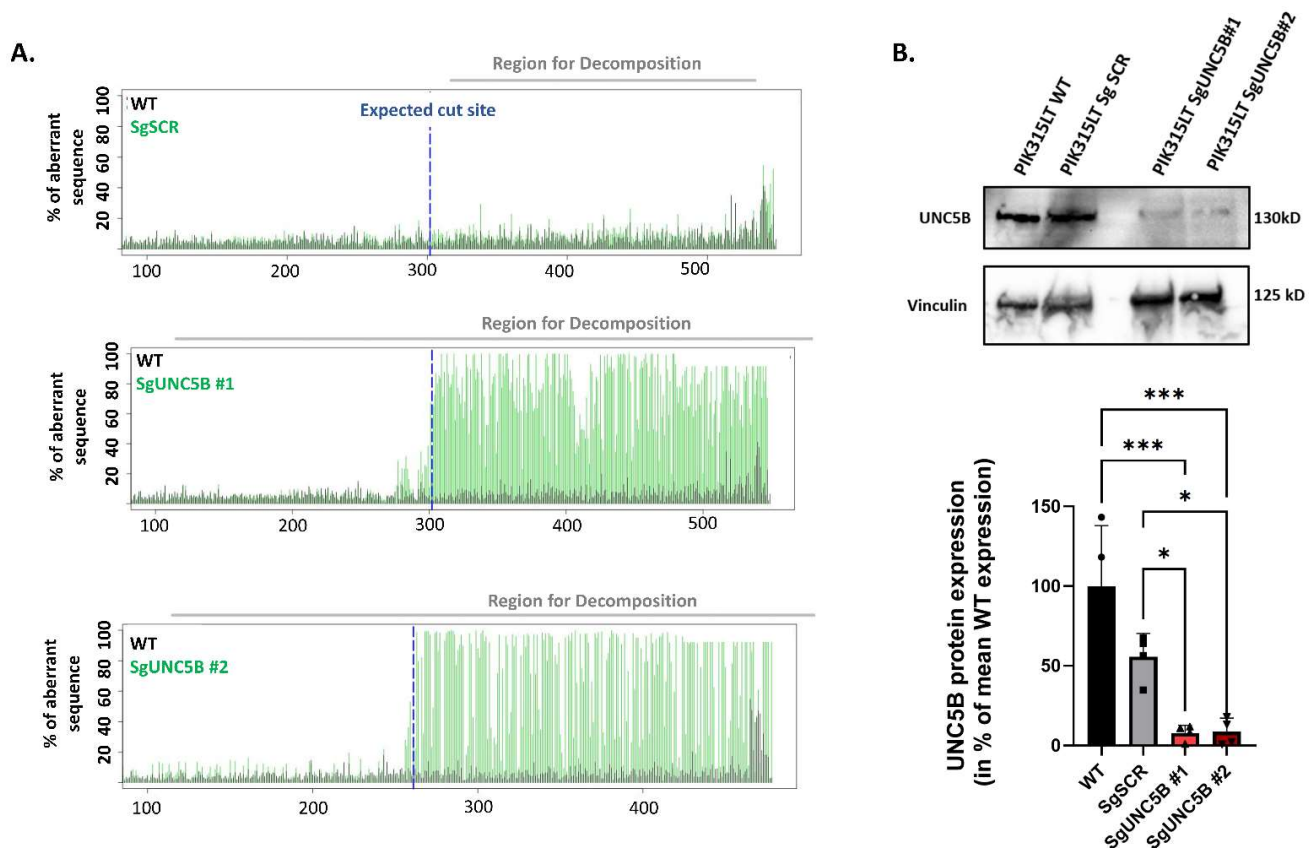


Figure 4.7 - CRISPR Cas9 Invalidation of *UNC5B* in PIK315LT mCRC organoid (A) Representative TIDE analyses comparing Sanger sequencing traces from wildtype PDOs versus scramble control (sgSCR) or CRISPR-edited (SgUNC5B#1 and SgUNC5B#2) organoid (B) Western Blot validation of *UNC5B* protein

knockout by looking at the protein level of *UNC5B* in wildtype, scramble control (sgSCR) and CRISPR-edited (SgUNC5B#1 and SgUNC5B#2) **PIK315LT**; For Indel spectrum: Green coloured lines represent the percentage of aberrant nucleotides between the sequence trace of the control and the experimental sample, black coloured lines represent noise; Statistical significance was determined using One-way Anova – Turkey’s multiple comparison test, Error bars indicate SD; ns = non-significant, * = $p < 0.05$, *** = $p < 0.001$, N=3

Surprisingly, despite the *UNC5B* gene invalidation in our organoid cell line PIK315LT, the difference in self-renewal between the wild-type (WT) and scramble control (SgSCR) organoid cell lines compared to the *UNC5B*-invalidated organoid cell lines cells (WT: 8.78 % $\pm$ 3.88; SgSCR: 8.89 % $\pm$ 4.925; SgUNC5B # 1: 6.31 % $\pm$ 3.12; SgUNC5B # 1: 6.04 % $\pm$ 3.01) was not significant (**Figure 4.7-A**). The result being non-significant might be due to the high variability observed across the experiments. However, several reasons are possible to explain this sustained self-renewal in the *UNC5B*-invalidated organoid cell lines, such as the invalidation of both *UNC5B* positive (survival) and negative (apoptotic) signalling or, since *UNC5B* invalidation is constitutive, cells resulting from this invalidation could have adapted other pathways that influence survival of CSCs.

To further characterize this model, we aimed to determine whether treatment with recombinant Netrin-1 would still be able to increase self-renewal in our PIK315LT organoid cell line despite the invalidation of *UNC5B* (**Figure 4.7-B**). Using recombinant Netrin-1 (100 ng/mL), we found a similar increase in self-renewal capability of cells in the WT PDO (WT control = 10.43 % $\pm$ 2.19; WT Net-1 = 17.82 % $\pm$ 4.95; $p < 0.05$, One-way ANOVA – Turkey’s multiple comparison test) and SgSCR PDO (Scr control = 11.06 % $\pm$ 1.189; Scr Net-1 = 22.10% $\pm$ 2.07), $p < 0.001$, One-way ANOVA – Turkey’s multiple comparison test). However, this increase was absent in *UNC5B*-invalidated organoids (SgUNC5B#1 control = 9.65 % $\pm$ 2.44 ; SgUNC5B#1 Net-1 = 8.47 % $\pm$ 1.47; SgUNC5B#2 control = 11.38% $\pm$ 2.88 ; SgUNC5B#2 Net-1 = 12.09 % $\pm$ 4.11). These results demonstrate that the effect of Netrin-1 on self-renewal is dependent on the presence of the *UNC5B* receptor.

To further validate these findings and confirm that the effect of Netrin-1 inhibition (using NP137) is also dependent on the *UNC5B* receptor, we investigated the impact of NP137 on self-renewal in our *UNC5B* invalidated PDO cell lines (**Figure 4.7-C**). We observed a similar decrease in self-renewal in both the WT organoid (WT control = 12.33 % $\pm$ 3.85;

WT NP001 = 12.47 % $\pm$ 1.54; WT NP137 = 3.042 % $\pm$ 1.08, $p < 0.01$ and 0.05 respectively, One-way ANOVA – Turkey’s multiple comparison test) and SgSCR control organoids (SgSCR control = 12.58 % $\pm$ 2.47; SgSCR NP001 = 14.4% $\pm$ 1.77; SgSCR NP137 = 3.99 % $\pm$ 2.46, $p < 0.01$, One-way ANOVA – Turkey’s multiple comparison test). However, we did not observe this effect in the two UNC5B-invalidated organoids (SgUNC5B #1 control = 11.7 % $\pm$ 1.65; SgUNC5B #1 NP001 = 12.24% $\pm$ 4.74; SgUNC5B #1 NP137 = 11.28 % $\pm$ 2.84; SgUNC5B #2 control = 11.5 % $\pm$ 0.42; SgUNC5B #2 NP001 = 11.76 % $\pm$ 2.66; SgUNC5B #2 NP137 = 9.48 $\pm$ 1.78). These results provide further evidence supporting the notion that the effect of Netrin-1 inhibition on self-renewal is indeed dependent on the presence of the UNC5B receptor.

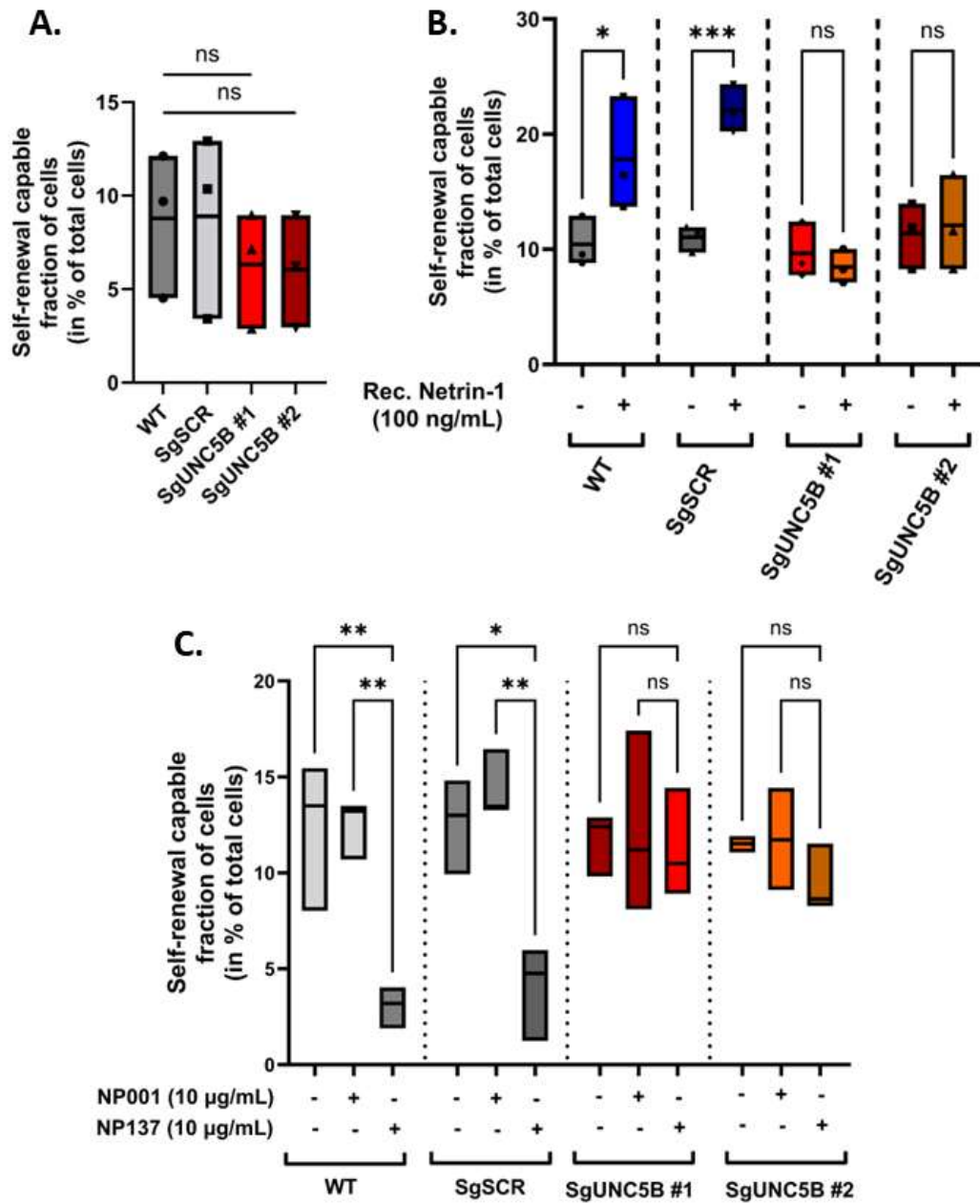


Figure 4.8 - Netrin-1 enhances self-renewal in mCRC organoids through the UNC5B dependence receptor (A) Self-renewal capable fraction of in wildtype, scramble control (sgSCR) and CRISPR-edited (SgUNC5B#1 and SgUNC5B#2) PIK315LT organoid cell line observed by ELDA (B) Self-renewal capable fraction of in wildtype, scramble control (sgSCR) and CRISPR-edited (SgUNC5B#1 and SgUNC5B#2) PIK315LT organoids, either left untreated (Control) or treated with recombinant Netrin-1 (100 ng/mL), observed by ELDA; (C) Self-renewal capable fraction of in wildtype, scramble control (sgSCR) and CRISPR-edited (SgUNC5B#1 and SgUNC5B#2) PIK315LT organoid cell line treated with either nothing (Control), NP001 (10µg/mL) or NP137 (10µg/mL) observed by ELDA; Statistical significance was determined using One-way Anova – Turkey’s multiple comparison test, ns = non-significant, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$; N=3

e. Targeting Netrin-1/UNC5B pathway in combination with chemotherapy

Our findings demonstrate the implications of the Netrin-1/UNC5B pathway in the regulation of self-renewal, a primary characteristic of CSCs, in mCRC. Since, as discussed previously, CSCs have a key role in tumour resistance to chemotherapy and post-treatment recurrence, the Netrin-1/UNC5B axis could represent an interesting therapeutic target in mCRC, in combination with chemotherapy. Indeed, by targeting this pathway, we aim to disrupt CSCs' ability to self-renew, potentially making chemotherapy more effective by reducing the chances of cancer recurrence. This could represent a significant advancement in the treatment of mCRC.

To explore this possibility, we continued our experiments using one of our mCRC organoid cell lines, specifically the one expressing the highest levels of UNC5B: ySAW190LT. Through quantitative PCR (qPCR), we observed a significant increase in *UNC5B* mRNA expression (approximately 3-fold, $p < 0.05$, paired t-test) and *NTN1* mRNA expression (approximately 3-fold, $p < 0.05$, paired t-test) upon treatment with low dose of FOLFOX (1 μ M for 24 hours) (**Figure 4.8-A and B**), confirming the upregulation of this pathway during FOLFOX treatment observed in the previous chapter. It is to be noted that FOLFOX treatment includes leucovorin in a clinical setting to diminish the toxicity of 5-FU, leucovorin being non-relevant in an *in vitro* setting, we used 5-FU/Oxaliplatin treatment in vitro experiments (referred to as FOLFOX treatment). Additionally, we noted that even after 72 hours of treatment with 1 μ M FOLFOX followed by 15 days of recovery, the expression of UNC5B at the protein level remained elevated in ySAW190LT organoids (approximately 3-fold, $p < 0.05$, paired t-test) (**Figure 4.8-C**). Considering the previously identified impact of Netrin-1/UNC5B inhibition on self-renewal, this sustained upregulation of Netrin-1 and the UNC5B receptor during chemotherapy treatment and after recovery suggests that targeting this pathway may represent a promising strategy in this type of tumour.

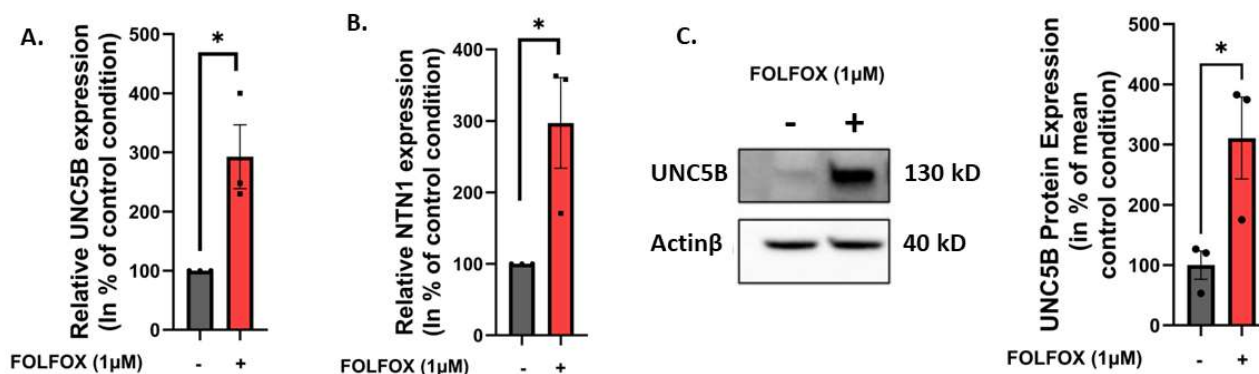


Figure 4.9 - Upregulation of UNC5B and Netrin-1 during FOLFOX treatment and recovery in recurrence capable PDOs (A) Quantification of relative **UNC5B** mRNA expression level in ySAW190LT PDO after 24h of treatment with either the vehicle or FOLFOX (1 µM) by real-time qPCR (B) Quantification of relative **NTN1** mRNA expression level in ySAW190LT PDO after 24h of treatment with either the vehicle or FOLFOX (1 µM) by real-time qPCR (C) Quantification of UNC5B protein expression level in ySAW190LT PDO after 72h of treatment with either the vehicle or FOLFOX (1 µM) and 15 days of recovery by western blot; Statistical significance was determined using paired t-tests, Error bar indicate SD ; * = $p < 0.05$.

Given the observed upregulation of both Netrin-1 and UNC5B following FOLFOX treatment, along with the maintained overexpression of the UNC5B receptor post-recovery, we hypothesized that the effects of Netrin-1 and its inhibition continue during and after FOLFOX treatment. To test this hypothesis, we assessed the post-chemotherapy self-renewal capability of ySAW190LT organoids treated with FOLFOX (1 µM for 72 hours), along with either recombinant Netrin-1 (100 ng/mL) and/or NP001 (10 µg/mL) and NP137 (10 µg/mL) (**Figure 4.9-A**). Our observations revealed that organoids treated with Netrin-1, with or without NP001, exhibited increased self-renewal (Netrin-1 = $8.65 \% \pm 5.15$; Netrin-1 + NP001 = $8.21 \% \pm 2.67$); compared to non-treated or NP001-treated cells (CTR = $4.34 \% \pm 0.87$; NP001 = $3.88 \% \pm 1.94$). Conversely, cells treated with NP137 showed a significant reduction in post-treatment self-renewal (NP137 = $0.98 \% \pm 0.11$; Netrin-1 + NP137 = $0.85 \% \pm 0.05$); compared to the other conditions, regardless of the presence or absence of recombinant Netrin-1. These findings indicate that Netrin-1 continues to regulate self-renewal and colorectal CSCs during FOLFOX treatment. The remarkable decrease of self-renewal observed in the NP137-treated conditions suggests a significant impact of Netrin-1 in inhibiting CSC apoptosis during chemotherapy treatment.

Therefore, we decided to investigate the potential effect of NP137 on the efficacy of FOLFOX treatment *in vivo*. We utilized a model of BALBc/nude mice, which retain an innate immune system, in which we performed engraftment with ySAW190LT organoids (**Figure 4.9-B**). We then monitored tumour growth in mice treated with a vehicle solution (non-treated), FOLFOX (a solution combining 5-Fluoro Uracil (50 mg/kg), oxaliplatin (6 mg/kg), and disodium levofolinate (90 mg/kg, daily for two days at week 0)), or FOLFOX + NP137 (20 mg/kg of NP137; three times a week). The experiment was first designed originally to observe a potential effect of NP137 on recurrence post FOLFOX-treatment, CSCs being known to be responsible for this type of recurrence due to their ability to recreate tumoral heterogeneity. However, the FOLFOX dose tested was insufficient to induce a significant tumour response, preventing the analysis of post-treatment recurrence. Nevertheless, we found that tumours treated with NP137 grew significantly slower than those in the non-treated or FOLFOX-treated groups. By week 9, tumours in the NP137 group exhibited considerably smaller sizes (n = 8; mean tumour size = 183.94 mm³) compared to those in the non-treated group (n = 8; mean tumour size = 523.94 mm³, p<0.01, Two-way Anova – Dunnett's multiple comparisons test) or the FOLFOX-treated group (n = 8; mean tumour size = 417.23 mm³, p<0.05, Two-way Anova – Dunnett's multiple comparisons test) (**Figure 4.9-C and E**). Additionally, we observed a similar effect on tumour weight (approximately 0.3 g/tumour for non-treated and FOLFOX-treated conditions vs approximately 0.15 g/tumour in the FOLFOX + NP137 condition) (**Figure 4.9-D**). These *in vivo* results demonstrate that targeting the UNC5B/Netrin-1 pathway to enhance chemotherapy treatment is relevant *in vivo* and that the NP137, beyond its possible effect on long-term self-renewal and relapse, has a strong effect in the potentiation of chemotherapeutic treatment. Overall, we illustrated the therapeutic potential of NP137 for the treatment of mCRC justifying further clinical studies.

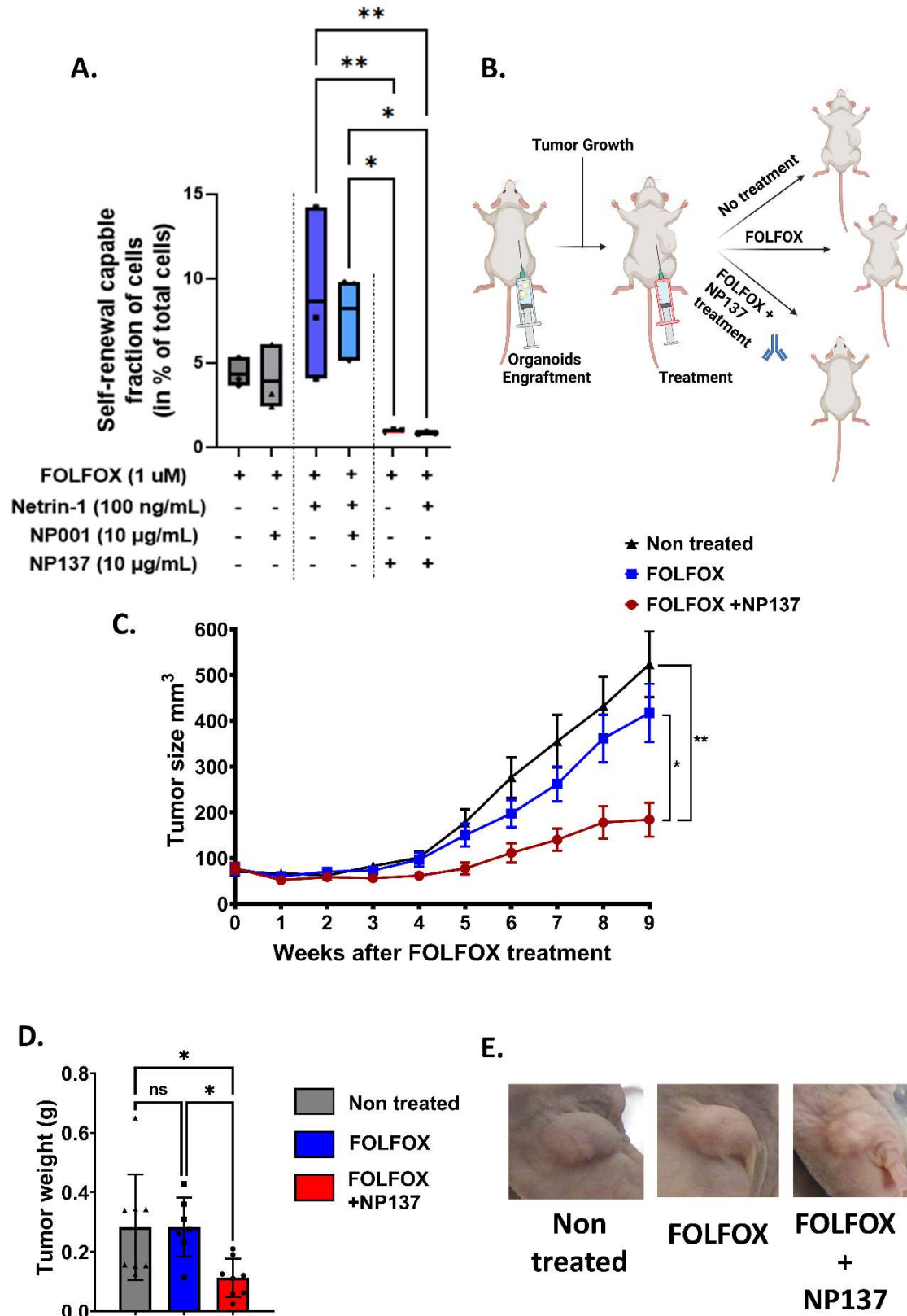


Figure 4.10 – Targeting Netrin-1 to enhance FOLFOX treatment in mCRC (A) Self-renewal capable fraction of ySAW190LT organoid cell line after 72h of FOLFOX treatment (72h ; 1 μM) in presence or absence of recombinant Netrin-1 (100 ng/mL), NP001 (10 μg/mL) and/or NP137 (10 μg/mL) observed by ELDA; Statistical test used : One-way Anova – Turkey's multiple comparison test **(B)** Schematic representation of In Vivo protocol to evaluate the therapeutic potential of NP137 in mCRC using PDO-

xenografted mice; (C) Evolution of tumour size in BALBc nude mice subcutaneously engrafted with ySAW190LT PDO after treatment with either PBS or FOLFOX (solution combining 5-Fluoro Uracil (50mg/Kg IP), oxaliplatin (6mg/Kg IP) and disodium levofolinate (90mg/Kg IP) daily for two days) or FOLFOX and NP137 (20 mg/kg ; three times a week); N = 8/group, Error bars indicate SEM; Statistical test used : Two-way Anova - Dunnett's multiple comparisons test (D) Tumour weight of engrafted tumours in mice treated with either PBS, FOLFOX or FOLFOX and NP137 at week 10 ; N = 8/group, Error bars indicate SD, Statistical test used : One-way Anova - Turkey's multiple comparisons test (E) Pictures of representative tumours in each treatment group (Non treated : mean tumour size = 523.94 mm³, tumour size in picture = 499.85 mm³; FOLFOX treated : mean tumour size = 417.23 mm³, tumour size in picture = 435.6675 mm³; FOLFOX + NP137 treated: mean tumour size = 183.94 mm³, tumour size in picture = 200.37 mm³)

III-Conclusion and discussion

Stemness represents a critical phenotypical feature of cancer cells that we need to apprehend to understand the complexity of tumoral heterogeneity and is pivotal in initiating and sustaining tumour growth, particularly in CRC and mCRC. These cells possess characteristics akin to non-pathological stem cells but are distinguished by their capacity to drive tumour growth, metastasis, and resistance to conventional therapies. Specifically, in CRC, CSCs demonstrate self-renewal abilities, enabling them to proliferate and generate new CSCs while maintaining an undifferentiated state, thereby contributing significantly to tumour malignancy. However, the precise molecular mechanisms governing self-renewal in colorectal CSCs remain incompletely understood. In mCRC, where conventional treatments like chemotherapy are often employed due to the impossibility of practising surgical resection alone, CSCs seem to play a significant role in therapeutic resistance and disease recurrence. Chemoresistance in CSCs involves diverse mechanisms such as quiescence, upregulation of anti-apoptotic proteins, enhanced drug efflux, increased detoxification enzyme activity, and improved DNA repair mechanisms. Understanding the modulation of CSC behaviour and their response to chemotherapies is crucial for developing more effective therapeutic strategies in mCRC.

The investigation into the involvement of Netrin-1 in CRC cell self-renewal revealed a significant decrease in self-renewal capacity upon Netrin-1 inhibition with the neutralizing antibody NP137, emphasizing the pivotal role of Netrin-1 in driving CRC cells' self-renewal potential. Moreover, the study explored the impact of UNC5B

overexpression on self-renewal potential, demonstrating a notable increase of self-renewal-capable fraction in UNC5B overexpressing cells, suggesting a positive regulatory role of UNC5B in CRC self-renewal. Interestingly, even in the absence of Netrin-1, UNC5B overexpression retained a non-significant impact on self-renewal, albeit to a lesser extent, implying either that endogen Netrin-1 is expressed at a concentration superior to 130 uM, a potential Netrin-1-independent effect of UNC5B, or just a slight effect of doxycycline.

We further investigated the effect of Netrin-1 supplementation or inhibition on a model more able to reconstitute the complex heterogeneity of cells observed in CRC. Showing that inhibition of Netrin-1 with NP137 led to a notable decrease in self-renewal capabilities in both UNC5B-high (ySAW190LT) and UNC5B-medium/low (PIK315LT) expressing organoid cell lines, indicating the importance of the Netrin-1 pathway in CRC self-renewal regulation. Additionally, supplementation with recombinant Netrin-1 resulted in enhanced self-renewal, further confirming its impact on CRC organoid behaviour. Importantly, NP137 effectively counteracted the effects of recombinant Netrin-1, further confirming the specificity of Netrin-1 inhibition. Further examination of CD133-positive cell populations, a marker of colorectal CSCs, revealed a significant increase in CD133-positive cells upon treatment with recombinant Netrin-1, which was reversed by NP137. These findings provide compelling evidence for the role of Netrin-1 in modulating colorectal CSC populations within mCRC, highlighting its potential significance in phenotypical characteristics of CRC tumours, and indicating a promising avenue for targeted therapeutic interventions.

Our studies concerning the role of Netrin-1 in mCRC organoids its impact on CSCs apoptosis and its relationship with self-renewal. Indeed, treatment with recombinant Netrin-1 led to a decrease in late apoptosis specifically within the CD133-positive CSC population, indicating a role for Netrin-1 in the inhibition of CSCs-apoptosis. Moreover, the inhibition of apoptosis with z-Vad-FMK restored self-renewal rates of NP137-treated cells to levels comparable to those seen in cells treated with Netrin-1, demonstrating that Netrin-1 signalling regulates colorectal CSC self-renewal through inhibition of apoptosis. Additionally, investigations into the mechanisms underlying apoptosis induced by Netrin-1 withdrawal revealed a significant decrease in MCL1 protein

expression, suggesting that the potential mechanism through which Netrin-1 inhibition mediates apoptosis in mCRC organoids relies on MCL1 active degradation.

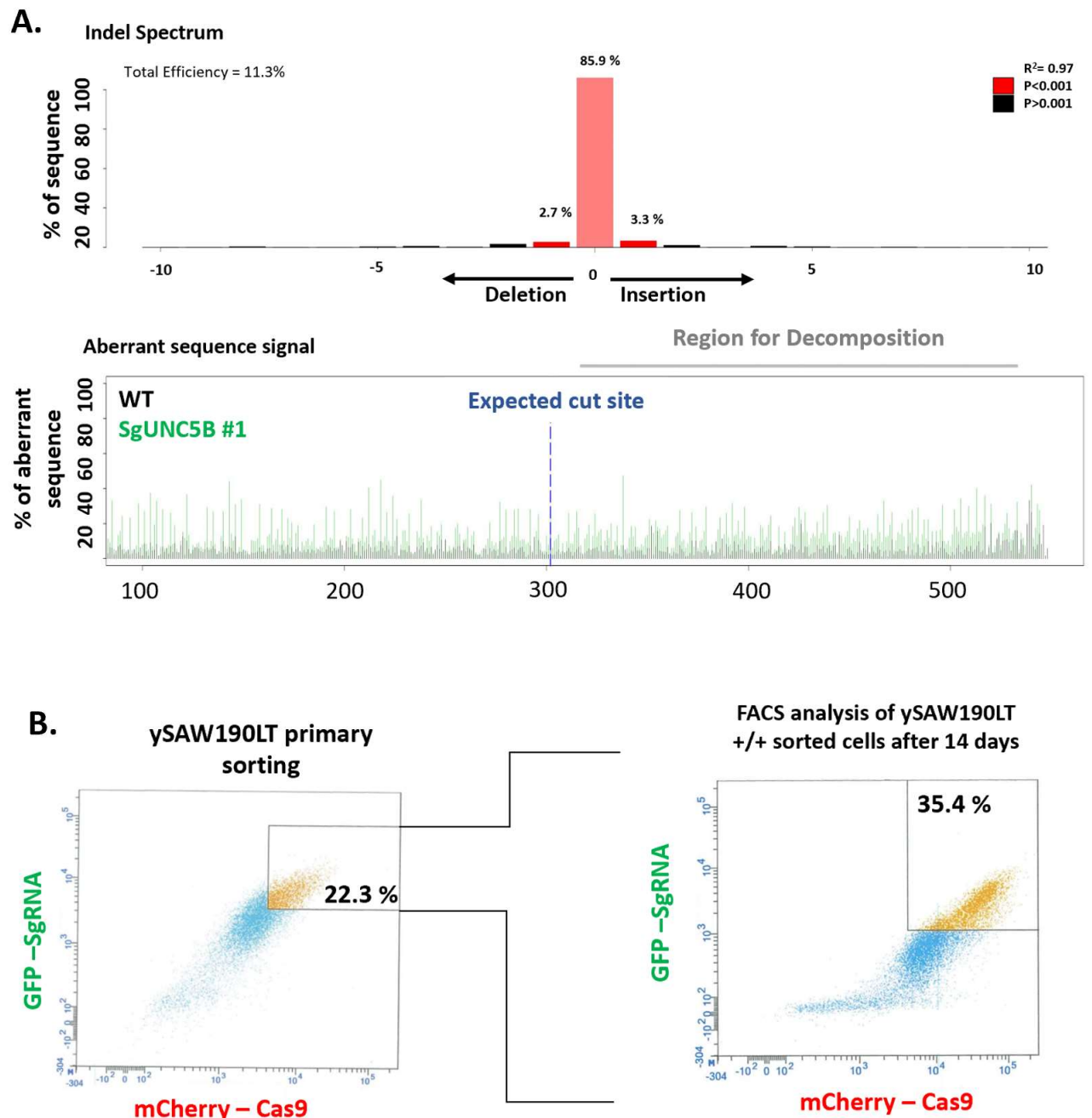
In our attempts to determine if the Netrin-1 effect was dependent on the UNC5B receptor, we observed that contrary to expectations, UNC5B gene invalidation did not lead to a substantial difference in self-renewal compared to wild-type and scramble control organoid cell lines. However, treatment with recombinant Netrin-1 significantly increased self-renewal in wild-type and scramble control organoids but had no effect in UNC5B-invalidated organoids, indicating the dependency of the Netrin-1 effect on the presence of the UNC5B receptor. Further validation using Netrin-1 inhibition with NP137 showed a decrease in self-renewal in wild-type and scramble control organoids but not in UNC5B-invalidated organoids, reinforcing the dependency of Netrin-1 impact on self-renewal on the UNC5B receptor.

Moreover, we explored the potential therapeutic implications of targeting the Netrin-1/UNC5B pathway in mCRC. RNA sequencing data from organoid cell lines treated with FOLFOX chemotherapy revealed a significant increase in UNC5B mRNA expression, suggesting a potential avenue for targeting this pathway in late-stage tumours. Further experiments confirmed the upregulation of UNC5B and Netrin-1 genes during and after FOLFOX treatment. Additionally, post-chemotherapy experiments demonstrated that Netrin-1 continues to regulate self-renewal and colorectal CSCs during FOLFOX treatment, with NP137, an inhibitor of Netrin-1, significantly reducing post-treatment self-renewal. Finally, *In vivo* experiments using a mouse model showed that tumours treated with NP137 alongside FOLFOX exhibited slower growth and smaller sizes compared to non-treated or FOLFOX-treated tumours, highlighting the therapeutic potential of targeting the UNC5B/Netrin-1 pathway in mCRC and justifying further clinical studies.

Finally, the significant effect of NP137 on self-renewal post-treatment suggests its potential as a post-chemotherapy treatment to prevent recurrence in metastatic colorectal cancer. Further *in vivo*, investigations using appropriate models are necessary to confirm the efficacy of NP137 in preventing recurrence and its potential as a therapeutic strategy in mCRC management. These findings collectively underscore the

complex interplay between Netrin-1, UNC5B, and chemotherapy in regulating CSC behaviour and highlight the therapeutic potential of targeting this pathway in mCRC.

Supplementary Figures



Supplementary Figure 4-1 Unsuccessful UNC5B invalidation in ySAW190LT PDO (A) Representative TIDE analyses comparing Sanger sequencing traces from wildtype PDOs versus CRISPR-edited (SgUNC5B#1) ySAW190LT organoid **(B)** (Left) FACS sorting of double-positive (Cas9-SgRNA) ySAW190LT cells (7 days after transduction, 72h after induction) (Right) FACS analysis of double-positive (Cas9-SgRNA) of cells sorted after 2 weeks in culture

Chapter 5 - Cellular heterogeneity of NTN1/UNC5B signaling

I- Introduction

In the previous chapter, we demonstrated that Netrin-1 significantly enhances self-renewal in mCRC cells along with an increased proportion of CSCs. This mechanism relies on apoptosis and is concomitant to the degradation of the anti-apoptotic protein MCL1 in our organoid model. We also showed that this effect of Netrin-1 is dependent on the UNC5B receptor. However, fully understanding the precise mechanism of how Netrin-1 influences the CSC subpopulation in mCRC requires a more detailed approach.

Indeed, the complexity and heterogeneity of CSCs in CRC present challenges in understanding their regulation. CSCs-related properties appear to be regulated through various mechanisms, though, it seems that these mechanisms do not always work in concert. Indeed, phenotypical characteristics such as self-renewal and tumour-forming abilities are sometimes not correlated, or even negatively influenced by the expression of markers or transcription factors normally associated with stemness[126]. For instance, experiments have been shown to influence sphere-forming abilities without impacting biomarker expression like CD133, highlighting the complex nature of CSC regulation [127].

Indeed, the complexity and heterogeneity inherent to the CSC phenotype necessitate moving beyond methods like the characterization of the CD133-positive population by FACS to fully apprehend the effect of Netrin-1 comprehensively. To address this, we performed single-cell sequencing on our organoid cell lines. This technique allowed us to investigate in greater detail the effects of Netrin-1 deprivation on mCRC cells using the NP137 antibody. Through this exploration, we aim to gain a deeper understanding of the molecular pathways governing CSC behaviour in mCRC and the mechanisms through which Netrin-1 and UNC5B couple modulate these cells.

II- Results

a. Netrin-1 inhibition impairs stemness in mCRC cells

The primary aim of this chapter was to investigate the impact of Netrin-1 deprivation on the CSC subpopulation within ySAW190LT and PIK315LT organoids at the single-cell level. We selected the ySAW190LT and PIK315LT organoid cell lines specifically for their distinct characteristics to understand how Netrin-1 deprivation affects relatively different mCRC organoid cell lines. ySAW190LT cell line expresses high levels of UNC5B (**Figure 3.7**), is chemosensitive but capable of recurrence after treatment, and is wild-type for the p53 gene. PIK315LT cell line expresses low to medium levels of UNC5B (**Figure 3.7**), is chemoresistant, and exhibits limited recurrence abilities. These divergent traits make these cell lines good candidates for studying the impact of Netrin-1 inhibition in different phenotypical contexts. This involved treating the organoids with either NP001 (10 ug/mL) or NP137 (10 ug/mL) for 72 hours, followed by scRNAseq analysis. After treatment and single-cell digestion of our organoids, cells were counted, and no decreased number of cells was observed in NP137 conditions. The concentration of cells per millilitre (c/mL; final volume 1 mL) for the ySAW190LT cell line treated with NP001 was 7.81×10^5 , and for the same cell line treated with NP137, it was 5.82×10^5 . On the other hand, for the PIK315LT cell line, the concentration with NP001 treatment was 1.04×10^6 c/mL, while with NP137 treatment, it was 1.22×10^6 c/mL. Cells were loaded and run to reach 7000 to 10,000 cells per sample and the Cell Ranger QC report of the samples estimated the median number of genes per cell between 5090 and 5,581 and a median number of reads per cell between 42,259 and 61,867 (**Supplementary figure 5.1**).

The clustering of organoid cells did not reveal the presence of a distinct CSC cluster. Instead, the analysis showed that mCRC cells of this organoid cell line predominantly clustered based on the expression of genes associated with various cellular processes such as Hypoxia, Wnt/Epithelial signalling, Cellular respiration, cytoplasmic translation, cell adhesion, and cellular division (**Figure 5.1 - A and Supplementary Figure 5.2**).

The lack of clustering based on stemness-related gene expression in the single-cell RNA sequencing data prevents us from analysing the effect of Netrin-1 deprivation on the specific CSC subpopulation. However, we can use this data to observe the impact of NP137 on stemness-related gene expression in ySAW190LT organoid cells. Through this analysis, we observed a global reduction in the expression of key CRC stemness-related

genes in ySAW190LT cells treated with NP137 (log2foldchange values and adjusted p-values : LGR5: -0.218, p.adj = 6.52e-56; SMOC2: -0.573, p.adj = 1.05e-123; PROM1: -0.288, p.adj = 1.97e-92; SOX4: -0.482, p.adj = 3.55e-230) (**Figure 5.1 - B & C; Table 5.1**).

It is to be noted that we obtained from our collaborators (The Apoptosis and Cancer Team in CRCL, France) RNA sequencing data from a patient suffering from Colon adenocarcinoma integrated in the Phase I clinical trial for the NP137. These RNA sequencing data from the patient tumour before and after treatment with NP137 allow us to observe a similar decrease in stemness-related genes (PROM1, LGR5, SMOC2, SOX4) after treatment (**Supplementary Figure 5.3**). Confirming the effect of Netrin-1 on stemness of CRC cells *in situ*.

Additionally, utilizing the UCell function of the clusterProfiler package to evaluate the transcription of several genes listed in our samples. UCell is an R package for scoring gene signatures in single-cell datasets. UCell scores, based on the Mann-Whitney U statistic, are robust for analysing dataset size and heterogeneity, enabling the processing of large datasets, and can be applied to any cell vs. gene data matrix, and includes functions to directly interact with Seurat, it calculates enrichment scores on the single-cell level and returns a single value per cell. Doing so, we observed a notable decrease in genes associated with stem cell proliferation (mean expression of genes in GO: Positive regulation of stem cells proliferation in NP001: 0.075 vs NP137: 0.069; p.adj = 1e-36) (**Figure 5-1.D**), further supporting the downregulation of stemness-related pathways in the ySAW190LT organoid upon NP137 treatment. These findings confirm observations described in the previous chapter and provide additional evidence of Netrin-1's influence on stemness in mCRC cells.

We also performed a similar scRNA sequencing experiment on organoids derived from PIK315LT; However, we did not observe a similar decrease in stemness-related genes upon NP137 treatment as seen in ySAW190LT. There was a slight increase in the expression of some genes (log2foldchange values and adjusted p-values : LGR5: 0.0683, p.adj = 7.4e-13; SMOC2: 0.105, p.adj = 2.1e-16; PROM1: 0.0367, p.adj = 2.1e-05).

This difference could be due to the fact that the baseline self-renewal of the ySAW190LT cell line is higher than the PIK315LT one, which could explain the relatively low

expression of genes like PROM1 in PIK315LT compared to ySAW190LT (ySAW190LT: 0.75, PIK315LT: 0.3). Moreover, UNC5B protein is considerably more expressed in the ySAW190LT cell line, therefore, ySAW190LT must be more sensitive to Netrin-1 inhibition than the PIK315LT organoid cell line. This hypothesis is coherent with our previous observation regarding UNC5B invalidation in PIK315LT cells and our hypothesis stating that, unlike ySAW190LT, PIK315LT cells possess mechanisms to adapt to the loss of UNC5B, whereas UNC5B invalidation completely inhibits ySAW190LT growth.

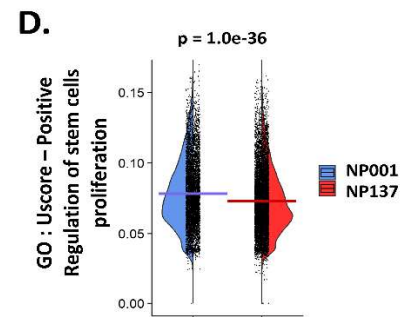
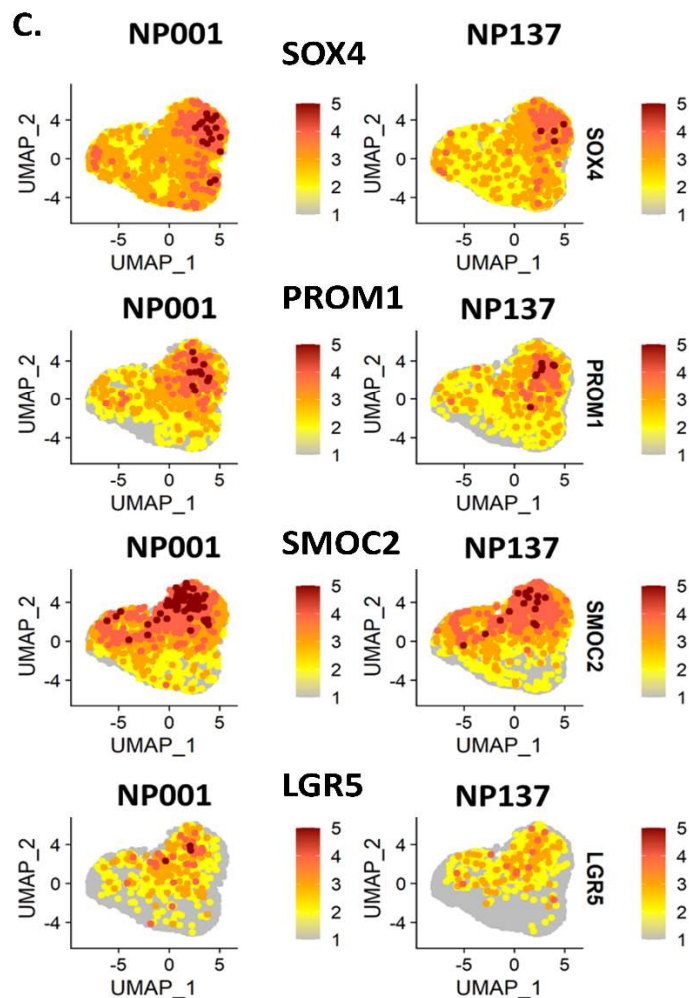
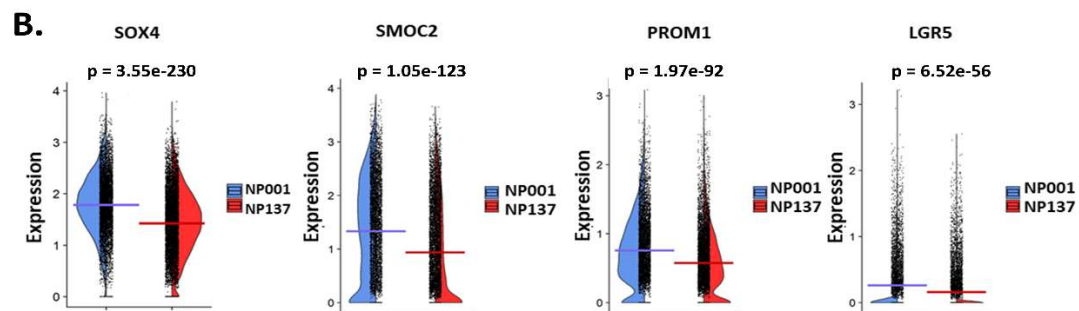
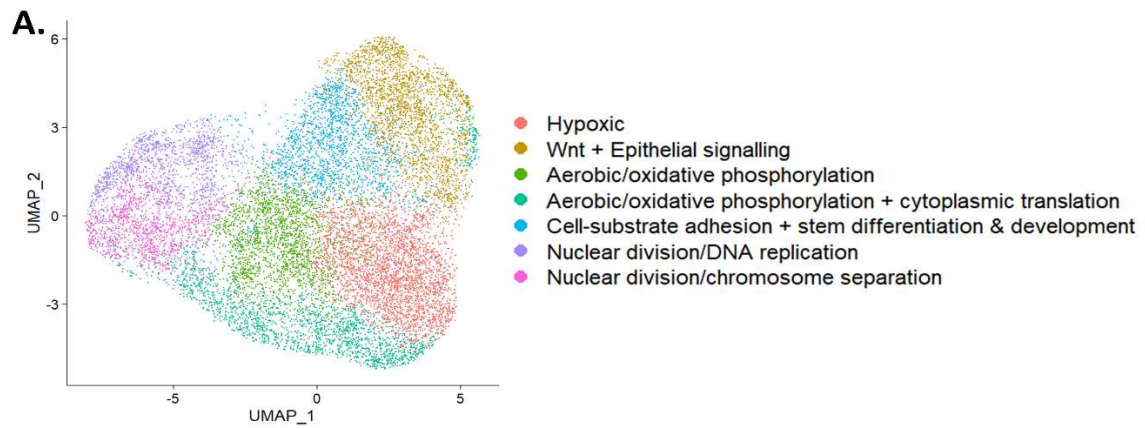


Figure 5.1 - NP137 impairs expression of stemness-related genes in ySAW190LT – scRNA sequencing analysis **(A)** UMAP of integrated data of ySAW190LT organoid treated with NP001 or NP137 – display of clusters defined using Findclusters and Findmarkers function of Seurat package ; **(B)** Violin plot of the expression of SOX4, SMOC2, PROM1 and LGR5 in ySAW190LT organoid treated with either NP001 or NP137 (Means and p.values displayed determined by stat_compare_means function; Bars indicate means, statistical test use : Wilcoxon test, precise values displayed in table 5-1); **(C)** UMAP plot of SOX4, SMOC2, PROM1 and LGR5 expression level ySAW190LT organoid cells treated with either NP001 or NP137; **(D)** Violin plot of global expression of genes related to the Positive regulation of stem cells proliferation in ySAW190LT organoid cells treated with either NP001 or NP137 (Score determined using UCell function and MSigDB GO: Positive regulation of stem cells proliferation gene list as reference; Means and p.values displayed determined by stat_compare_means function; Bars indicate means, statistical test use : Wilcoxon test, Each dot represent an event (cell))

Stemness related genes				
ySAW190LT				
Downregulated				
Gene	Expression level (NP001)	Expression level (NP137)	Log2FoldChange	Corrected p value
SOX4	1.784758	1.303090083	-0.481667917	3.55E-230
SMOC2	1.330902	0.757979342	-0.572922658	1.05E-123
PROM1	0.756295	0.468400053	-0.287894947	1.97E-92
LGR5	0.377681884	0.1597075	-0.217974384	6.52E-56

Table 5.4; Effect of NP137 on stemness related genes in ySAW190LT (Corrected p value and Log2FoldChange calculated using Findmarkers function of Seurat package; Wilcoxon Rank Sum test)

In the process of analysing the impact of NP137 on stemness genes in ySAW190LT organoids, we unexpectedly observed that cells displaying robust UNC5B expression did not appear to be the same that expressed high levels of stemness markers such as PROM1 (**Figure 5.2-A**). Additionally, the reduced expression of stemness markers like PROM1 was not specific to UNC5B-expressing cells (PROM1 in UNC5B^{neg}: 0.7651495 vs 0.4777741, p = 1.1e-53; PROM1 in UNC5B^{pos}: 0.7344532 vs 0.4428986, p = 2.5e-47 ; SMOC2 in UNC5B^{neg}: 1.5800644 vs 1.0288429, p = 3.6e-85; SMOC2 in UNC5B^{pos}: 0.9629583 vs 0.5604800, p = 2.5e-43 ; SOX4 in UNC5B^{neg}: 1.826728 vs 1.364448 , p = 8.3e-143; SOX4 in UNC5B^{pos}: 1.681229 vs 1.168886, p = 1.092030e-95 ; LGR5 in UNC5B^{neg}: 0.3942218 vs 0.1856880, p = 1.5e-28; LGR5 in UNC5B^{pos}: 0.3138779 vs 0.1026887, p = 2.757059e-17) (**Figure 5.2-B**). This data suggests that the effect of netrin-1 deprivation on self-renewing cells may not be directly triggered by UNC5B activation in these cells but rather involves an indirect mechanism. It is to be noted though, that the high expression of UNC5B, NTN1 and PROM1 genes seems correlated in PIK315LT organoid cells (**Supplementary Figure 5.4**).

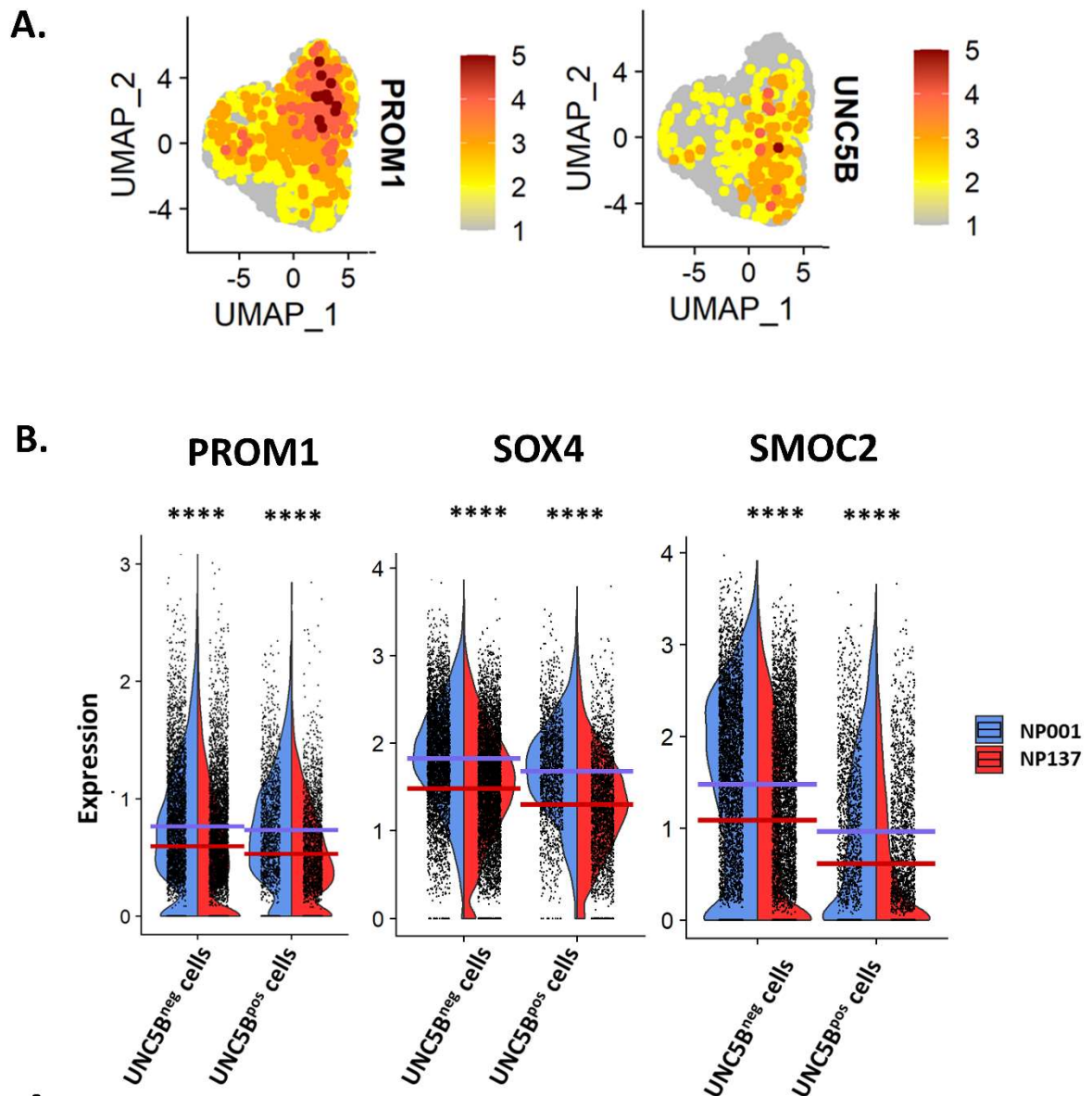


Figure 5.2 – *PROM1* expression in *UNC5B* expressing cells (A) UMAP plot of *PROM1* and *UNC5B* expression level in control ySAW190LT organoid cells (NP001-treated); (B) Violin plot of the expression of *PROM1*, *SOX4* and *SMOC2* genes in *UNC5B^{neg}* and *UNC5B^{pos}* cells treated with either NP001 or NP137 (Each dot represent an event (cell), Means and p.values displayed determined by *stat_compare_means* function; Bars indicate means, statistical test use: Wilcoxon test, **** = $p < 0.0001$)

Finally, using UCell, we observed a decrease in genes associated with negative regulation of apoptosis, suggesting the implication of apoptosis in NP137 (NP001: 0.173 vs NP137: 0.170; $p_{adj} = 6.5e-40$) (Table 5.2). This allowed us to see that the inhibition of apoptosis signalling wasn't especially impaired in *UNC5B^{pos}* cells than in *UNC5B^{neg}* cells, though it was particularly impaired in *PROM1^{high}* cells (top 25% of cells expressing high levels of *PROM1*) compared to *PROM1^{low}* cells (Table 5.2). This reinforces the observation that

Netrin-1 inhibition affects particularly the CD133-positive population, though, it implies that the apoptosis triggered in CSCs is not a direct result of the apoptosis triggered by the UNC5B dependence receptor.

Score - Negative Regulation of Apoptosis				
ySAW190LT				
Population	Score (NP001)	Score (NP137)	Difference (in % of NP001 condition)	Adjusted p value
General population	0.1728221	0.1705482	-1.316%	6.50E-40
UNC5B defined populations				
UNC5B neg	0.1726868	0.170111	-1.492%	1.40E-33
UNC5B pos	0.1731557	0.1715076	-0.952%	6.90E-09
PROM1 defined populations				
PROM1 low	0.1732197	0.1709746	-1.296%	3.11E-15
PROM1 high	0.1720074	0.1688616	-1.829%	1.99E-30

Table 5.5; Change of expression of genes related to the Negative regulation of apoptosis in ySAW190LT organoid cells treated with either NP001 or NP137 (Score determined using UCell function and MSigDB GO: Negative regulation of apoptosis gene list as reference; Cells having an expression inferior to 0.1 was defined as UNC5Bneg, others as UNC5Bpos; the top 25% of the cells expressing PROM1 (expression > 0.95) were considered as PROM1high, the others as PROM1low. Means and p.values displayed are determined by stat_compare_means function; Bars indicate means, statistical test use: Wilcoxon test)

b. TFF3: the link between Netrin-1 and CSCs ?

To elucidate the mechanisms on which Netrin-1 relies to modulate mCRC CSCs, we attempted to identify genes for which expression would be affected by NP137 treatment specifically in UNC5B-expressing cells, with a particular interest in genes involved in cell-to-cell communication. This led us to identify Trefoil Factor 3 (TFF3) as a potential intermediate for the effect of Netrin-1 on self-renewal. TFF3 is a member of the trefoil factor family, small secretory peptides playing crucial roles in mucosal protection of the gastrointestinal tract, wound healing, and cancer progression. In the context of cancer, TFF3 has been implicated in promoting EMT, as well as in enhancing stemness traits in

cells of several cancer types including colorectal cancer [352-356] and self-renewal in breast cancer [357]. Additionally, TFF3 degradation has been shown to trigger apoptosis in cancer cells through the dysregulation of anti-apoptotic proteins such as BCL2 or MCL1 [358-361]. Interestingly, our analysis revealed that cells expressing high levels of TFF3 were predominantly UNC5B-expressing cells (**Figure 5.3-A**). Furthermore, a significant decrease in TFF3 expression was observed specifically in UNC5B-expressing cells upon NP137 treatment (TFF3 in UNC5B^{pos}: 4.3058 vs 4.1386, $p = 2.1 \times 10^{-11}$; TFF3 in UNC5B^{neg}: 3.8272 vs 3.8144, $p = 0.5$;) (**Figure 5.3 - B & C**). These findings suggest a potential link between Netrin-1 signalling through UNC5B and the regulation of TFF3 expression in mCRC.

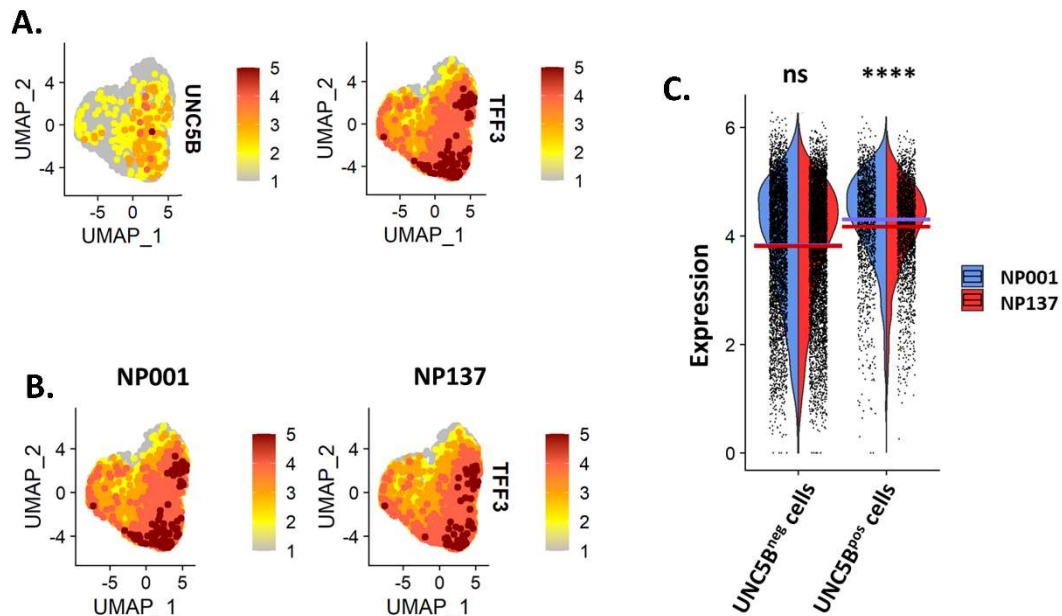


Figure 5.3 – NP137 decrease TFF3 expression in UNC5B^{pos} cells (A) UMAP plot of UNC5B and TFF3 expression level in control ySAW190LT organoid cells (NP001-treated); (B) UMAP plot of TFF3 expression level ySAW190LT organoid cells treated with either NP001 or NP137; (C) Violin plot of the expression of TFF3 treated with either NP001 or NP137 in UNC5B^{neg} and UNC5B^{pos} cells (Means and p.values displayed determined by `stat_compare_means` function; Bars indicate means, statistical test use: Wilcoxon test)

To further investigate the potential relationship between TFF3 and Netrin-1 in mCRC cells, we used our RNA sequencing data to evaluate potential correlations between TFF3, UNC5B and stemness-related genes. In late-stage CRC tumours (n=58), TFF3 mRNA expression was positively correlated with PROM1 and SOX4 expression (**Figure 5.4-B &**

C). Additionally, TFF3 mRNA expression was correlated with UNC5B mRNA levels (**Figure 5.4-A**). Similarly, in mCRC organoids (n=28) TFF3 mRNA expression strongly correlated with SMOC2 and PROM1 mRNA levels (**Figure 5.4-D & E**). These correlations suggest the plausibility of TFF3 implications in the modulation of the Netrin-1 effect on mCRC CSCs.

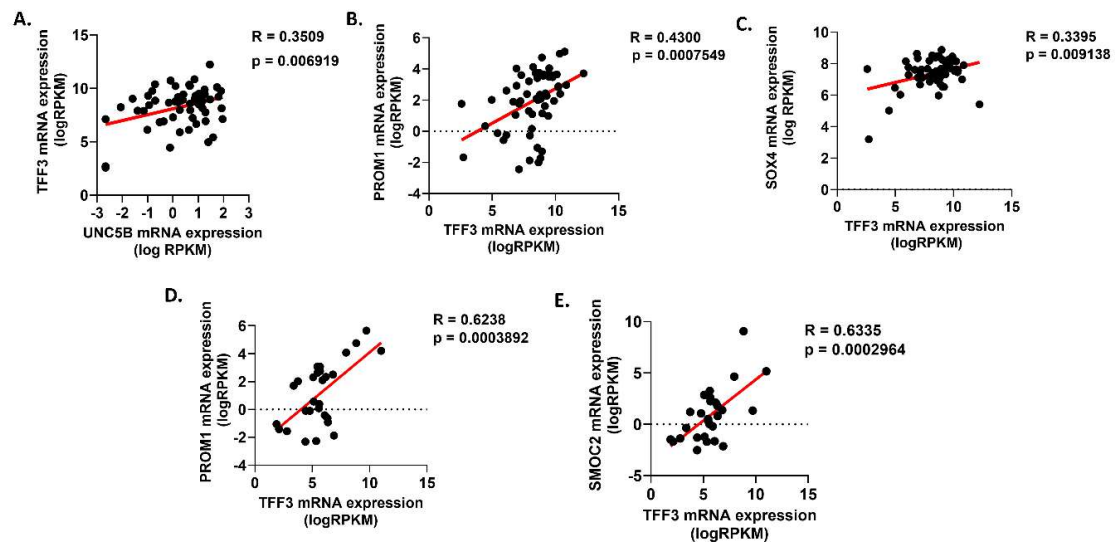


Figure 5.4 – TFF3 mRNA expression correlates with UNC5B and stemness related genes in late-stage CRC tumours and mCRC organoids (A) Correlation between UNC5B mRNA and TFF3 in late-stage CRC tumours (B) Correlation between PROM1 mRNA and TFF3 in late-stage CRC tumours (C) Correlation between SOX4 mRNA and TFF3 in late-stage CRC tumours (D) Correlation between PROM1 mRNA and TFF3 mRNA in mCRC organoids (E) Correlation between SMOC2 mRNA and TFF3 mRNA in mCRC organoids; (late stage tumours cohort: n=58, mCRC organoids: n=28); Statistical tests: Spearman's rank correlation coefficient (All values are displayed)

Unfortunately, due to time restrictions, we were not able to explore further the possible link between UNC5B, TFF3 and stemness-related features. A set of experiments has been designed to evaluate the possible causal relationship of UNC5B and TFF3 using recombinant TFF3 and a TFF3 inhibitor (AMPC) to revert effects observed with recombinant Netrin-1 and NP137.

c. Impact of NP137 treatment on other biological processes in mCRC organoids

We further analysed our scRNAseq data to gain a more exhaustive view of the effect of netrin-1 deprivation on mCRC cell signalling and biology. We utilized the `FindMarkers`

function from the Seurat package to analyse differential gene expression between our samples, helping us identify genes that were significantly differentially expressed. Additionally, we employed the `EnrichGO` function to perform gene ontology enrichment analysis on these differentially expressed genes, providing information into the biological processes associated with these gene expression changes. We identified differentially expressed between the NP001 and NP137 treated conditions and observed effects that were both common and specific to the two analysed organoid lines, PIK315LT and γSAW190LT (**Figure 5.5 & 5.6**). A common observation in both cell lines was the impaired expression of genes related to cytoplasmic translation following treatment with NP137, as well as an impact on mitochondrial chain components, oxidative phosphorylation, and cellular respiration (**Figure 5.5 & 5.6**). There was also a significant decrease in genes involved in development and morphogenesis in NP137-treated γSAW190LT organoids, such as BMP4, CTNNB1, STARD13, PROX1, NRP1, PDE4D, MYADM, SOX4, SEMA3A, and NRP1, aligning with the observed effects of Netrin-1 on CSCs. Furthermore, a modification of the expression of genes related to cell adhesion was observed in both organoid cell lines, though genes differentially expressed related to these processes are different in both organoids and seem mostly upregulated in PIK315LT and downregulated in γSAW190LT. Finally, NP137 treatment also affected the regulation of GTPases in the PIK315LT organoid cell line.

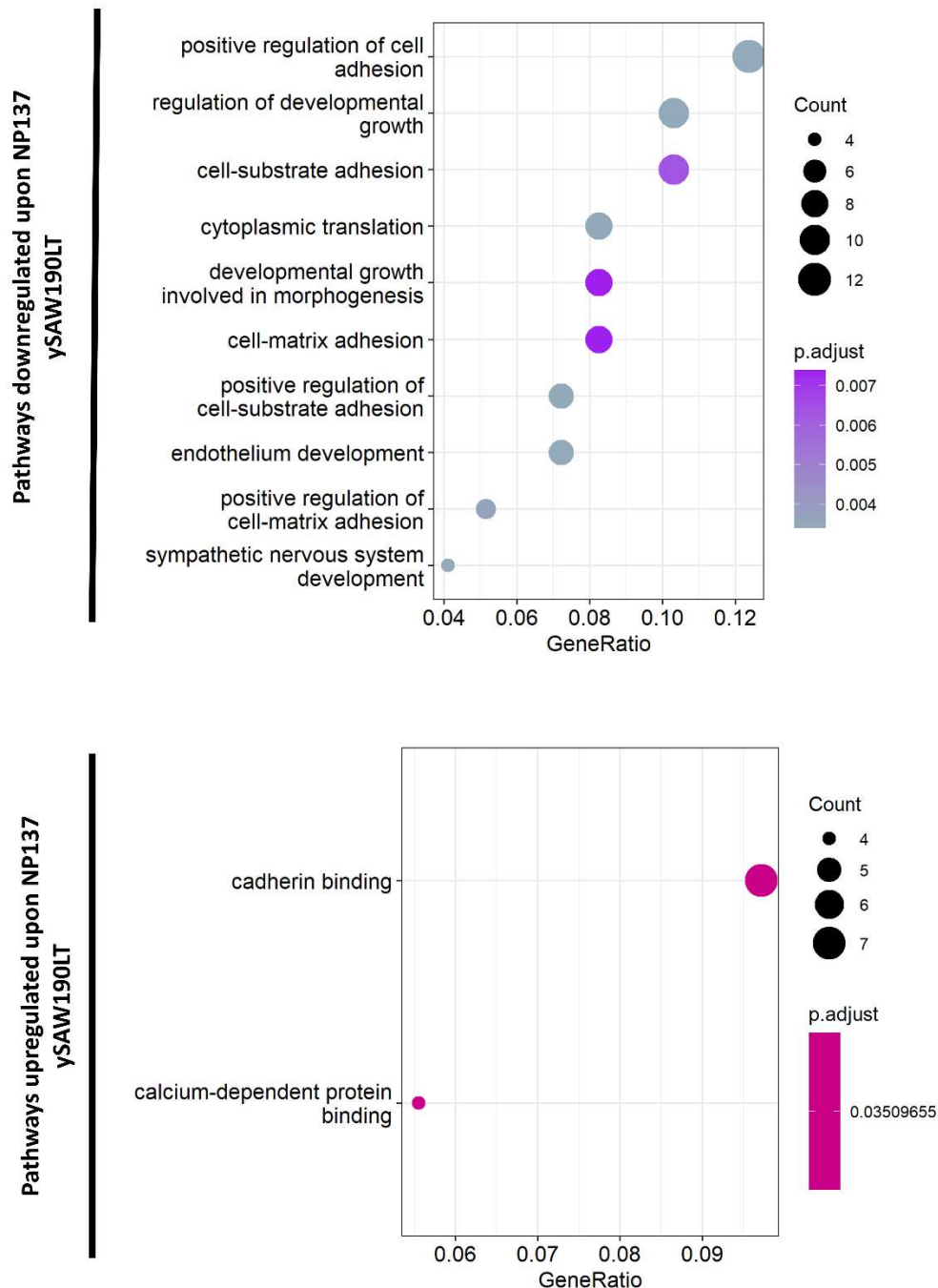


Figure 5.5 – Pathways impacted by NP137 treatment in ySAW190LT organoids –Pathway enrichment analysis for genes (up) downregulated and (down) upregulated in ySAW190LT upon NP137 treatment (Genes differentially expressed determined using FindMarkers function of Seurat package, pathways determined using enrichGO function of clusterProfiler package, Gene Ratio is a metric used to measure the degree of enrichment of a gene set within a larger gene list. It is calculated by dividing the number of genes from the gene set that are present in the input gene list by the total number of genes in the gene set; Genes were considered differentially expressed if $\log_2\text{foldchange} > 0.15$; $p\text{-value cut. off} = 0.04$; Statistical test used: Benjamini-Hochberg)

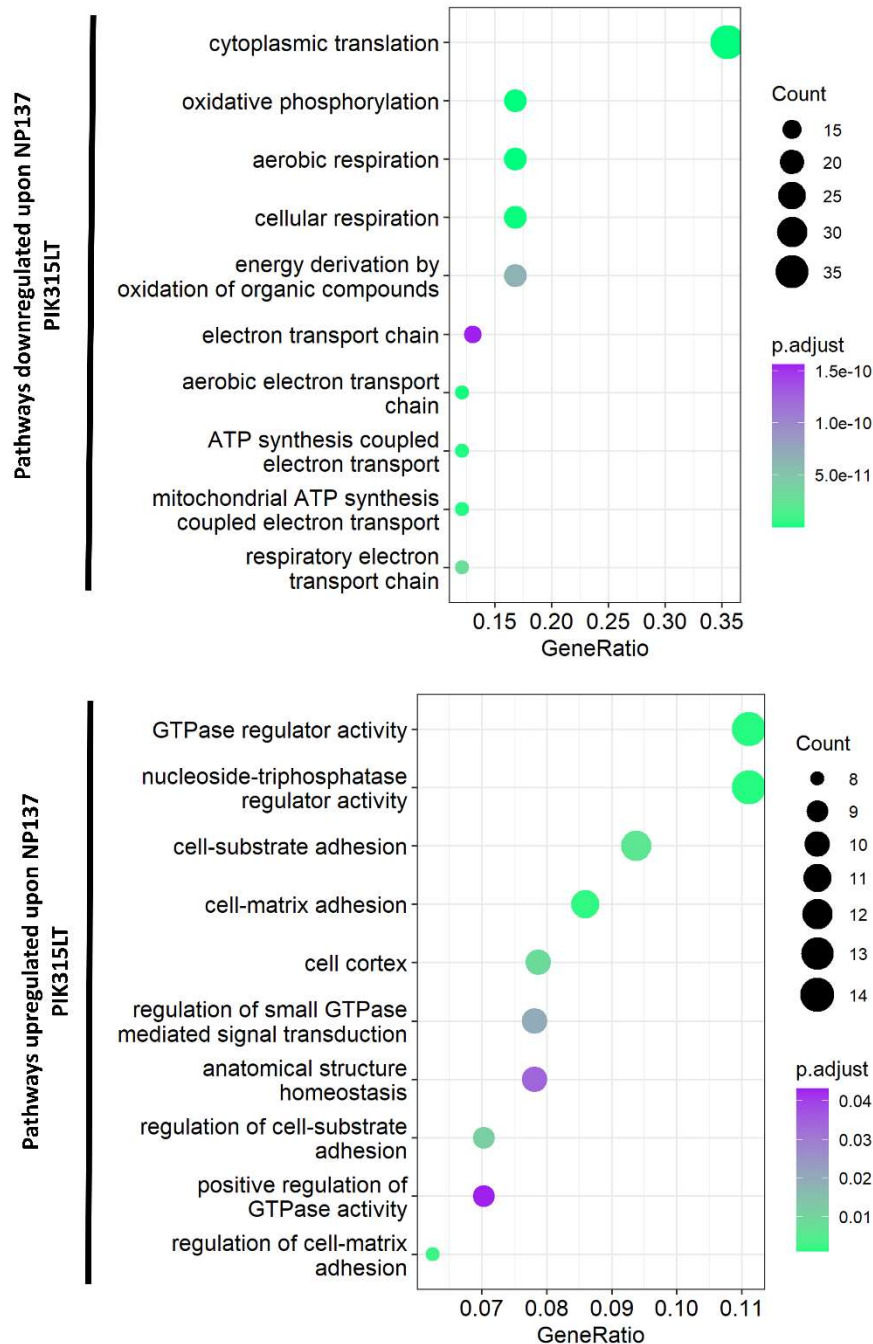


Figure 5.6– Pathways impacted by NP137 treatment in PIK315LT organoid –Pathway enrichment analysis of genes downregulated (Top) and Upregulated (Down) in PIK315LT upon NP137 treatment (Genes differentially expressed determined using FindMarkers function of Seurat package, pathways determined using enrichGO function of clusterProfiler package, Gene Ratio is a metric used to measure the degree of enrichment of a gene set within a larger gene list. It is calculated by dividing the number of genes from the gene set that are present in the input gene list by the total number of genes in the gene set; Genes were considered differentially expressed if $\log_2\text{foldchange} > 0.15$; Statistical test used: Benjamini-Hochberg)

d. NP137 influences cell adhesion

Given the established effects of Netrin-1 on EMT and the identified correlation between UNC5B, Netrin-1, and EMT-related genes in CRC and mCRC tumours, our focus turned to examining the impact of Netrin-1 on EMT. Utilizing the UCell function and referencing the MSigDB list Hallmark: EMT, we did not observe a significant effect of NP137 on EMT in γSAW190LT (NP001: 0.1075 vs NP137: 0.1073; $p_{\text{adj}} = 0.72$). However, a notable decrease in genes associated with EMT was observed in PIK315LT (NP001: 0.107 vs NP137: 0.105; $p_{\text{adj}} = 2.2 \times 10^{-16}$) (**Figure 5.7-A & B**). Notably, among the most differentially expressed genes in PIK315LT, we identified **VCL** (**Figure 5.7-C, Table 5.3**), which codes for vinculin, a protein implicated in CRC EMT through its role in cell-cell and cell-matrix adhesion. The upregulation of VCL in PIK315LT upon treatment with NP137 suggests a potential downregulation of EMT in this cell line. Expanding on these results, in PIK315LT, we observed a clear upregulation of genes involved in the regulation of cell adhesion to the matrix, substrate, or other cells (NF1, VCL, ITGA6, ROCK2, CDH13, CASK, KANK1, MACF1, PTPRK, RASA1, DISC1, FREM1). These gene expressions have been mainly described as detrimental to the EMT process, suggesting that Netrin-1 may promote EMT in mCRC cells and that NP137 reverses this effect. In γSAW190LT, there was also an effect on processes related to cell adhesion, albeit in a different manner. We noted the downregulation of genes related to cell adhesion to the substrate and extracellular matrix, notably CTNNB1, SKAP1, ACTN1, CDH13, DISC1, CDK6, NRP1, SORBS1, MYADM, OLFM4. These genes have a less documented role in EMT, though they confirm an effect of Netrin-1 on the regulation of cell adhesion in mCRC cells. These findings suggest cell-specific responses to Netrin-1 deprivation in terms of EMT regulation within different mCRC cell lines.

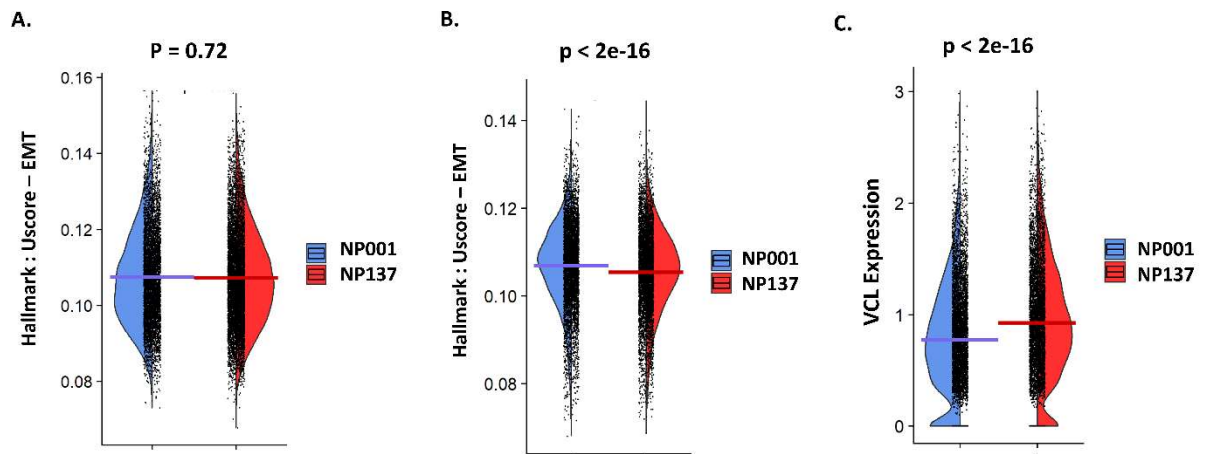


Figure 5.7– NP137 impairs EMT in PIK315LT mCRC organoid (A) Violin plot of global expression of genes related to the *Epithelial to Mesenchymal Transition* in *ySAW190LT* organoid cells treated with either NP001 or NP137 (Score determined using UCell function and MSigDB Hallmark : EMT gene list as reference; Means and p.values displayed determined by stat_compare_means function; Bars indicate means, statistical test use : Wilcoxon test); (B) Violin plot of global expression of genes related to the *Epithelial to Mesenchymal Transition* in *PIK315LT* organoid cells treated with either NP001 or NP137 (Score determined using UCell function and MSigDB Hallmark : EMT gene list as reference; Means and p.values displayed determined by stat_compare_means function; Bars indicate means, statistical test use : Wilcoxon test); (C) Violin plot of the expression of *VCL* in *PIK315LT* organoid cells treated with either NP001 or NP137 (Means and p.values displayed determined by stat_compare_means function; Bars indicate means, statistical test use : Wilcoxon test)

We can also observe that some of these genes related to cell adhesion are directly linked to GTPase regulation, and it's indeed another effect observed in our organoids. Notably, the upregulation of genes related to GTPase regulation (NF1, FARP1, ARHGAP32, RABGAP1L, RALGAPA2, STARD13, AGAP1, RASA1) observed in PIK315LT organoids is significant.

Finally, we can observe an upregulation of several genes (S100A14, S100P, S100A10 and ANXA2) involved in calcium-binding in the *ySAW190LT* organoid cell line, notably of several genes coding for the S100 proteins. The S100 protein is involved in various cellular processes including the regulation of cell movement, invasion, cytoskeleton interactions as well as programmed cell death to a lesser extent [362].

Genes involved in Cell adhesion				
ySAW190LT				
Upregulated				
Gene	Expression level (NP001)	Expression level (NP137)	Log2FoldChange	Corrected p value
CIB1	1.275485	1.483511857	0.208026857	4.24E-62
RRAS	0.647543	0.830842527	0.183299527	4.38E-45
S100A10	2.291375	2.505070661	0.213695661	9.77E-45
DNM2	0.6530268	0.805284456	0.152257656	1.51E-35
CEACAM6	0.235589	0.409266192	0.173677192	7.85E-21
Downregulated				
Gene	Expression level (NP001)	Expression level (NP137)	Log2FoldChange	Corrected p value
SOX4	1.784758	1.303090083	-0.481667917	3.55E-230
SKAP1	1.288778	0.963611229	-0.325166771	2.02E-65
MAGI1	1.050124	0.834880103	-0.215243897	4.35E-50
DISC1	0.632724	0.44463253	-0.18809147	1.97E-37
CDK6	1.433954	1.270827761	-0.163126239	2.62E-36
RUNX1	0.7965611	0.62111319	-0.17544791	2.15E-34
NRP1	0.71651	0.533760451	-0.182749549	8.40E-24
FRMD5	1.050124	0.841752459	-0.208371541	4.63E-23
SYK	0.6664472	0.512765556	-0.153681644	7.14E-19
MYADM	0.9203041	0.764652403	-0.155651697	1.26E-17
OLFM4	0.2519953	0.068917482	-0.183077818	6.59E-11
PIK315LT				
Upregulated				
Gene	Expression level (NP001)	Expression level (NP137)	Log2FoldChange	Corrected p value
NF1	1.240096842	1.44105	0.200953158	7.51E-50
VCL	0.684219471	0.9279132	0.243693729	1.64E-46
ITGA6	1.564461849	1.725786	0.161324151	5.71E-42
ROCK2	1.821785335	2.009835	0.188049665	7.02E-42
CASK	1.10907826	1.28107	0.17199174	1.42E-35
KANK1	0.698071938	0.902763	0.204691062	3.93E-31
MACF1	0.875758561	1.034995	0.159236439	1.39E-28
PTPRK	1.437260985	1.605271	0.168010015	2.68E-26
RASA1	0.737679227	0.8905196	0.152840373	4.51E-22
FREM1	0.20775884	0.3807414	0.17298256	5.89E-13
Downregulated				
Gene	Expression level (NP001)	Expression level (NP137)	Log2FoldChange	Corrected p value
BSG	1.377324	1.213416147	-0.163907853	5.01E-42
PFN1	1.40858	1.256509428	-0.152070572	2.55E-26

Table 5.3 ; Effect of NP137 on genes related to cell adhesion in mCRC organoids (ySAW190LT & PIK315LT)
(Corrected p value and Log2FoldChange calculated using Findmarkers function of Seurat package; Wilcoxon Rank Sum test)

e. NP137 influences mitochondrial respiration

Mitochondria are vital organelles within cells, responsible for coordinating energy production through oxidative phosphorylation (OXPHOS) based on the availability of oxygen and substrates. They also facilitate crucial metabolic processes like the citric acid cycle, fatty acid oxidation, and glutaminolysis. Given their role in fundamental cellular functions, mitochondria have been associated with various aspects of tumorigenesis and CSCs [363]. Even if more recent research is especially describing an increased mitochondrial activity in CSCs [364-366], the consensus seems to indicate that CSC decrease the activity of mitochondrial chain components towards activation of glycolysis to support proliferation (Warburg effect) [364, 367].

Moreover, mitochondria play a crucial role in controlling cell death by initiating mitochondrial outer membrane permeabilization, which triggers the release of specific proteins from the mitochondrial intermembrane space. These released proteins then activate caspases, initiating the apoptotic pathway.

Interestingly, in our scRNAseq analysis, we observed a significant downregulation of genes coding for key components of the mitochondrial oxidative chain in both ySAW190LT and PIK315LT organoids. Specifically, in ySAW190LT, genes like ATP5ME, NDUFB1, NDUFA3, ATP5F1E, and IMMP2L were downregulated, while in PIK315LT, genes such as COX7C, ATP5F1E, ATP5ME, NDUFA1, COX6B1, ATP5MG, COX5B, COX7B, UQCRI1, UQCRIQ, COX7A2, NDUFB11, ATP5MF, NDUFA7, UQCRB, and COX6C showed decreased expression levels (**Table 5.4**).

The significance of this observation of the effect of NP137 on mitochondria oxidative chain is not anodyne when put in the context of colorectal CSCs. Indeed, mitochondria are crucial for CSCs as they provide the energy and metabolic intermediates necessary for their survival, proliferation, and maintenance of stemness properties. Therefore, the downregulation of mitochondrial oxidative chain genes by NP137 may contribute to its effect on the self-renewal of mCRC cells by disrupting the energy production and metabolic processes essential for CSC function. Indeed, despite the well-known

preferences of CSCs towards glycolytic pathways, recent studies demonstrate the importance of the mitochondrial chain for CSCs-related features [363-366]. For instance, in melanoma, inhibition of enzymes of mitochondrial oxidative ATP synthesis leads to the elimination of drug-resistant slow-cycling subpopulation [368]. Or in hematopoietic stem cells where respiratory chain components are needed to sustain HSC differentiation and maintenance of HSC quiescence [369]. The mitochondrial chain disruption by NP137 could potentially impair CSC viability and reduce their ability to proliferate or resist therapy in our mCRC organoid cell lines.

Genes involved in Aerobic respiration/Oxidative phosphorylation				
ySAW190LT				
Downregulated				
Gene	Expression level (NP001)	Expression level (NP137)	Log2FoldChange	Corrected p value
ATP5ME	1.500912	1.28576	-0.310517516	3.67E-198
NDUFB1	0.8277972	0.554725116	-0.273072084	1.68E-168
NDUFA3	1.101196	0.873979183	-0.227216817	1.35E-99
ATP5F1E	2.008018	1.847786081	-0.160231919	1.07E-74
IMMP2L	0.7794634	0.620791458	-0.158671942	1.03E-22
PIK315LT				
Downregulated				
Gene	Expression level (NP001)	Expression level (NP137)	Log2FoldChange	Corrected p value
COX7C	1.778479	1.540909649	-0.237569351	1.77E-120
ATP5F1E	1.703996	1.458401702	-0.245594298	2.01E-104
ATP5ME	0.9534393	0.706021175	-0.247418125	7.25E-102
NDUFA1	1.175137	0.929585212	-0.245551788	9.42E-101
COX6B1	1.378956	1.153822042	-0.225133958	2.91E-91
ATP5MG	1.822714	1.633415293	-0.189298707	2.50E-75
COX5B	1.844534	1.643355464	-0.201178536	5.32E-75
COX7B	1.491874	1.28019327	-0.21168073	5.59E-74
UQCR11	1.321453	1.100963712	-0.220489288	1.14E-65
UQCRQ	1.027264	0.825143699	-0.202120301	1.22E-63
COX7A2	1.822714	1.631058903	-0.191655097	1.33E-59
NDUFB11	1.330357	1.154967259	-0.175389741	1.41E-49
ATP5MF	1.030808	0.851223329	-0.179584671	1.05E-40
NDUFA7	0.9853423	0.822953097	-0.162389203	1.18E-40
UQCRB	1.370642	1.207108405	-0.163533595	4.85E-40
COX6C	1.267693	1.114956104	-0.152736896	3.93E-34

Table 5.6 : Effect of NP137 on genes related to aerobic respiration and oxidative phosphorylation in mCRC organoids (ySAW190LT & PIK315LT); Corrected p-value and Log2FoldChange calculated using Findmarkers function of Seurat package; Wilcoxon Rank Sum test)

f. NP137 influences cytoplasmic translation

NP137 treatment had an important impact on cytoplasmic translation in both of our organoid cell lines. Specifically, we observed the downregulation of multiple ribosomal proteins (RPs) including RPS29, RPS28, RPS27, RPS21, RPL37, RPL38, and RPL37A in both

organoids, as depicted in **Figure 5.8** and detailed in **Table 5.5**. The effect is more pronounced in the PIK315LT organoid cells, where additional RPs such as RPL11, RPL12, RPL19, RPL22, RPL22L1, RPL28, RPL29, RPL30, RPL32, RPL34, RPL35, RPL35A, RPL36, RPL36A, RPL36AL, RPL39, RPL41, RPL8, RPLP1, RPS12, RPS13, RPS15, RPS15A, RPS19, RPS23, RPS24, RPS25, RPS26, RPS27A, RPS5, and RPS8 are also downregulated upon NP137 treatment in similar proportion. These findings highlight the significant impact of NP137 on the translational machinery within mCRC cells.

Apart from their primary function in protein biosynthesis, many RPs have been identified to have extra ribosomal functions. These additional roles include a wide range of cellular processes such as apoptosis, cell cycle, neoplastic transformation and cell adhesion and migration (**Supplementary Table 5.1**) [370]. These differential expression patterns of RPs are closely associated with CRC's differentiation, progression, or metastasis [371]. It is to be noted that several ribosomal proteins have been described as upregulated in colorectal cancer notably RPS6, RPS8, RPS12, RPL5, and RPP0 [372].

Several studies point towards a link between high expression of RPs and high self-renewal capacity [373-375]. Moreover, several of these proteins have been shown to be involved in apoptotic processes [376, 377]. Given the implication of cytoplasmic translation in self-renewal and apoptosis we can wonder if this effect of NP137 on RP genes participates to the effects we observed on self-renewal and CSCs apoptosis.

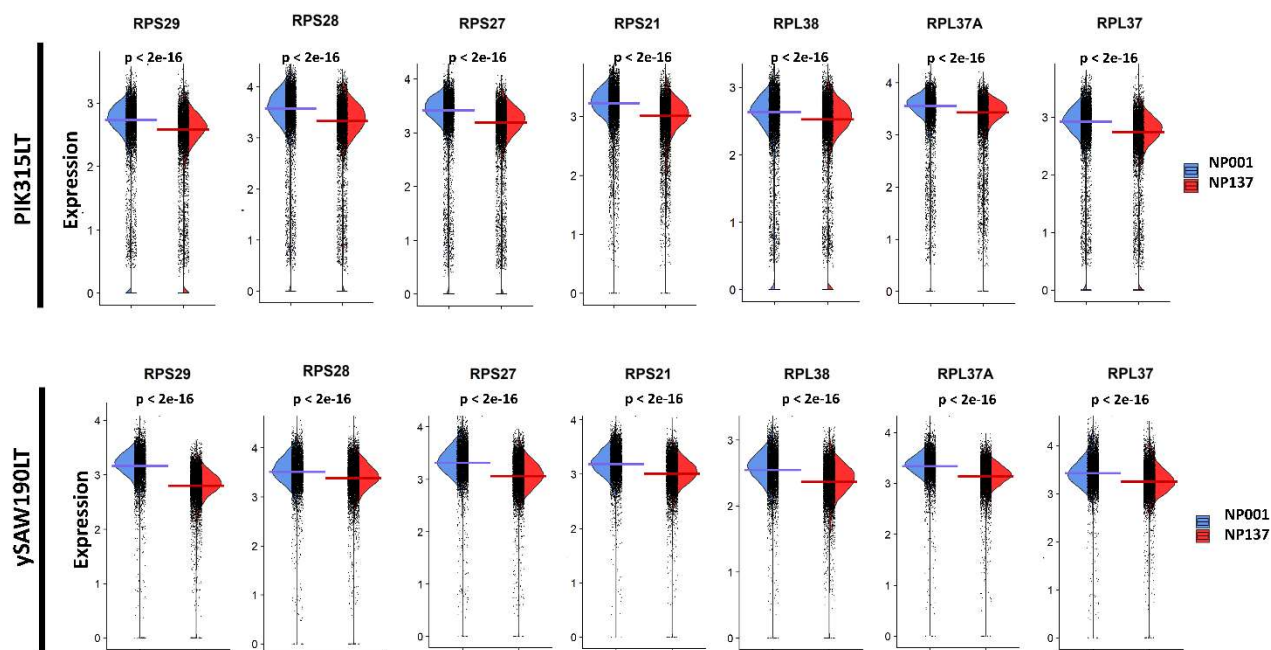


Figure 5.8 –NP137 impairs cytoplasmic translation in mCRC organoids - Violin plot of the expression of commonly downregulated ribosomal proteins (RPS29, RPS28, RPS27, RPS21, RPL38, RPL37A, RPL37) in (Top) PIK315LT organoid cells and (Down) ySAW190LT organoid cells treated with either NP001 or NP137 (Means and p.values displayed determined by `stat_compare_means` function; Bars indicate means, statistical test use: Wilcoxon test)

Ribosomal proteins coding genes										
ySAW190LT					PIK315LT					
Downregulated										
Gene	Expression level (NP001)	Expression level (NP137)	Log2FoldChange	Corrected p value	Expression level (NP001)	Expression level (NP137)	Log2FoldChange	Corrected p value		
RPS29	3.161653	2.641532203	-0.520120797	< 1E-300	2.614280481	2.383585	-0.230695481	2.71E-153		
RPS28	3.513644	3.322101958	-0.191542042	6.87E-142	3.486969456	3.131716	-0.355253456	7.73E-219		
RPS27	3.430159102	3.062368	-0.367791102	< 1E-300	3.315436576	2.979749	-0.335687576	1.17E-283		
RPS21	3.263225863	3.01082	-0.252405863	< 1E-300	3.064855201	2.910713	-0.154142201	8.04E-68		
RPL38	2.547532	2.289967912	-0.257564088	< 1E-300	1.531093394	1.215425	-0.315668394	2.85E-293		
RPL37A	3.451860044	3.166034	-0.285826044	< 1E-300	2.712428108	2.522151	-0.190277108	1.00E-131		
RPL37	3.423678	3.169047754	-0.254630246	2.44E-237	3.521172834	3.25711	-0.264062834	2.12E-181		

Table 5.7 Ribosomal proteins-related genes expression upon NP137 treatment in mCRC organoids (ySAW190LT & PIK315LT); Corrected *p*-value and Log2FoldChange calculated using Findmarkers function of Seurat package; Wilcoxon Rank Sum test)

III- Conclusion

The primary goal of our scRNA sequencing analysis was to assess the impact of the Netrin-1 blockade on metastatic organoids at the single-cell level. We identified several cell populations displaying differences in gene expression associated with various cellular processes, notably hypoxia, Wnt/Epithelial signalling, cellular respiration, cytoplasmic translation, cell adhesion, and cellular division.

We used this data to observe the effects of NP137 on stemness-related genes in our mCRC organoid models. Our analysis revealed a global reduction in the expression of stemness-related genes, including SOX4, PROM1, SMOC2, and LGR5, upon NP137 treatment in our ySAW190LT organoid. Furthermore, genes associated with stem cell proliferation displayed a significant decrease in expression in a general manner upon treatment with NP137, supporting the downregulation of stemness-related pathways. These findings align with our previous observations and provide additional insights into Netrin-1's influence on stemness in mCRC cells. In contrast, our scRNA sequencing experiment on PIK315LT organoids did not show a similar decrease in stemness-related genes upon NP137 treatment despite the assessed effect of NP137 treatment on the self-renewal of this cell line. This difference may be due to an uncoupling between self-renewal abilities and transcription of stemness-associated markers and transcription factors in the PIK315LT cells.

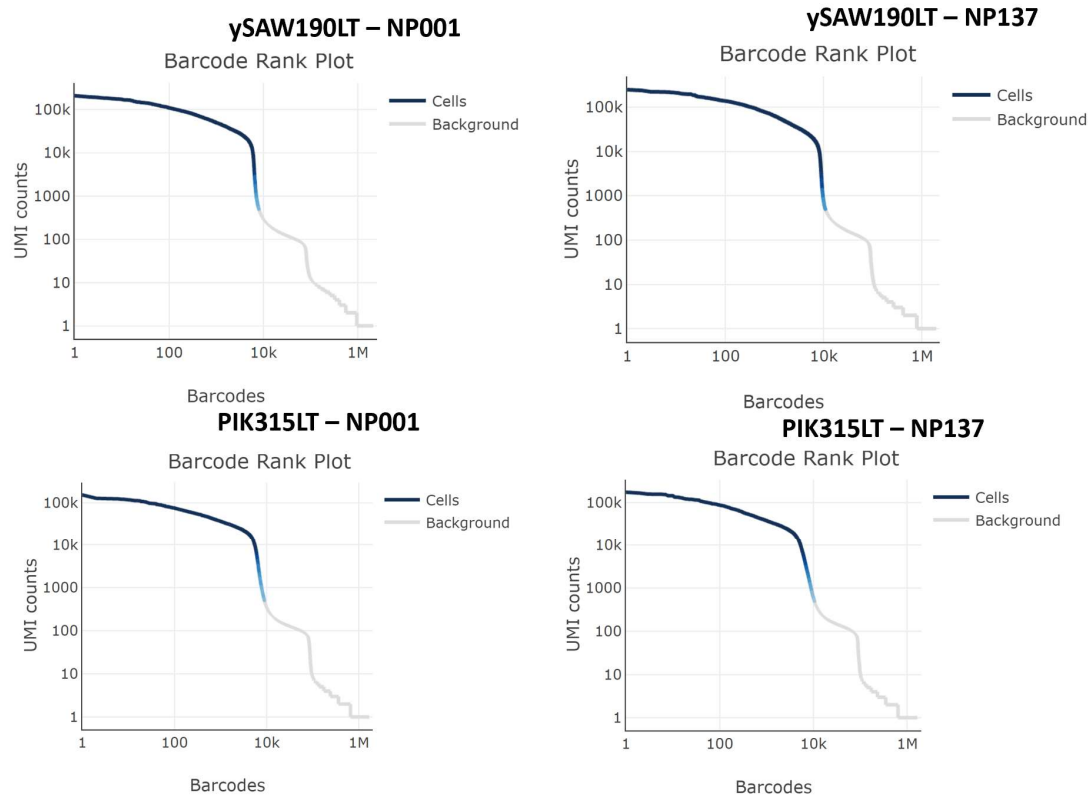
Furthermore, our analysis revealed a decrease in genes associated with negative regulation of apoptosis upon NP137 treatment, further suggesting the involvement of apoptosis in Netrin-1 mechanisms of action.

Our initial hypothesis regarding UNC5B receptor expression triggering direct apoptosis upon Netrin-1 withdrawal was not fully supported by the data. The lack of correlation between UNC5B expression and high stemness marker levels, along with the non-specific reduction in stemness markers in UNC5B-expressing cells, suggests that apoptosis in mCRC CSCs following Netrin-1 deprivation might involve another mechanism than UNC5B-dependent apoptosis. To explore possible mechanisms explaining this, we focused on genes correlating with UNC5B expression and affected by NP137 treatment specifically in UNC5B-expressing cells. This led us to identify TFF3, a

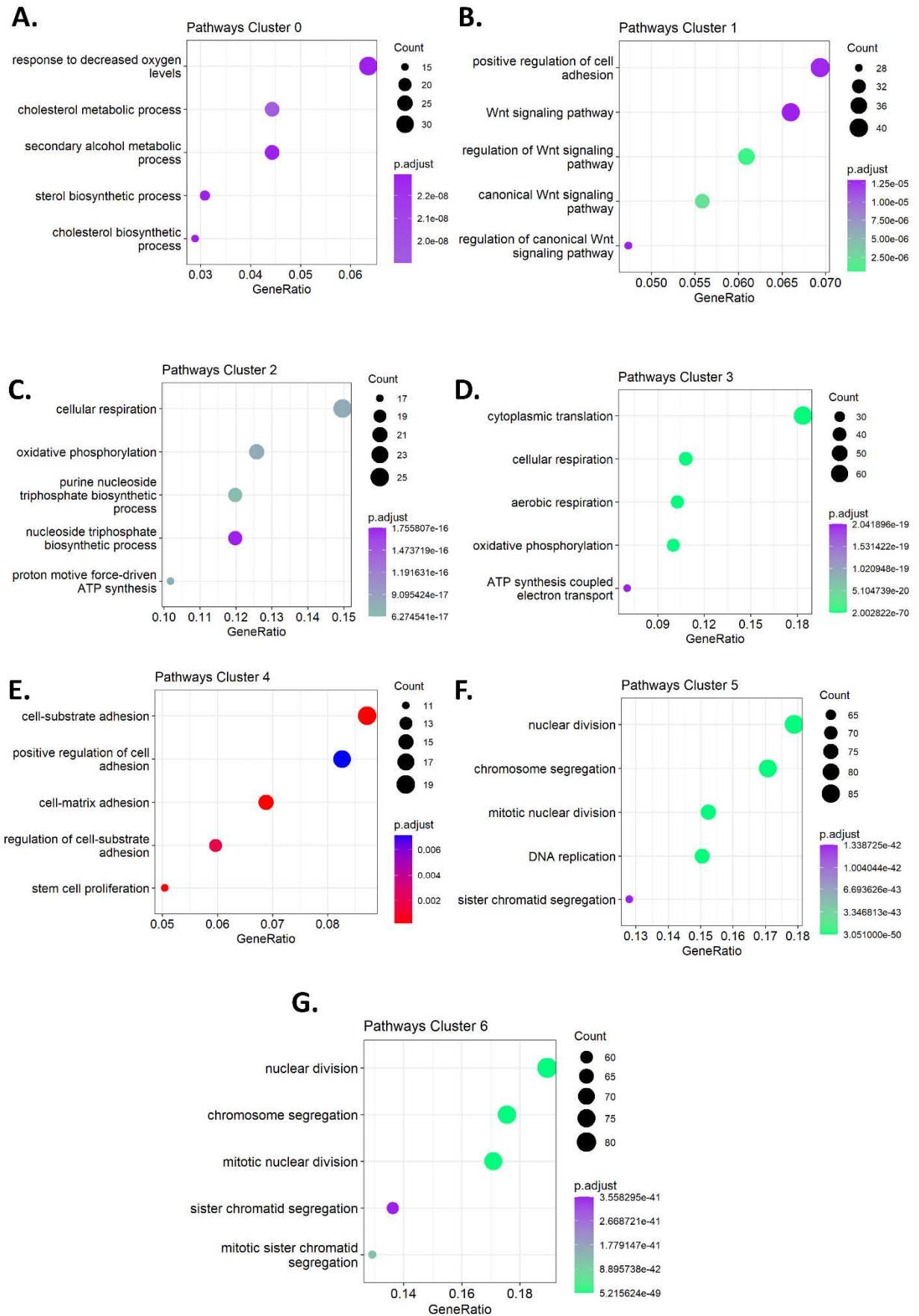
peptide implicated in EMT, stemness and apoptosis in cancer cells. The observed decrease in TFF3 expression upon NP137 treatment in UNC5B-expressing cells suggests a potential link between Netrin-1 signalling, UNC5B, and TFF3 regulation in mCRC.

Our scRNA sequencing analysis also reveals the broader impact of Netrin-1 deprivation on mCRC cell signalling and biology. Especially, effects on cytoplasmic translation, oxidative phosphorylation, cellular respiration, and cell adhesion.

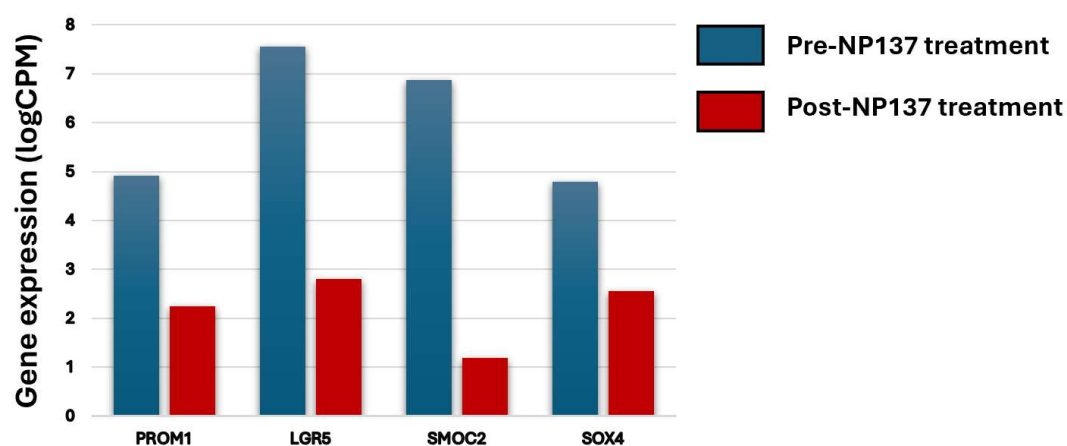
Supplementary Material – Chapter 5



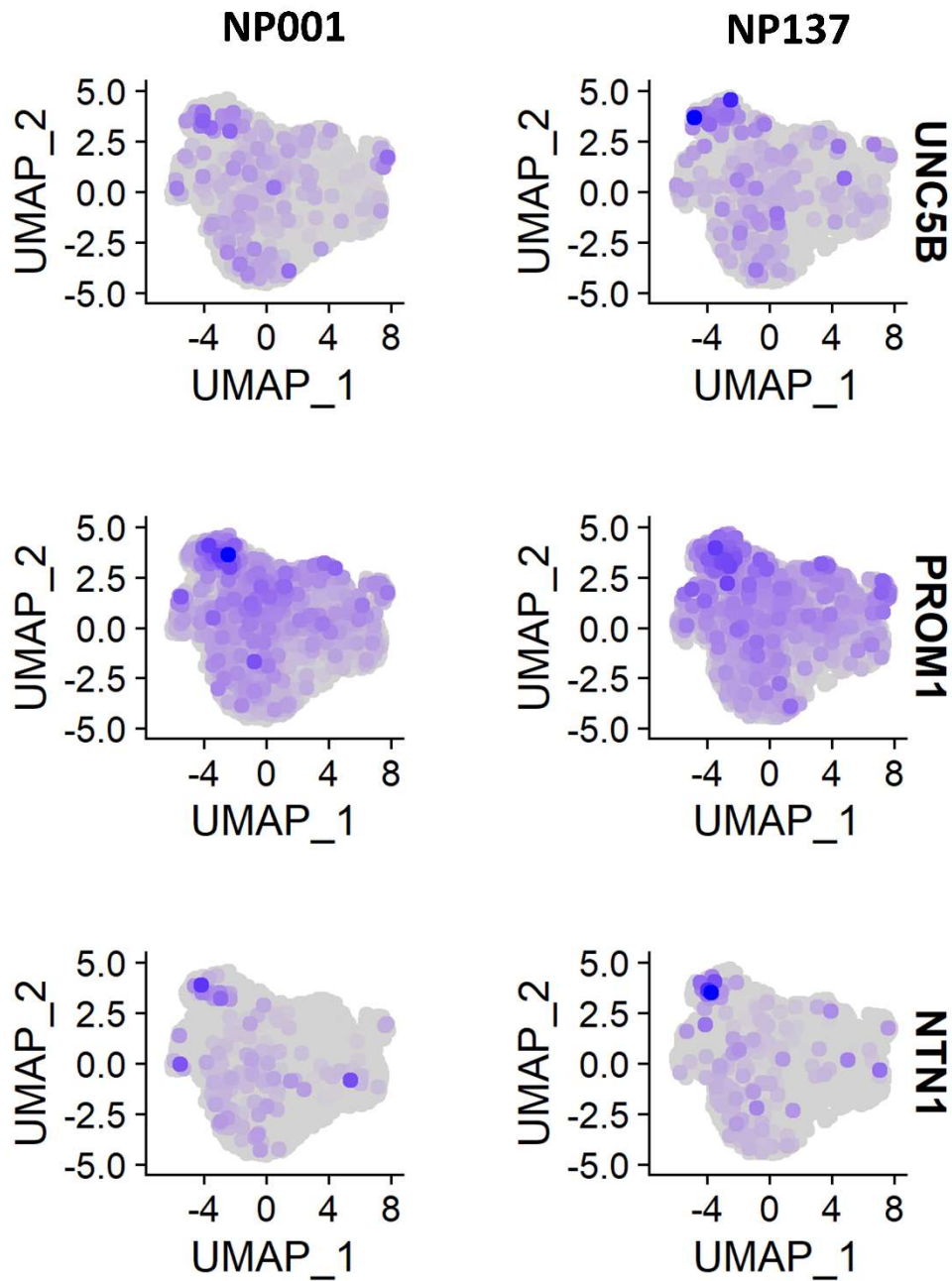
Supplementary Figure 5.1 – Barcode Rank Plot for Single-cell RNA sequencing of ySAW190LT and PIK315LT organoids cells treated with either NP001 (10 ug/mL) or NP137 (10 ug/mL)



Supplementary Figure 5.2 - Pathways differentially expressed by clusters in ySAW190LT organoid – Pathway enrichment analysis of genes upregulated clusters defined by FindClusters function; the genes differentially expressed have been determined using FindConservedMarkers function of Seurat package, pathways determined using enrichGO function of clusterProfiler package, Gene Ratio is a metric used to measure the degree of enrichment of a gene set within a larger gene list. It is calculated by dividing the number of genes from the gene set that are present in the input gene list by the total number of genes in the gene set; Statistical test used: Benjamini-Hochberg)



Supplementary Figure 5.3 – Expression of stemness-related genes in Colon Adenocarcinoma patient treated with NP137 (Clinical trial phase I) – PROM1, LGR5, SMOC2 and SOX4 mRNA in CRC tumour of a patient treated with NP137 levels determined by RNA sequencing (Raw data provided from Apoptosis and Cancer Team in CRCL, France; Data analysed using DESeq2 package in R)



Supplementary Figure 5.4 – *UNC5B*, *PROM1* and *NTN1* genes expression in PIK315LT mCRC PDO - (A) UMAP plot of *UNC5B* expression level PIK315LT organoid cells treated with either NP001 or NP137; **(B)** UMAP plot of *PROM1* expression level PIK315LT organoid cells treated with either NP001 or NP137; **(C)** UMAP plot of *NTN1* expression level PIK315LT organoid cells treated with either NP001 or NP137

Extraribosomal functions	RPs
Apoptosis	S3, S3a, S6, S7, S14, S25, S27, S27L, S29, L3, L6, L7, L8, L23, L26
Cell cycle	S3, S5, S6, S7, S14, S15, S19, S20, S25, S26, S27, S27L, S27a, L3, L5, L6, L7, L11, L13, L23, L26, L31, L34, L37, L41
Cell proliferation	S6, S9, S13, S14, S15a, S24, S27, L6, L8, L11, L15, L17, L26, L29, L31, L34, L36a
Neoplastic transformation	P1, S3a, S14, L5, L22, L41
Cell migration and invasion	S3, p-S6, S7, S15a, S24, S27, L15

Supplementary Table 5.1 – Extraribosomal functions of Ribosomal proteins (Adapted from Xu, X., Xiong, X. & Sun, Y. The role of ribosomal proteins in the regulation of cell proliferation, tumorigenesis, and genomic integrity. *Sci. China Life Sci.* 59, 656–672 (2016). <https://doi.org/10.1007/s11427-016-0018-0>)

Chapter 6 - Crosstalk between UNC5B/Netrin-1
pathway and other dependence receptors :
NOTCH3

I- Introduction

We characterized in the previous chapters of this thesis how the UNC5B dependence receptor and its ligand, Netrin-1, were modulating self-renewal and other stemness-related features such as expression of transcription factors or dysregulation of cell adhesion.

In this chapter, we sought to examine whether other dependence receptors could potentially interfere with the role of UNC5B in metastatic CRC. Indeed, the literature suggests that other dependence receptors may be involved in processes that may impact the function of UNC5B in stemness.

Indeed, dependence receptors seem to act on similar cellular processes. For example, it has been observed that the dependence receptors have functions in processes like modulation of embryogenesis, neurogenesis, and angiogenesis since these mechanisms necessitate a fine-tuning of growth and development that the dependence receptors can provide. Moreover, it is also apparent that different dependence receptors can modulate overlapping pathways, which renders the understanding of their downstream pathways more difficult to apprehend. Indeed, PI3K/AKT and MAPK/ERK seem to be the principal targets of all these receptors towards the regulation of different cellular responses such as maintenance of pluripotency and promotion of EMT but also proliferation, angiogenesis and immunomodulation[165, 176, 213, 214, 378, 379]. In addition, there are previously reported examples of dependence receptors regulating others or their protein partner. For instance, PlexinD1 is directly regulated by NOTCH3 activation [380]. p75^{NTR} is involved in the regulation of other neurotrophin receptors and cooperates with them to induce different biological responses[381-383]. As previously discussed, UNC5B and Neogenin can collaborate in several cancer types [176, 213, 384, 385]. Finally, dependence receptors both influence and are influenced by several proteins such as p53 or NFκB [281, 386-391].

We therefore sought to better understand whether the UNC5B/Netrin-1 pathway could interact with other dependence receptors. Among possible candidates for such an interaction, NOTCH3 was particularly appealing. Indeed, NOTCH3 regulates the stem cell phenotype in several cancer cell types such as ovarian cancer and hepatocellular

carcinoma, directly influencing clinical outcomes [228-230]. Its role in cancer cell EMT is less well understood and may vary depending on the type of cancer cell, acting as a tumour suppressor in small cell lung cancer (SCLC) whilst promoting EMT in NSCLC [230-233].

Overall, NOTCH signalling is a key regulator of stemness and self-renewal in colorectal CSCs. Activation of NOTCH receptors, notably NOTCH1 and NOTCH3 by the ligands JAG1 and Dll4 results in the inhibition of differentiation through repression of expression of genes such as MUC2 or ATOH1 and inhibition of apoptosis triggered by cell cycle arrest [392-395]. Moreover, Notch pathways have a major role in the asymmetrical division of colorectal CSCs and the maintenance of several pools of pluripotent stem cell [330, 396].

Due to the known implications of NOTCH3 in regulating stemness and EMT processes in CRC cells, coupled with the potential interactions between dependence receptor pathways, we thought examining the potential implications of NOTCH3 on stemness in mCRC cells could be interesting.

More specifically, we aimed to investigate how NOTCH3 influences stemness properties in mCRC cells and to explore the potential cross-regulations or crosstalk between UNC5B/Netrin-1 and NOTCH3 pathways. To do so, we first used the TCGA cohort and RNA sequencing data obtained from stage 4 tumours and mCRC organoid analysis to study NOTCH3 expression patterns and their correlation with stemness markers, EMT-related genes and UNC5B expression in CRC patients.

Additionally, we investigated the expression of NOTCH3 protein upon FOLFOX treatment. It involved examining NOTCH3 expression levels following FOLFOX treatment. Understanding how FOLFOX influences NOTCH3 expression can provide valuable information regarding chemotherapy resistance mechanisms and potential therapeutic targets.

Furthermore, we used CRISPR-Cas9 to invalidate NOTCH3 in mCRC organoids, enabling us to study the effect of NOTCH3 on FOLFOX efficacy, to assess the impact of NOTCH3 on chemotherapy sensitivity in mCRC cells, and to study the relationship between

NOTCH3 and UNC5B. Lastly, we used NP137 to observe the effect of Netrin-1 inhibition on the NOTCH3 receptor expression and its ligand Jagged-1.

II- Results

a. Expression pattern of UNC5B and NTN1 in mCRC tumours

To explore the expression of NOTCH3 in stage 4 colorectal tumours, we first assessed the expression of Netrin-1 in 8 metastatic hepatic CRC tumours (PIK315LT, yPTM089LT, SJB486LT, PSH095LT, yPDW886LT, yPPM845LT, yPDC275LT, and yPPJ102LT) using IHC (obtained by Dr. Behrenbruch), enabling us to confirm the expression of NOTCH3 proteins in liver metastasis samples and the heterogeneity of this expression among patients (**Figure 6-1**). Indeed, we observe a strong expression of the protein in tumours like yPDC275LT, PIK315LT or yPTM089LT, whereas we observe no to low expression in the yPPJ102LT tumour. Our immunohistochemical staining confirmed the predominant localization of NOTCH3 expression in both the cytoplasmic and membranous compartments of tumour cells, as expected since the antibody used is designed to recognize both the Full-length (FL) and the cleaved Intracellular domain (ICD) form of the protein.

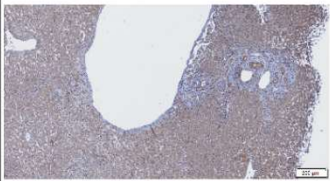
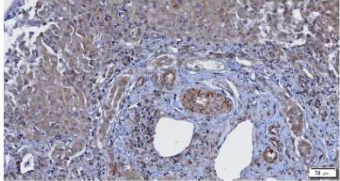
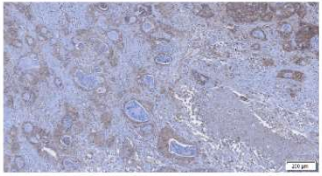
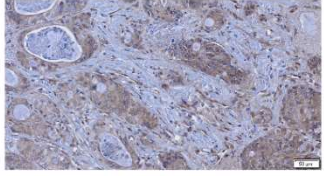
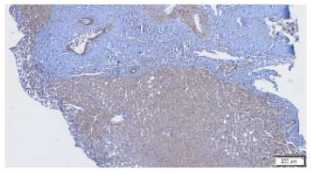
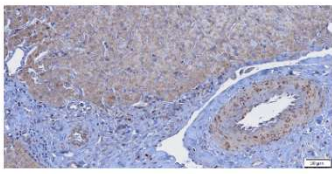
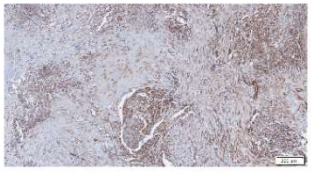
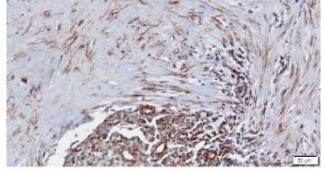
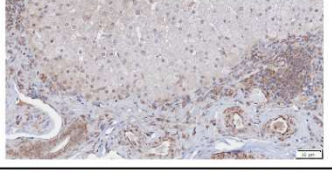
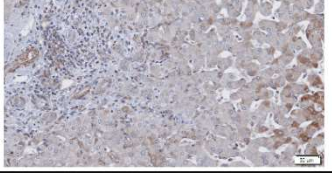
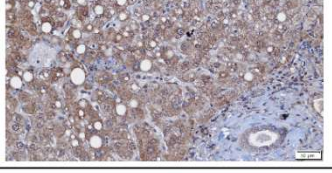
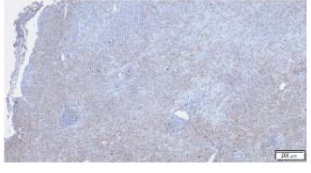
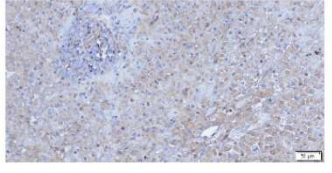
Tumor ID		
PIK315LT		
yPTM089LT		
SJB486LT		
PSH095LT		
yPDW886LT		
yPPM845LT		
yPDC275LT		
yPPJ102LT		

Figure 6.3 **Immunohistochemical Staining of NOTCH3 protein in a panel of Stage 4 Colorectal Tumour Sections** (left: scale = 200 um, right: scale = 50 um; tumour tissues were fixated with formalin, embedded with paraffin, sectioned and stained with H&E and NOTCH3 antibody (Cell Signalling; Ref D11B8))

We also analysed the expression of NOTCH3 RNA using RNA sequencing data, both publicly available through the Integrated TCGA Pan-Cancer Clinical Data as well as generated within our research team. The TCGA cohort analysis revealed the progressively higher expression of NOTCH3 mRNA within increasing stages of colorectal cancer (**Figure 6-2.A**). NOTCH3 mRNA expression was significantly higher in the T4 stage CRC samples compared to those from early T stages (T1, T2) ($p < 0.01$ and $p < 0.05$, respectively, One-way ANOVA, Tukey's multiple comparisons test). This increased expression in T4 samples is reminiscent of that observed for UNC5B and NTN1.

We then further investigated the possible associations between NOTCH3 mRNA expression and EMT-related genes, as well as stemness-related gene expression, using data from the TCGA cohort ($n=521$), our library of metastatic CRC samples ($n=58$), and mCRC organoid cell lines ($n=28$).

We found strong positive correlations between the expression levels of NOTCH3 mRNA and genes associated with EMT in the TCGA cohort (**Figure 6.2-B**), particularly of several key EMT transcription factor genes such as *ZEB1*, *ZEB2*, and *SNAI2*, as well as the gene encoding Vimentin (*VIM*). To further investigate the correlation between the EMT-associated phenotype and NOTCH3 mRNA expression in stage 4 CRC, we employed the same "song score" method, and the same EMT-related genes signature (JP.Thiery) that we employed to study the relationship between UNC5B and EMT [321, 322]. This approach allowed us to reveal a significant and positive correlation between NOTCH3 mRNA expression and EMT-related gene signature in stage 4 CRC (**Figure 6.2-C**), confirming the strong association observed in the TCGA cohort. Specifically, we found a Pearson correlation coefficient (R) of 0.71 and a p -value inferior to 2×10^{-16} , indicating a robust relationship between NOTCH3 expression and the EMT process in stage IV CRC.

Simultaneously, our analysis of stage 4 colorectal tumours ($n=58$), PDO cell lines ($n=14$) and non-pathogenic adjacent tissues ($n=34$) allowed us to observe an interesting point about NOTCH3 expression in stage 4 CRC tumours. Indeed, we observed a marked overexpression of NOTCH3 mRNA expression levels within tumour samples (mean = 0.6541 ± 1.41 logRPKM) compared to normal adjacent tissues (mean = -1.132 ± 1.228 logRPKM) ($p < 0.0001$, unpaired t-test) (**Figure 6.2-D**), though, in our mCRC organoid cell lines (mean = -1.677 ± 1.074 logRPKM), we observe a lower expression of NOTCH3 mRNA

than the one observed in tumour tissues. This disparity between the tissues analysed can be either due to culture conditions or to the fact that some of the cells from the tumoral microenvironment express high levels of NOTCH3 or promote the expression of tumour cells in the tissue. Nevertheless, the increased expression of NOTCH3 in mCRC tumours suggests a functional role for NOTCH3 in mCRC cells.

In addition, we observed a correlation between NOTCH3 mRNA expression and the expression of the intestinal stem cell marker LGR5 in our PDO mCRC cell lines (n=14, Pearson correlation $R = 0.646$, $p = 0.013$) (**Figure 6.2-E**). These correlations raise the possibility of an interplay between NOTCH3, stemness, and EMT in CRC and mCRC, just like its dependence receptor counterpart UNC5B.

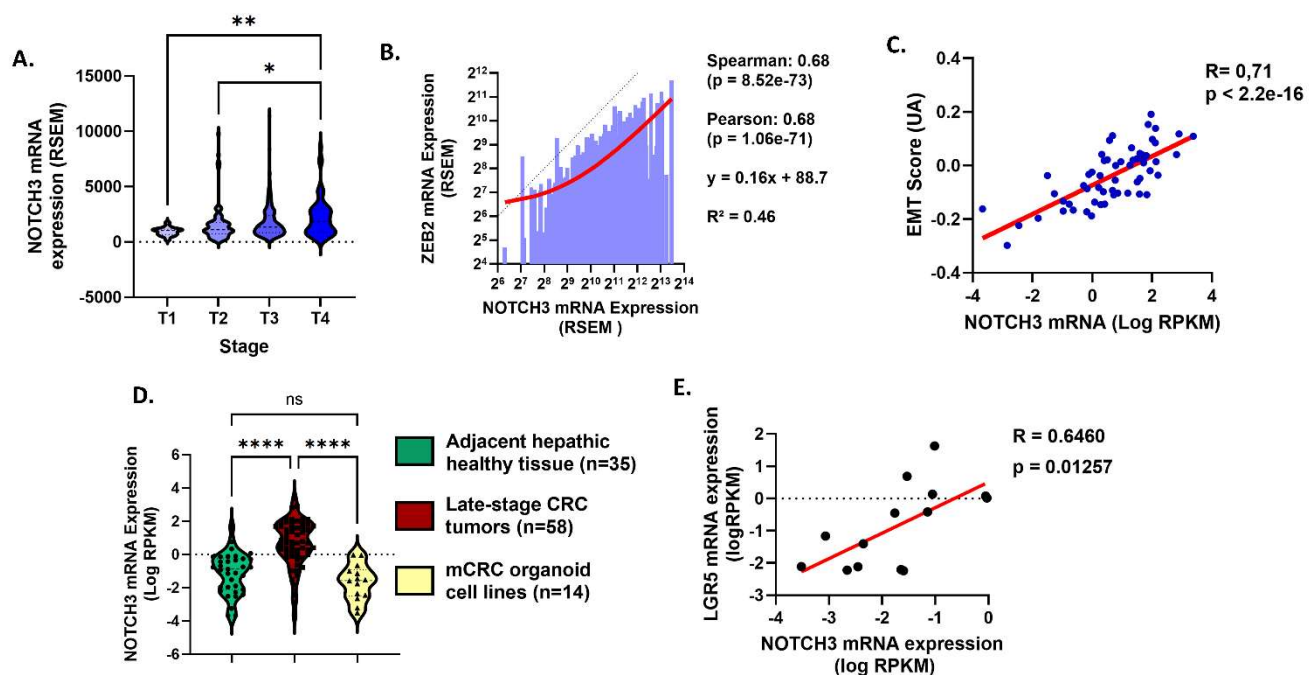


Figure 6.2 – Characterisation of NOTCH3 expression in CRC tumours and mCRC organoids (A) Expression Profiles of NOTCH3 mRNA in Colorectal Cancer TCGA according to T stage (B) Correlation between NOTCH3 mRNA and ZEB2 mRNA expression in TCGA cohort; (C) Correlation between NOTCH3 mRNA and EMT-signature Score (JP.Thiery EMT signature) in stage 4 CRC tumours; (D) Comparison of NOTCH3 mRNA Expression in Healthy adjacent liver, Stage 4 colorectal tumour tissues and mCRC organoid cell lines; (E) Correlation between NOTCH3 mRNA and LGR5 mRNA expression in mCRC organoid lines; (TCGA cohort: n=521; late stage tumours cohort: n=58, Adjacent healthy tissue cohort: n=34, mCRC organoid cell lines: n= 14); p Values: * = <0.05, ** = <0.01, **** = <0.0001; Statistical test: One-Way ANOVA - Tukey's multiple comparisons test for (A) and (D), Pearson for (C) and (E)

b. NOTCH3 upon FOLFOX treatment

To explore the therapeutical potential of targeting of NOTCH3 pathway to enhance the efficacy of chemotherapy like FOLFOX, we first assessed the expression of the NOTCH3 receptor upon chemotherapy. To do so, we investigated the expression of the NOTCH3 receptor in our mCRC PDO cell lines upon treatment with FOLFOX using RNA sequencing. The first observation was that NOTCH3 is still expressed in mCRC organoids at the end of FOLFOX exposure (n=14), supporting the potential interest of further investigations regarding its potential roles in chemoresistance and post-treatment recurrence. Moreover, we observed a noticeable upregulation of the NOTCH3 mRNA expression upon FOLFOX treatment (25uM, 72h) in our PDO cell lines (**Figure 6.3-A**), notably in our two organoid cell lines previously characterised as resistant to FOLFOX chemotherapy (PIK315LT and VJR275LT) (**Figure 6.3-B & C**). It is to be noted that FOLFOX treatment includes leucovorin in a clinical setting to diminish the toxicity of 5-FU, leucovorin being non-relevant in an *in vitro* setting, we used 5-FU/Oxaliplatin treatment in *in vitro* experiments (referred to as FOLFOX treatment).

We also observed an upregulation of *JAG1* mRNA levels in our organoid cell lines upon treatment with FOLFOX (25uM, 72h; mean control = 3.425 ± 0.971 logRPKM; FOLFOX treated mean = 4.003 ± 0.983 logRPKM) ($p < 0.001$, paired t-test) (**Figure 6.3-D**). *JAG1* encodes the Jagged-1 protein, a ligand described as responsible for the inhibition of apoptosis triggered by the NOTCH3 dependence receptor [227]. However, we don't observe a similar overexpression of the two other receptors of Jagged-1, NOTCH1 and NOTCH2 (**Figure 6.3-E & F**), suggesting that NOTCH3 could be an important partner of Jagged-1 ligand during chemotherapy. This suggests that increased JAG1/NOTCH3 expression may play a role in the resistance of mCRC organoids to apoptosis after FOLFOX treatment and that the dependence receptor activity of NOTCH3 might therefore be a pathway to exploit to enhance chemotherapy sensitivity.

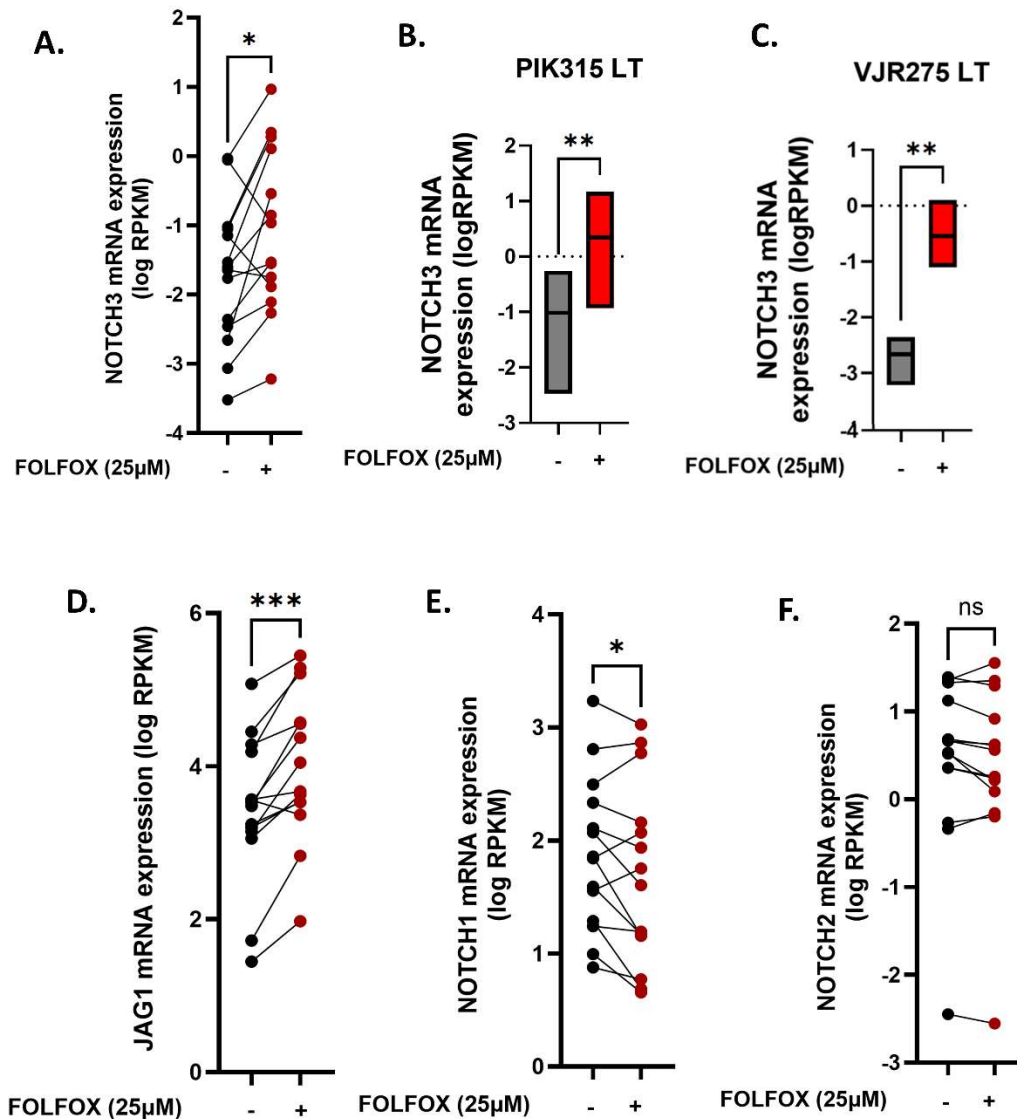


Figure 6.4 - Upregulation of NOTCH3 and JAG1 mRNA expression upon FOLFOX treatment in resistant mCRC PDOs (A) NOTCH3 mRNA expression in mCRC PDOs (n=14) after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; (B) NOTCH3 mRNA expression in PIK315LT PDO after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; (C) NOTCH3 mRNA expression in VJR275LT PDO after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; (D) JAG1 mRNA expression in mCRC PDOs (n=14) after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; (E) NOTCH1 mRNA expression in mCRC PDOs (n=14) after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; (F) NOTCH2 mRNA expression in mCRC PDOs (n=14) after 72h of treatment with either the vehicle or FOLFOX (25 μM) quantified by RNA sequencing; Statistical significance was determined using paired t-tests, *** = $p < 0.001$, ** = $p < 0.01$; tumours N=14; VJR275LT : N=3; PIK315LT : N=4.

Additionally, we noted that even after 72 hours of treatment with 10 μM FOLFOX followed by 15 days of recovery, the expression of NOTCH3-ICD (Intracellular domain)

at the protein level was elevated in both PIK315LT and VJR275LT organoids (approximately 6-fold compared to vehicle-treated samples for PIK315LT and 4-fold for VJR275LT, $p < 0.01$, paired t-test) (**Figure 6.4**). We can conclude with an overexpression of the NOTCH3 receptor upon chemotherapy treatment and recovery. Moreover, observing the PIK315LT cell line, we can observe not only an increase of NOTCH3-ICD but also, cannot observe the full-length form of the protein in the treated sample, suggesting that the overexpression of the protein is concomitant with an over-activation of the receptor, resulting in the cleavage of the full-length receptor into intracellular form of the protein. Considering the studies suggesting an impact of NOTCH3 on self-renewal [229, 330, 397, 398], this sustained upregulation of NOTCH3 receptor during chemotherapy treatment and after recovery suggests that targeting this pathway may represent a promising strategy in resistant mCRC tumours.

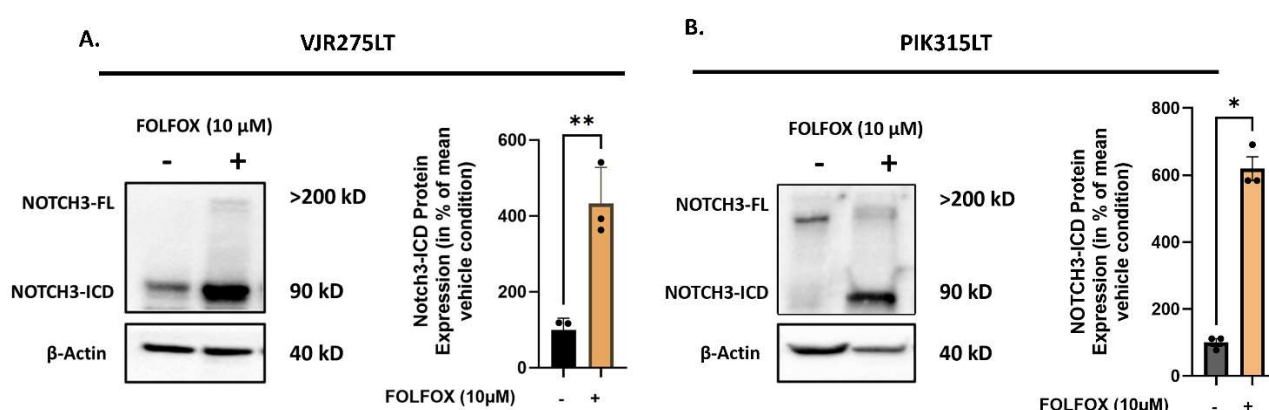


Figure 6.4 – Upregulation of NOTCH3 after recovery in chemoresistant organoid cell lines (A) Quantification of **NOTCH3** protein expression level in **VJR275LT** PDO after 72h of treatment with either the vehicle or FOLFOX (10 μ M) and 15 days of recovery by Western blot (N=3); **(B)** Quantification of **NOTCH3** protein expression level in **PIK315LT** PDO after 72h of treatment with either the vehicle or FOLFOX (10 μ M) and 15 days of recovery by Western blot (N=3); Statistical significance was determined using paired t-tests, ** = $p < 0.01$, * = $p < 0.01$.

It is to be noted that we also observed a significant upregulation of the NOTCH3 mRNA expression in the chemosensitive organoid cell line ySAW190LT (**Figure 6.5-A**). Though, contrarily to PIK315LT and VJR275LT chemoresistant organoids, the over-activation of the NOTCH3 receptor, characterized by the increase of the NOTCH3-ICD expression, is not significant after FOLFOX treatment (1 μ M, 72h) and recovery (15 days) (**Figure 6.5-**

B). This suggests that the effect of NOTCH3 activity upon FOLFOX treatment might be less important in chemosensitive organoids than in chemoresistant organoid cell lines.

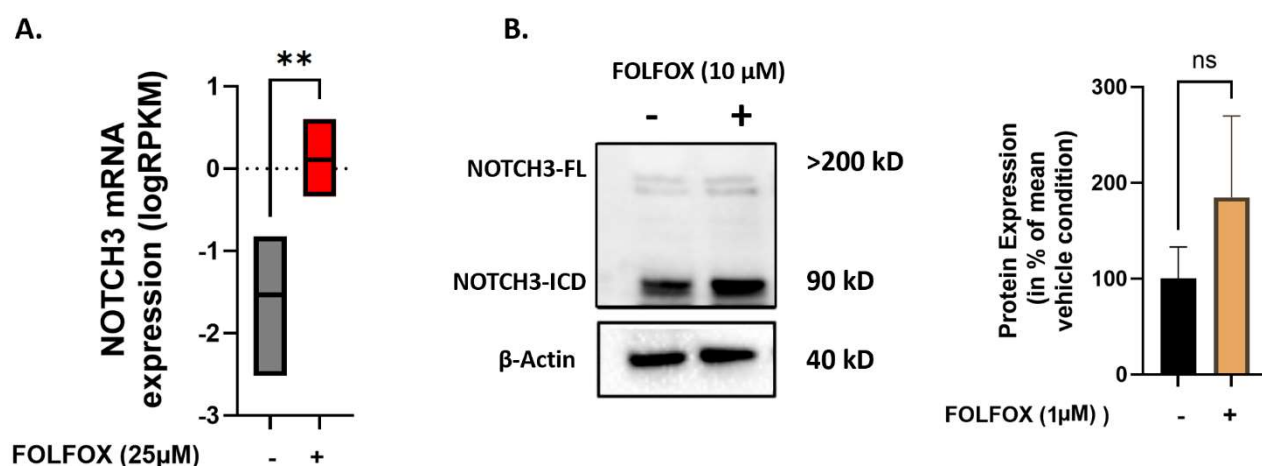


Figure 6.5 –NOTCH3 expression upon FOLFOX and after recovery in ySAW190LT organoid cell lines (A) NOTCH3 mRNA expression in ySAW190LT PDO after 72h of treatment with either the vehicle or FOLFOX (25 µM) quantified by RNA sequencing; **(B)** Quantification of NOTCH3 protein expression level in ySAW190LT PDO after 72h of treatment with either the vehicle or FOLFOX (1 µM) and 15 days of recovery by Western blot (N=3); Statistical significance was determined using paired t-tests, ** = $p < 0.01$, N=3

To investigate the implication of the NOTCH3 receptor in chemoresistant mCRC tumours, we then used CRISPR Cas9 technology to invalidate the NOTCH3 gene in our VJR275LT organoid cell line. This cell line was selected due to the high increase of NOTCH3 mRNA expression observed upon FOLFOX treatment (2,1 log fold-change) (Figure 6.3-C). Analysis of Sanger sequencing traces comparing the parental organoid with the scramble control (sgRNA) or sgNOTCH3 using Tracking of Indels by Decomposition analysis (TIDE) [346] confirmed that genome editing occurred at the predicted Cas9 cut site (Supplementary Figure 6.1). We further confirmed the NOTCH3 protein knockout by Western blot (Figure 6.6-A). Using this NOTCH3-invalidated model, we assessed the effect of NOTCH3 invalidation on the efficacy of FOLFOX treatment in VJR275LT organoids (Figure 6.6-B). We selected a wide range of FOLFOX concentrations (0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM, 33 µM, 100 µM) and quantified the viability of the different VJR275LT organoid lines (WT, SCR, SgNOTCH3) after 72 hours of treatment using CellTiter-Glo 3D, a luminescent cell viability assay used to determine the number of viable cells present in culture. Contrary to our expectations, there was no significant

effect of NOTCH3-invalidation on the sensitivity to FOLFOX (IC50 WT: 1.628 ± 0.1320 μ M, IC50 SgSCR: 0.6567 ± 0.1413 μ M, IC50 SgNOTCH3: 3.505 ± 0.1511 μ M).

Finally, considering the implications of NOTCH3 on self-renewal described in several studies, we investigated the effect of NOTCH3-invalidation on self-renewal in VJR275LT organoids. We did not observe any significant effect of NOTCH3 invalidation on the self-renewal capability of this organoid cell line (**Figure 6.6-C**), though we noticed that the post-treatment self-renewal was affected (**Figure 6.6-D**). Surprisingly, the NOTCH3 protein invalidation resulted in an increase in self-renewal capability after 72 hours of treatment with 10 μ M of FOLFOX (mean = $3.427 \% \pm 3.232$) compared to wild-type PDO (mean = $0.277 \% \pm 0.222$) and to control (SgSCR) PDO (mean = $0.275 \% \pm 0.209$). This result is surprising due to the well-accepted role of NOTCH receptors in the positive modulation of stemness. The result though is not statistically significant ($p=0.18$; One-way ANOVA, Turkey's multiple comparison test), but this is probably due to a large variability observed in the NOTCH3 knockout samples after treatment.

Overall, these results suggest that the NOTCH3 pathway is stimulated during chemotherapy, notably in chemoresistant mCRC. Paradoxically though, our results seem to indicate a potentially negative role of NOTCH3 in chemoresistance and post-treatment self-renewal features. These results contrast with those of the literature since most studies exploring the role of NOTCH3 in stemness find a positive role for this receptor in CSCs-related phenotypes[398].

However, since our project demonstrated the implication of both the UNC5B and NOTCH3 dependence receptors in the self-renewal abilities of mCRC, it suggests the possibility of an interaction between pathways related to these two receptors.

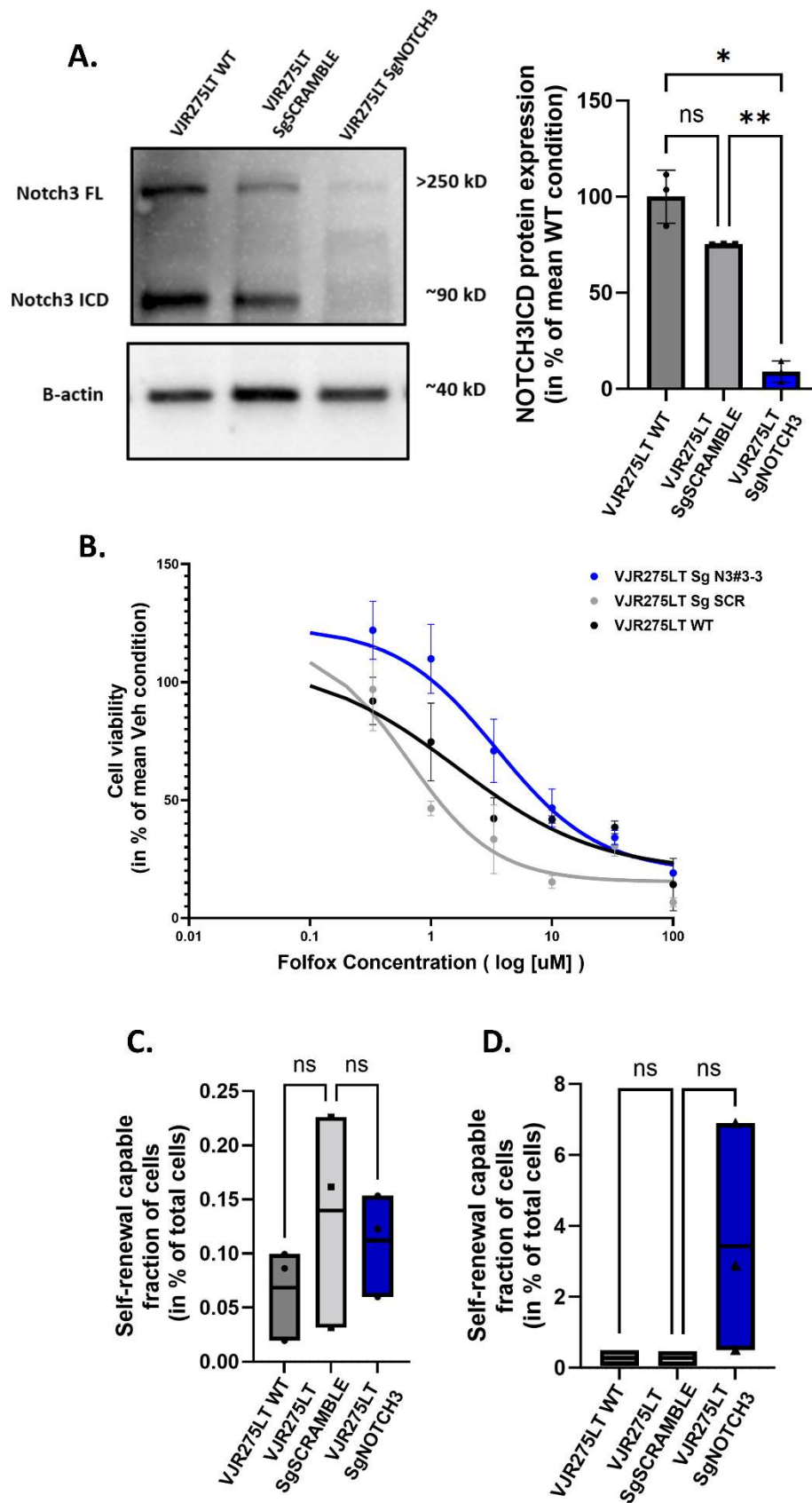


Figure 6.6– NOTCH3 invalidation decreases FOLFOX treatment efficacy in resistant mCRC organoid (A) Western Blot validation of **NOTCH3** protein knockout by looking at the protein level of NOTCH3 in wildtype, SgSCRAMBLE control (sgSCR) and CRISPR-edited (SgNOTCH3) **VJR275LT**; **(B)** Effect of **NOTCH3**

invalidation on FOLFOX treatment efficacy (FOLFOX: 0.1 to 100uM concentration range, wild-type (WT) vs. SgSCRAMBLE (SgSCR) vs SgNOTCH3 (SgN3)) using Cell-Titer-Glo3D; **(C)** Effect of **NOTCH3 invalidation** on self-renewal capable fraction of **VJR275LT** organoid cell line observed by ELDA; **(D)** Effect of **NOTCH3 invalidation** on self-renewal capable fraction of **VJR275LT** organoid cell line after 72h of FOLFOX treatment (72h; 10 μ M observed by ELDA; Statistical test used: One-way Anova- Tukey's multiple comparison tests For (A) and (C)

c. NOTCH3 and UNC5B pathways interactions

To explore the possible links between UNC5B and NOTCH3 in CRC, we used once again TCGA Pan-Cancer Clinical Data and our internal RNA sequencing data. The TCGA cohort analysis revealed a strong correlation between the expression of NOTCH3 mRNA and UNC5B expression (**Figure 6.7-A**). This correlation was similar in metastatic tumours, as shown in our stage IV CRC cohort (n=58) (**Figure 6.7-B**).

These analyses of CRC tumours seem to indicate a positive association between the expression of these two dependence receptors. However, analysis of the RNA sequencing data obtained from our organoid lines indicated the opposite. Indeed, in our PDO cohort, we observed a negative correlation between the expression of NOTCH3 and UNC5B mRNA (**Figure 6.7-C**). These results therefore seem contradictory, although, the positive correlations observed in tumours could be conditioned by the presence of surrounding non-tumoral cells such as hepatocytes.

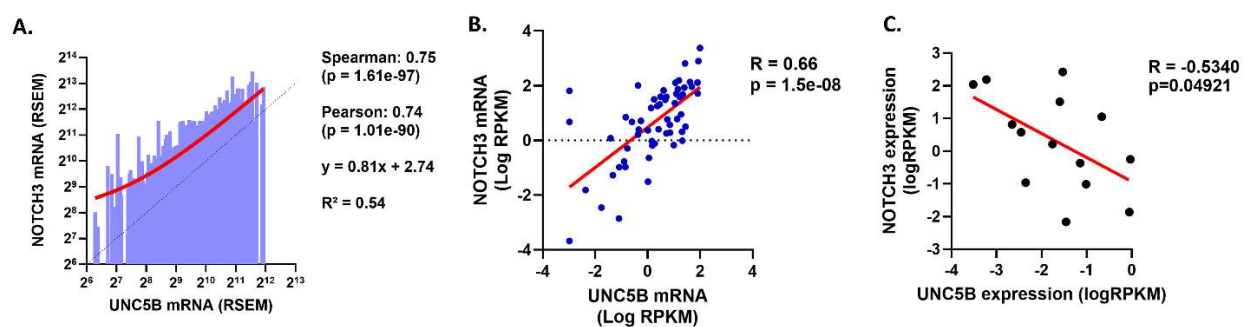


Figure 6.7 – Characterisation of NOTCH3 and UNC5B relationship in CRC tumours and mCRC organoids (A) Correlation between **NOTCH3** mRNA and **UNC5B** mRNA expression in TCGA cohort; **(B)** Correlation between **NOTCH3** mRNA and **UNC5B** mRNA in stage 4 CRC tumours (n=58); **(C)** Negative correlation between **NOTCH3** mRNA and **UNC5B** mRNA expression in mCRC organoid cell lines; (TCGA cohort: n=521; late stage tumours cohort: n=58, Adjacent healthy tissue cohort: n=34, mCRC organoid cell lines: n= 14); p Values are displayed; Statistical test: Pearson correlation test

To determine whether the negative relationship observed in our PDO cell lines reflected a direct cell-intrinsic effect, we investigated the expression of the NOTCH3 receptor in our PIK315LT organoid model invalidated for UNC5B. Doing so, we observed an overexpression of the NOTCH3 intracellular domain in SgUNC5B invalidated cell lines (**Figure 6.8-A**), demonstrating a negative impact of the UNC5B protein on NOTCH3 receptor expression and activation. To investigate the implication of the NOTCH3 receptor in the UNC5B receptor expression in the same PDO cell line, we used CRISPR Cas9 technology to knock out the NOTCH3 gene in our PIK315LT organoid cell line, since it is the cell line where we successfully invalidated UNC5B. Though, even though the analysis of Sanger sequencing traces comparing the parental organoid with the scramble control (sgRNA) or sgNOTCH3 using TIDE [346] confirmed that genome editing occurred at the predicted Cas9 cut site (**Figure 6.8-A and B**), Western Blot analysis (**Figure 6.8-C**) detected the presence of the Full-length form of the protein, though no longer generating the intracellular (ICD) fragment of the protein.

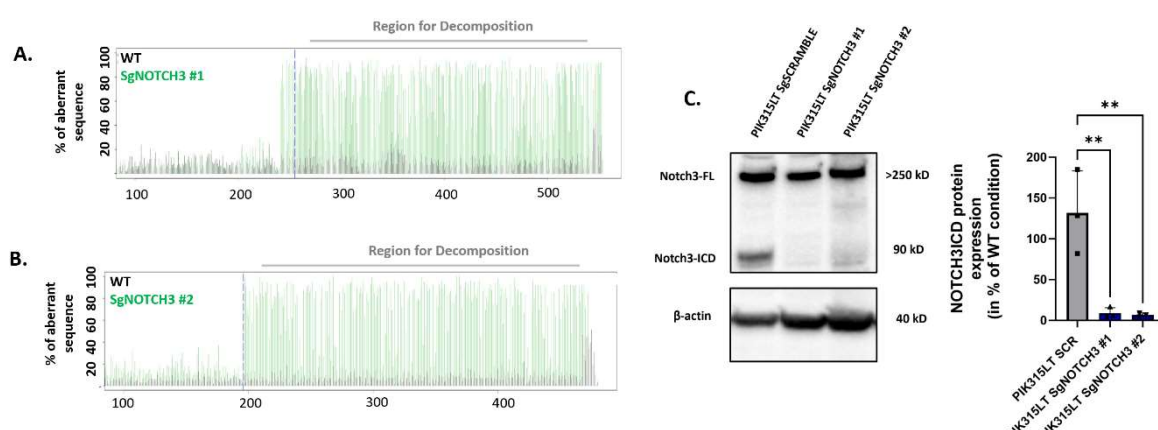


Figure 6.8 – NOTCH3 invalidation in PIK315LT PDO (A) Representative TIDE analyses comparing Sanger sequencing traces from wildtype PDOs versus CRISPR-edited (SgNOTCH3 #1) in PIK315LT organoid; **(B)** Representative TIDE analyses comparing Sanger sequencing traces from wildtype PDOs versus CRISPR-edited (SgNOTCH3 #2) in PIK315LT organoid **(C)** Western Blot validation of NOTCH3 intracellular-domain protein knockout by looking at protein level of NOTCH3 protein, scramble control (sgSCR) and CRISPR-edited (SgNOTCH3#1 and SgNOTCH3#2) **PIK315LT**; Statistical significance was determined using One-way Anova – Turkey’s multiple comparison test, Error bars indicate SD; ***= $p < 0.01$, $N=3$

In these PIK315LT cell lines with this modified NOTCH3 receptor, we observed a clear upregulation of the UNC5B receptor compared to the SgSCRAMBLE organoid cell line, suggesting that NOTCH3 activation may repress UNC5B expression (**Figure 6.9-B**). Conversely, in the PIK315LT organoid cell line invalidated for UNC5B, we observed the

upregulation of NOTCH3-ICD protein expression (**Figure 6.9-A**). Suggesting that UNC5B receptor expression inhibits activation of the NOTCH3 receptor.

These experiments demonstrate that these two receptors exert a negative pressure on each other's expression levels, suggesting possible antagonistic roles for them. It is to be noted though that this experiment was made in the absence of exogenous Netrin-1, therefore, we're not able to evaluate properly if this effect of the UNC5B receptor on NOTCH3 is rather due to its positive or apoptotic signalling. Further experiments in the presence of recombinant Netrin-1 could help us to distinguish between these.

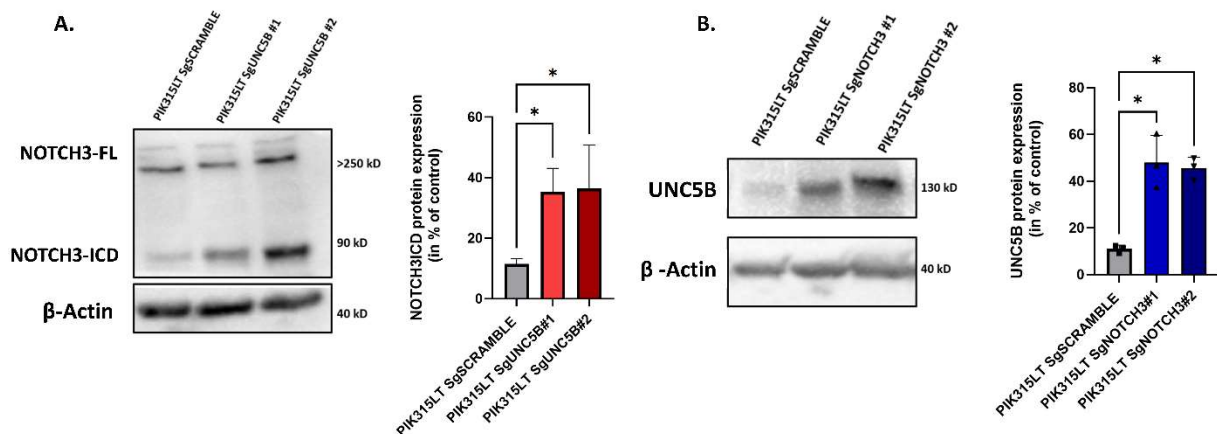


Figure 6.9 – UNC5B and NOTCH3 proteins expression is mutually exclusive (A) Expression of **NOTCH3** protein expression in **UNC5B-invalidated PIK315LT** organoid (control (sgSCR) vs. CRISPR-edited (SgUNC5B#1 and SgUNC5B#2), (Left) Western Blot, (Right) Histogram representation of NOTCH3 intracellular domain (ICD) protein expression ; **(B)** Expression of **UNC5B** protein expression in **NOTCH3-invalidated PIK315LT** organoid (control (sgSCR) vs. CRISPR-edited (SgUNOTCH3#1 and SgNOTCH3#2), (Left) Western Blot, (Right) Histogram representation of UNC5B protein expression; Statistical significance was determined using One-way Anova – Turkey's multiple comparison test, Error bars indicate SD; ns = non-significant, * = $p < 0.05$, $n = 3$

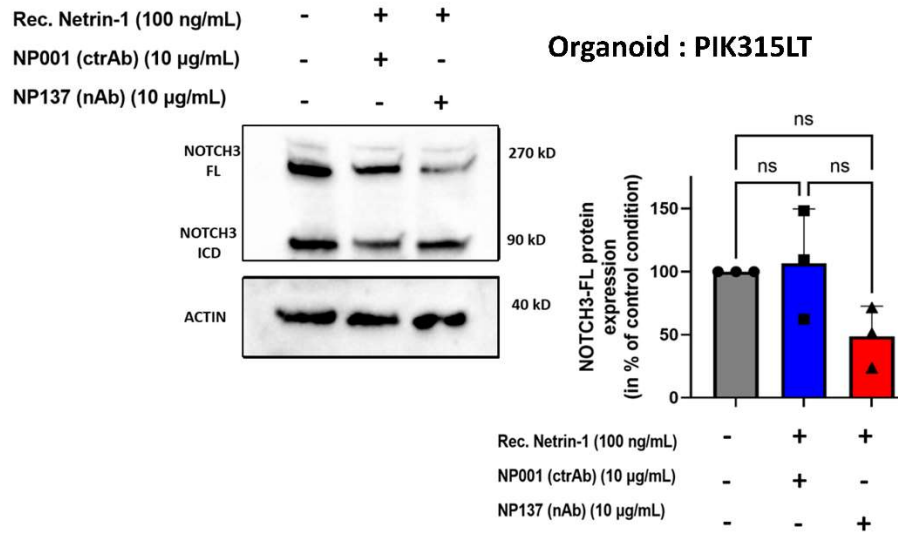
To further analyze the impact of the Netrin-1/UNC5B activity on the NOTCH3 pathway, we investigated the effect of NP137 on the expression of the NOTCH3 receptor in our PIK315LT organoid line (**Figure 6.10-A**). We did not observe any significant effect of recombinant Netrin-1 (100 ng/mL) in the presence or absence of NP137 (10 ug/mL) in the expression of the NOTCH3 receptor protein. However, we observed that NP137 tends to lower the expression of the full-length form of the protein. Since ySAW190LT is the organoid expressing the highest levels of UNC5B, we evaluated if we found a similar

or stronger effect in Netrin-1 inhibition on NOTCH3 Full-Length protein expression (**Figure 6.10-B**). In the presence of recombinant Netrin-1 (100ng/mL), we detected a significant decrease in the expression of the Full-length NOTCH3 receptor upon treatment with NP137 (10ug/mL) compared to cells treated with NP001 control antibody (10ug/mL) and to non-treated cells. However, the intracellular form of the NOTCH3 protein did not appear to be affected by NP137 treatment, suggesting an effect of Netrin-1 on the expression of the NOTCH3 receptor but not necessarily on its activation. Moreover, the supplementation of recombinant Netrin-1 alone did not modify NOTCH3 expression, indicating that the endogenous level of Netrin-1 is sufficient to have a complete effect on the NOTCH3 receptor. Finally, the fact that this effect appears stronger in the chemosensitive ySAW190LT organoid cell line than in the chemoresistant PIK315LT organoid cell line suggests a difference in behaviour for the NOTCH3 receptor according to the phenotype of the mCRC cells.

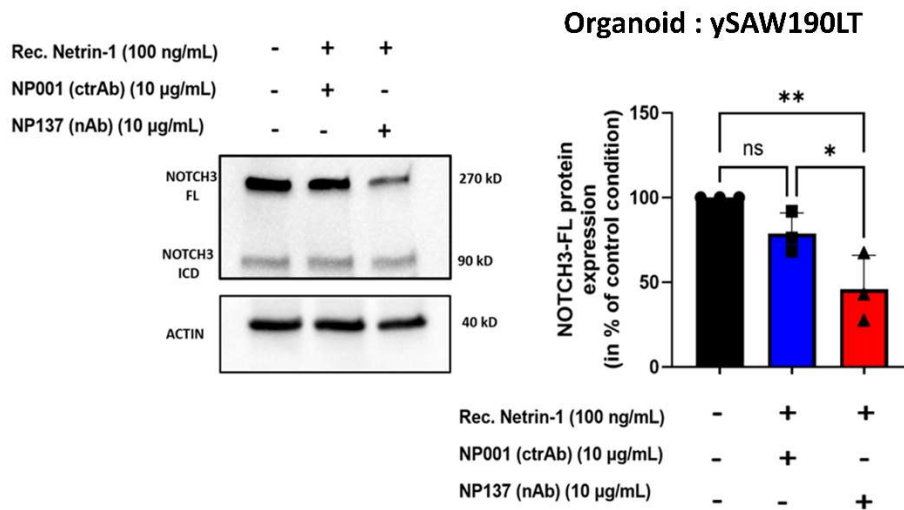
Similarly, we observed that JAG1 expression tends to be decreased in ySAW190LT organoid treated with recombinant Netrin-1 and NP137 compared to non-treated cells and cells treated with recombinant Netrin-1 and NP001 control antibody, though this result is not statistically significant when compared to the Netrin-1 and NP001 condition, probably due to the high variability observed in the latter condition (**Figure 6.10-C**). As for the NOTCH3 protein, we don't observe any effect on cells treated with recombinant Netrin-1.

These results demonstrate interactions between the UNC5B/Netrin-1 and NOTCH3/Jagged-1 pathways., Netrin-1 seems to influence positively the expression of the protein belonging to this pathway whereas UNC5B receptor seems to decrease the activation of the NOTCH3 receptor. We can also note that NOTCH3 receptor modulation is phenotype-dependent in mCRC cells. Indeed, we can observe that the increased activation of the NOTCH3 receptor upon FOLFOX treatment and recovery is weaker in the ySAW190LT organoid than in the PIK315LT and VJR275LLT organoid cell lines.

A.



B.



C.

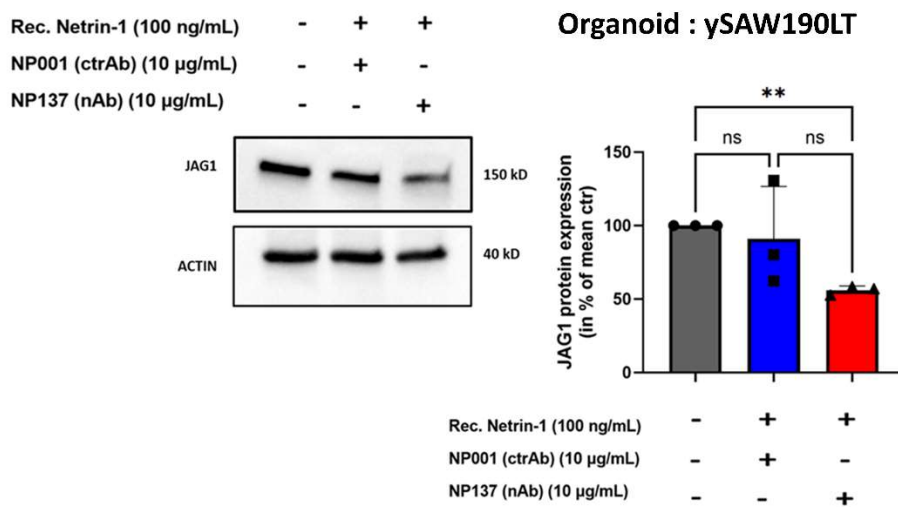


Figure 6.10 – Netrin-1 inhibition dysregulates NOTCH3/JAG1 pathway (A) Expression of *NOTCH3* protein expression in *PIK315LT* organoid after 72h of treatment with either nothing (Control), Netrin-1 (100ng/mL)

& NP001 (10µg/mL) or Netrin-1 (100ng/mL) & NP137 (10µg/mL) observed by Western blot, (Left) Western Blot, (Right) Histogram representation of NOTCH3 Full Length (FL) protein expression ; **(B)** Expression of **NOTCH3** protein expression in **ySAW190LT** organoid after 72h of treatment with either nothing (Control), Netrin-1 (100ng/mL) & NP001 (10µg/mL) or Netrin-1 (100ng/mL) & NP137 (10µg/mL) observed by Western blot, (Left) Western Blot, (Right) Histogram representation of NOTCH3 Full Length (FL) protein expression ; **(C)** Expression of **JAG1** protein expression in **ySAW190LT** organoid after 72h of treatment with either nothing (Control), Netrin-1 (100ng/mL) & NP001 (10µg/mL) or Netrin-1 (100ng/mL) & NP137 (10µg/mL) observed by Western blot, (Left) Western Blot, (Right) Histogram representation of **JAG1** protein expression; Statistical significance was determined using One-way Anova – Turkey’s multiple comparison test, Error bars indicate SD; ns = non-significant, * = $p < 0.05$, ** = $p < 0.01$, N=3

To further explore the effect of Netrin-1 inhibition on the NOTCH3 pathway, we observed the effect of NP137 on the transcriptional targets of the NOTCH3 receptor, notably HES1, a basic helix-loop-helix transcription factor that plays a crucial role in various cellular processes, including development, differentiation, and stem cell maintenance. Doing so we observed a decrease in the expression of this transcriptional target in both PIK315LT (log2foldchange values: -0.141, p.adj = 1.62e-16) (**Figure 6.11-A**) and ySAW190LT (log2foldchange values: -0.150, p.adj = 5.69e-16) (**Figure 6.11-B**). This demonstrates further the effect of Netrin-1 inhibition on the NOTCH3 pathway and seems to indicate that a similar effect of NP137 on the NOTCH3 receptor is found in the two phenotypically different organoid cell lines PIK315LT and ySAW190LT.

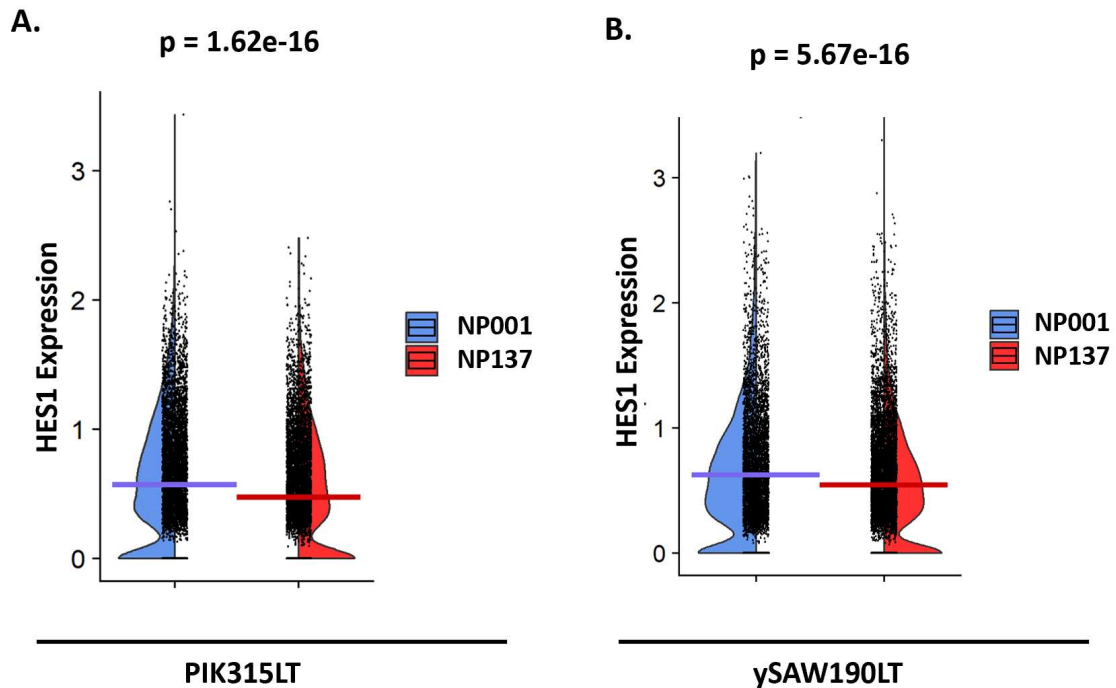


Figure 6.11 – HES1 is downregulated upon NP137 treatment in mCRC organoids (A) Violin plot of the expression of HES1 in PIK315LT organoid treated with either NP001 (10 ug/mL) or NP137 (10 ug/mL) ; **(B)** Violin plot of the expression of HES1 in ySAW190LT organoid treated with either NP001 (10 ug/mL) or NP137 (10 ug/mL) (Means displayed determined by `stat_compare_means` function; Corrected p-value displayed calculated using `Findmarkers` function of `Seurat` package; Bars indicate means, statistical test use: Wilcoxon Rank Sum test, Each dot represent an event (cell))

III-Conclusions

Using immunohistochemistry and RNA sequencing data, we described NOTCH3 expression patterns in CRC and observed a correlation between expression of *NOTCH3* and genes associated with EMT and stemness.

Further exploration revealed a marked increase in *NOTCH3* mRNA expression levels in mCRC tumours when compared to normal adjacent liver tissues, suggesting potential roles in tumour modulation. Of note, *NOTCH3* expression in our organoid lines was lower than in stage 4 CRC tumours, which might indicate a role for microenvironment in the upregulation of NOTCH3 in tumours.

NOTCH3 is overexpressed during FOLFOX treatment through its over-activation post-recovery seems to be exacerbated in resistant organoid cell lines (VJR275LT and PIK315LT). We interpreted this upregulation of NOTCH3 as a clue towards its potential

role in chemoresistance. However, contrary to expectations, no significant effects on FOLFOX sensitivity or self-renewal capabilities were observed in our model of VJR275LT organoid invalidated for *NOTCH3*. Moreover, post-treatment self-renewal was positively affected by *NOTCH3* invalidation, suggesting a negative modulation of post-treatment self-renewal by the NOTCH3 receptor in chemoresistant mCRC organoids.

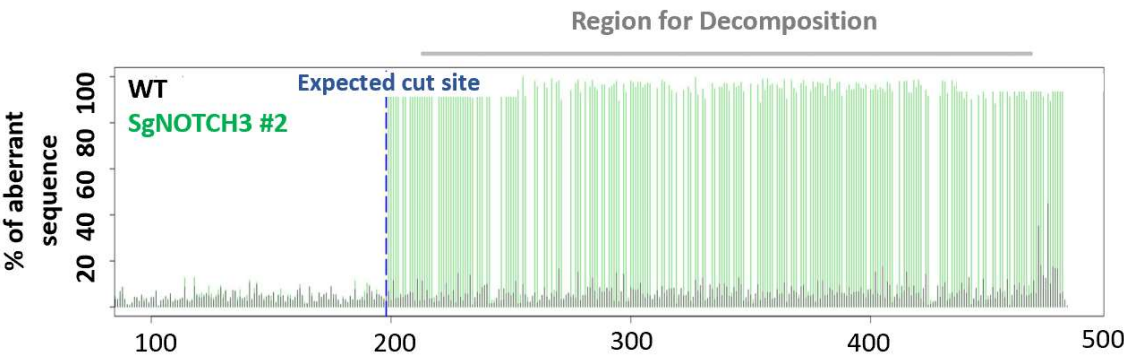
Exploring the relationship between NOTCH3 and the dependence receptor UNC5B revealed seemingly contradicting correlations between the expression of these receptors in CRC tumours versus organoid cell lines, suggesting potential tissue-specific or microenvironment-driven interactions. Knocking out *NOTCH3* in our PIK315LT model of organoid triggered a clear upregulation of UNC5B, whereas the inactivation of UNC5B in the same organoid line resulted in an overactivation of the NOTCH3 receptor characterised by increased expression of the NOTCH3-ICD fragment, suggesting that their relationship may be mutually exclusive, at least in our PIK315LT organoid cell line. Indeed, because of our inability to grow a ySAW190LT model invalidated for UNC5B, we were not able to see if this relationship was similar in these chemosensitive organoid cell lines.

Finally, we observed that the inhibition of Netrin-1 was leading to a decrease of NOTCH3-Full Length expression and a decrease in *HES1* mRNA expression, though the inhibition of NOTCH3 full-length protein was more significant in the ySAW190LT organoid.

This seems to indicate some difference in the modulation and activity of the NOTCH3 receptor depending on the phenotypical characteristics of mCRC cells. Several hypotheses could explain these. For instance, the difference of expression of UNC5B, higher in the ySAW190LT organoid compared to PIK315LT and VJR275LT could explain the stronger effect of NP137 on NOTCH3-FL expression. The fact that ySAW190LT is *TP53* wild-type whereas both PIK and VJR are mutated for *TP53* could explain the difference regarding the over-activation of the NOTCH3 receptor observed in chemoresistant organoids but not significantly in ySAW190LT organoid. Indeed, these organoid cell lines are representative of very different phenotypical behaviours potentially explaining the differences observed. Nevertheless, further investigations are needed to have a precise

idea of the mechanisms involved in the interactions of the UNC5B/Netrin-1 and NOTCH3/Jagged-1 pathways.

Supplementary Material – Chapter 6



Supplementary Figure 6.1 - Representative TIDE analyses comparing Sanger sequencing traces from wildtype PDOs versus CRISPR-edited (SgNOTCH3 #2) in VJR275LT organoid

Discussion & Conclusion

Colorectal cancer (CRC) ranks as the fourth most common cancer worldwide, and, alarmingly, nearly a third of CRC patients are diagnosed or will progress to metastatic disease, where the 5-year survival rate drops dramatically [1-3]. This observation underscores the urgent need for more effective therapeutic approaches, especially given the poor efficacy of current chemotherapy used in metastatic colorectal cancer (mCRC) treatment [4-6]. Because of the small number of available treatment options, scientists have been looking for alternatives by exploring targets on tumour cells but also in the microenvironment [7]. As part of this effort, the emergence of dependence receptors as potential targets for solid tumour therapy has gained considerable scientific interest [8-10]. Our project study aimed to investigate the involvement of one of these receptors, the UNC5B receptor and its ligand Netrin-1, in the regulation of mCRC cell self-renewal, and to assess the therapeutic potential of targeting this signalling pathway to potentiate existing chemotherapy treatments. During the course of this work, we explored the regulation of netrin-1 and UNC5B expression in mCRC, characterised the role of Netrin-1 and thereafter of UNC5B in the regulation of self-renewal, provided insight into the cellular biology and the therapeutic leveraging of these effects, and studied the potential crosstalk between this pathway and another dependence receptor, NOTCH3.

a. Regulation of Netrin-1 and receptor expression in mCRC

We first explored the expression patterns of Netrin-1 and UNC5B across different CRC tumours and stages. Our findings highlighted the higher expression of both NTN1 and UNC5B mRNA levels in later stages of CRC and we confirmed the expression of both protein and mRNA for both in mCRC tumours using RNA sequencing and immunohistochemistry. Interestingly, we also found that the expression of Netrin-1 was significantly higher in healthy liver tissues than in the hepatic mCRC tumours or mCRC organoid cell lines.

This suggests a role for non-tumoral cells from the tumour microenvironment in the secretion of Netrin-1. This hypothesis is further reinforced by the study from Sung et al. in 2019 demonstrating that CRC-associated fibroblasts were secreting Netrin-1, enhancing tumour cell stemness-related features [11]. This data is also in agreement with previous results demonstrating that the induction of Netrin-1 expression in metastatic pancreatic adenocarcinoma cells enhanced metastatic seeding in the liver, acting through a feedforward loop whereby Netrin-1 on the surface of PDAC-secreted extracellular vesicles prepares the metastatic niche by inducing hepatic stellate cell activation and retinoic acid secretion [12]. Together with the results of this work, this leads us to speculate that the high expression of Netrin-1 in the liver likely contributes to the high prevalence of liver metastasis in CRC [1-3]. It is to be noted that we unsuccessfully tried to observe the expression of Netrin-1 at the protein level in our organoid models. This is consistent with the RNA sequencing data showing that NTN1 mRNA is lowly expressed in PDOs. However, the effects observed with NP137 suggest an endogenous expression of Netrin-1 in organoids. We're currently optimising our protocols to observe this supposed expression.

In parallel, we also found that UNC5B was the predominant Netrin-1 receptor expressed in mCRC organoid lines and tumour samples, suggesting a very limited role for other Netrin-1 receptor signalling in driving the effects of Netrin-1 in mCRC cells.

Further characterisation of the Netrin-1/UNC5B pathway in mCRC revealed a sustained or increased expression of UNC5B mRNA upon treatment with chemotherapy in Wild-Type *TP53* organoids. The observed UNC5B overexpression may be attributed to the activation of P53 following chemotherapy treatment and its subsequent upregulation of UNC5B and Netrin-1. Indeed, previous studies in other models have reported the activation of P53 by 5-FU or Oxaliplatin, and the regulation of the Netrin-1 and UNC5B by P53 has also been documented [8, 13, 14]. The upregulation of Netrin-1 and of its receptors, notably UNC5B, upon treatment with different chemotherapeutic molecules (Doxorubicin, Cisplatin, 5-Fluorouracil and Taxol) has been observed in a large variety of cancer cell lines (Breast cancer, lung cancer, ovarian cancer, pancreatic cancer, neuroblastoma, glioblastoma and colorectal cancer)[15], and our results now corroborate this evidence in a more clinically relevant mCRC model.

Accordingly, we observed the expression of *CDKN1A* mRNA (which encodes P21), a usual marker of P53 activation in our mCRC organoid model following FOLFOX treatment. However, increased expression of this gene was detected in all organoid cell lines tested, even those carrying a *TP53* mutation. This suggests that the expression of *CDKN1A* mRNA is not ideal as a control for P53 activation in this model, at least in the context of treatment with chemotherapies. Secondly, it also indicates that P21 expression is induced in *TP53*-mutated mCRC cells upon chemotherapy, demonstrating that in this context, P21 may be regulated independently from p53.

b. Netrin1 pathway activation regulates self-renewal.

Our study then explored the possibility of Netrin-1 and UNC5B modulating stemness in mCRC, detecting correlations between the expression of this ligand/receptor pair, the expression of genes related to EMT and cancer stem cells, and phenotypical features such as self-renewal in mCRC cells.

We further explored this modulation of stemness-related features by demonstrating that Netrin-1 is promoting CRC self-renewal, its inhibition using the NP137 antibody resulting in a decrease of self-renewal in mCRC organoid. The fact that the inhibition of Netrin-1 led to decreased self-renewal capabilities in organoids that express different levels of UNC5B (low to high) and different phenotypes (*TP53* mutation or chemosensitivity status), underscores the importance of the Netrin-1 pathway in CRC self-renewal regulation. Moreover, it is coherent with the fact that the different cell lines used to investigate the Netrin-1 effect on self-renewal (HCT116 cell line and ySAW190LT and PIK315LT PDOs) express endogenous Netrin-1 (detected at protein level in HCT116 and mRNA levels in PIK315LT and ySAW190LT). However, the fact that the supplementation of Netrin-1 in the medium of ySAW190LT and PIK315LT cell lines triggers self-renewal upregulation indicates that endogenous levels of Netrin-1 are not sufficient to obtain an optimal effect of Netrin-1 and that liver microenvironment-derived Netrin-1 may enhance these effects *in vivo*.

This study also explored the impact of Netrin-1 on apoptosis, showing a decrease in late apoptosis specifically in the CD133-positive CSC population, with implications for CSC inhibition. Furthermore, although the difference in levels of apoptosis detected in CD133-positive cells appears to be small, it's noteworthy that a reduction in the CD133+ cell population already occurred at this early time point. It's important to consider that this measurement is taken at 72 hours, whereas the extended duration of the ELDA assay spans 15 days, during which treatment with Netrin-1 and/or NP137 is consistently applied. Thus, despite the initial observation of limited apoptosis, the longer-term effects of Netrin-1 and NP137 on CSC self-renewal may become more pronounced over time. Another explanation would be that the decrease in self-renewal does not necessarily equate to CSC apoptosis.

c. UNC5B is the key mediator of netrin-1 role in mCRC

Our results clearly point to UNC5B as the key receptor mediating the effect of Netrin-1 in CRC cells. UNC5B overexpression notably increased self-renewal in the HCT116 CRC cell line, indicating a positive regulatory role for UNC5B in CRC cell self-renewal. HCT116 cells are derived from the primary tumour of a patient with metastatic CRC, and these cells have been shown to express Netrin-1 [16]. Yet, while they are commonly used in the literature, the HCT116 cell line has been cultured in 2D for a long time and is therefore not very representative of patient tumours *in situ*. Therefore, for the bulk of this work, we decided to use patient-derived organoids as a working model with higher clinical relevance.

Interestingly, despite evidence of expression of the Netrin-1 ligand in the ySAW190LT and PIK315LT organoids (at mRNA level) and the HCT116 cell line (at protein level), the experimental manipulation of the UNC5B receptor seems to result in slightly different effects in these different models. Indeed, we observed an obvious increase in self-renewal upon UNC5B overexpression in the HCT116 cell line, whereas, in contrast, the invalidation of UNC5B in PIK315LT organoids did not alter their self-renewal in the absence of exogenous Netrin-1. This lack of effect upon UNC5B invalidation is intriguing.

In the absence of Netrin-1 one would expect UNC5B to function purely as a dependence receptor, and therefore to see an increase of self-renewal upon its invalidation, due to inhibition of apoptosis triggered by the UNC5B receptor. However, the expression of Netrin-1 by PIK315LT organoids suggests that UNC5B signalling should positively influence self-renewal, and we would therefore expect to see a decrease in self-renewal upon its invalidation. The stable self-renewal in the UNC5B-invalidated organoid model doesn't allow us to clearly establish the baseline function of UNC5B in the PIK315LT organoid line. This could be explained by the fact that Netrin-1 both inhibits intracellular signal-promoting apoptosis, and stimulates cell survival, proliferation, and migration through the UNC5B receptor as seen in endothelial cells [17]. This would suggest that at a low concentration of Netrin-1, an equilibrium can exist between the positive and apoptotic signalling. Then, if the endogenous netrin-1 levels in mCRC cells are low this equilibrium between positive and negative signals can exist in the PIK315LT cell line. UNC5B-invalidation in this cell line would then not result in a modification of self-renewal. It is to be noted that this is likely an issue in vitro but that the corresponding tumour in situ would receive Netrin-1 from the tumour microenvironment from either hepatic cells or CAFs [11, 12], and therefore the absence of UNC5B in those conditions would most likely decrease self-renewal.

Given that the UNC5B invalidation in this organoid cell line is constitutive, it is also possible that alternative pathways may compensate for the absence of UNC5B, suggesting the potential existence of an adaptive process. The fact that our attempts to invalidate UNC5B in ySAW190LT organoids resulted in the impossibility to grow cells after selection, suggests a potentially essential role for UNC5B in this specific line. We could verify this hypothesis further using short-term siRNA-mediated UNC5B downregulation to study the potential modulation of self-renewal and apoptosis in this context. Another possibility would be that the ySAW190LT cell line relies on TFF3 than the PIK315LT cell line, this is coherent with the fact that we observe a much higher expression of TFF3 in ySAW190LT comparatively to PIK315LT (Data not shown).

We then looked to understand the effects of Netrin-1 blockade on metastatic organoids at a single-cell level using single-cell RNA sequencing. NP137 treatment in ySAW190LT organoids resulted in a global reduction in the expression of stemness-related genes,

including SOX4, PROM1, SMOC2, and LGR5, indicating a downregulation of stemness pathways. However, PIK315LT organoids did not show a similar decrease in stemness genes upon NP137 treatment. This difference could be linked to the significantly higher baseline self-renewal rate and/or UNC5B receptor expression in ySAW190LT organoids, which may render ySAW190LT more sensitive to Netrin-1 inhibition than the PIK315LT line. However, since we showed that PIK315LT organoid self-renewal is also regulated by Netrin-1/UNC5B, another explanation would be the uncoupling between self-renewal abilities and stemness marker transcription in PIK315LT mCRC cells. Indeed, several previous studies identified processes resulting in the positive regulation of self-renewal abilities of CRC cells but having negative impacts on the expression of genes related to stemness such as PROM1 or EpCAM [18-21]. Finally, we can also consider the possibility that CSCs of the PIK315LT cell line express different stemness-related markers than the ySAW190LT. Indeed, in CRC cells, several types of CSCs, exhibiting the expression of different markers, have been described. This includes co-existing populations of fast-cycling CSCs expressing stemness markers like LGR5 and CD133 and depending on MYC activity to proliferate and of slow-cycling CSCs characterized by other markers such as BMI1 and hTERT, which are independent of MYC [22, 23].

d. Netrin1/UNC5B effects on self-renewal may be indirect.

In addition, although our result clearly demonstrated that Netrin-1 has a broad effect on the mCRC self-renewal, our single-cell RNA sequencing results suggest that the underpinning mechanisms may differ depending on the phenotype of tested organoids. Indeed, different cell subpopulations within ySAW190LT organoids were enriched for UNC5B expression or stemness markers such as PROM1. This comes in contradiction with our initial hypothesis which proposed a direct regulation by Netrin-1 of the self-renewal and apoptosis in UNC5B-expressing CSCs. When mining our scRNA sequencing data to identify a factor that may be responsible for mediating the communication between UNC5B-expressing cells and CSCs, we detected a significant decrease in the expression of the gene encoding Trefoil Factor 3 (*TFF3*) specifically in UNC5B-expressing

cells upon NP137 treatment of ySAW190LT organoid. This raised our attention since TFF3 expression is highly related to CSCs-related features in several cancer types including colorectal cancer [24-29]. Moreover, TFF3 degradation has been shown to trigger apoptosis in cancer cells through the downregulation of anti-apoptotic proteins such as BCL2 or MCL1 [30-33]. Altogether this data points to TFF3 as a potential link between Netrin-1 signalling, UNC5B, and self-renewal regulation in the ySAW190LT mCRC cell line. However, the co-expression of UNC5B and PROM1 could indicate that NP137 inhibit UNC5B positive signalling in mCRC cell lines, not necessarily triggering apoptosis but inhibiting important pathways for cell proliferation and survival. This effect would affect indirectly PROM1 expressing cells in ySAW190LT through TFF3 regulation and apoptosis of CD133-positive cells, whereas, in the PIK315LT cell line, this effect would affect directly the PROM1-expressing cells, these cells expressing higher levels of UNC5B in this cell line. Further experiments will help us to explore these hypotheses, notably by assessing whether TFF3 is essential to the effect of Netrin-1/UNC5B on self-renewal.

In addition, our single-cell RNA sequencing analysis provided insights into the broader impact of Netrin-1 blockade on mCRC cell signalling, including effects on cytoplasmic translation, respiration, and adhesion. Genes related to these cellular processes are mostly downregulated upon treatment with NP137.

Several studies point towards a link between high expression of ribosomal proteins and high self-renewal capacity [34-36]. Moreover, several of these proteins have been shown to be involved in apoptotic processes [37, 38]. For instance, the deletion of RPL29 impairs cellular differentiation, proliferation regulation, and tumorigenesis [39][40]. Interestingly, RPL29, which we found downregulated upon NP137 treatment in PIK315LT, has been found to have a significantly elevated expression in human colorectal carcinomas, reinforcing the idea of ribosomal protein contribution to tumorigenesis [41]. Given the implication of cytoplasmic translation in self-renewal and apoptosis it appears plausible that the impact of NP137 on ribosomal protein-coding genes participates in the effects we observed on mCRC organoid self-renewal.

We can also observe that some of the genes related to cell adhesion upregulated upon NP137, notably NF1, RASA1, ROCK2, and KANK1, are directly linked to GTPase

regulation [42-45]. Other several genes regulating the activity of GTPase were found upregulated in our organoid cell lines upon treatment with NP137. Notably the upregulation of NF1, FARP1, ARHGAP32, RABGAP1L, RALGAPA2, STARD13, AGAP1. A large proportion of these genes (NF1, RABGAP1L, RALGAPA2, AGAP1, RASA1) are linked to the regulation of RAS signalling, especially its inhibition [45-51]. Their downregulation of RAS signalling can lead to aberrant cell behaviour and may contribute to the observed effects on cell adhesion in the PIK315LT organoids. Moreover, GTPase has been shown to be involved in the modulation of the mitochondrial division [52, 53], and accordingly, we found that mitochondrial genes were also downregulated upon NP137 treatment of mCRC organoids.

This downregulation of mitochondrial oxidative chain genes by NP137 may contribute to its effect on the self-renewal of mCRC cells as well. Indeed, despite the well-known preferences of CSCs towards glycolytic pathways in order to produce nucleotides and phospholipids necessary for proliferation, recent studies demonstrate the importance of the mitochondrial chain for CSCs-related features [54-58]. However, when we confront this well-known fact with the intratumoral CSC heterogeneity composed of slow- and fast-cycling CSC discussed previously, we can think that the Warburg effect would only be beneficial to a subset of CSC cells [22]. Indeed, slow-cycling CSCs being way less proliferative, they would be less dependent on the Warburg effect. It is coherent with the observation that, in melanoma for instance, mitochondrial oxidative-ATP-synthesis enzyme inhibition leads to the elimination of drug-resistant slow-cycling subpopulation [59]. The mitochondrial chain disruption by NP137 could potentially impair CSC viability and reduce their metabolic capabilities in our mCRC organoid cell lines.

The fact that NP137 affect mitochondrial chain components in our organoid cell lines may have other implications than energy metabolism. Indeed, recent research emphasizes the link between the expression of mitochondrial chain components and ECM function, highlighting the importance of mitochondrial function in preserving ECM composition, collagen content, and mechanical stability [61]. This study Following this logic, the impact of NP137 on mitochondrial chain components in our organoid cell lines could perturb cellular processes involved in ECM maintenance and cell adhesion.

These effects of Netrin-1 blockade on mCRC cells' cytoplasmic translation, respiration, and adhesion could directly or indirectly contribute to the effect observed on self-renewal. Further investigations are needed to fully apprehend the impact of these effects on self-renewal though.

e. Therapeutic implications

In this work, we also explored the potential therapeutic implications of targeting the Netrin-1/UNC5B pathway in mCRC using NP137, a therapeutical antibody. NP137 inhibits the interaction between Netrin-1 and UNC5B by binding Netrin-1 at the UNC5B-binding site, preventing fixation of Netrin-1 to its receptor [62]. Using a mouse model, we showed that tumours treated with NP137 alongside FOLFOX exhibited slower growth and smaller sizes compared to non-treated or FOLFOX-treated tumours, highlighting the high therapeutic potential of targeting the UNC5B/Netrin-1 pathway in mCRC and justifying further clinical studies. We should note that it is most likely that this effect we observed on tumour growth is not related to the effect of Netrin-1 that we observed on self-renewal. Indeed, the effect on tumour growth is probably linked to an effect on proliferation and/or apoptosis of tumour cells such as observed in breast and endometrial cancer [10, 63].

Although, we can note that RNAseq data from patients in NP137 phase A clinical trial, confirm a decrease of stemness-related (LGR5, PROM1, SOX4, SMOC2) genes expression in the only colon adenocarcinoma patient of the study, the tumour of this patient exhibiting Netrin-1 expression. This *in situ* effect of NP137, demonstrates that the targeting of Netrin-1 to modulate self-renewal of mCRC cells is still relevant in a clinical setting and may open the door for long-term beneficial effects of this treatment in the future.

Additionally, post-chemotherapy *in vitro* experiments demonstrated that Netrin-1 continues to regulate self-renewal after FOLFOX treatment, with NP137 significantly reducing post-treatment self-renewal. This significant effect of NP137 on self-renewal post-treatment suggests its potential as a post-chemotherapy treatment to prevent

recurrence in metastatic colorectal cancer. Further *in vivo* investigations are necessary to confirm the efficacy of NP137 in preventing recurrence and its potential as a therapeutic strategy in mCRC management. To design this experiment, we would either need to establish what is the ideal protocol of chemotherapy treatment (concentration and schedule) needed to observe tumoral recurrence of our mCRC organoid cell line in mice model or to use a different type of assay, such as an *In Vivo* ELDA after treatment of organoid cells with FOLFOX that would give us an indication on post-treatment self-renewal *In Vivo*, allowing us to draw conclusion on the therapeutical potential of NP137 to prevent tumour recurrence in mCRC patients.

Finally, the increased UNC5B expression and increased efficacy of NP137 in inhibiting self-renewal in FOLFOX-treated ySAW190LT organoids (*TP53* WT), suggests that CRC patients with tumours carrying wild-type *TP53* could particularly derive great benefit from NP137 treatment, concurrent with or following FOLFOX treatment.

These findings collectively underscore the relevance of targeting the Netrin-1/UNC5B pathway for the treatment of mCRC, with a particular advantage in combining NP137 treatment with common chemotherapies such as FOLFOX for a patient with *TP53* WT tumours.

f. Possible interference/synergies of Netrin-1/UNC5B with other DRs

In the last part of this project, our research extended to investigate the potential roles of another dependence receptor, NOTCH3, in the regulation of mCRC phenotypes and response to chemotherapy. NOTCH3 overexpression was observed during FOLFOX treatment, particularly in FOLFOX-resistant organoids, hinting at a potential role for this receptor in chemoresistance.

However, the impact of NOTCH3 invalidation on post-treatment self-renewal was different to our expectations. Indeed, NOTCH3 protein invalidation resulted in an increase in post-chemotherapy self-renewal capability in PIK315LT organoids, in apparent paradox with the previous literature concerning the relationship between NOTCH3 and stemness. Indeed, in colorectal cancer, NOTCH3 receptor activation was

shown to result in the overexpression of stemness markers such as MUSASHI-1 and ALDHA1, and inhibition of the NUMB protein in both colorectal cancer cell lines and primary cultures of colorectal cancer metastases [64-67]. This suggests that the role of NOTCH3 in colorectal CSCs is contextual and more ambiguous than suggested by previous studies. Moreover, this surprising result could be due to the dependence receptor characteristics of the NOTCH3 receptor, which is rarely taken into consideration in studies [8].

Finally, our exploration of NOTCH3 in relation to the UNC5B receptor and Netrin-1 highlighted some aspects of the relationship between these two pathways. This includes an apparent mutual exclusivity between the UNC5B and NOTCH3 dependence receptors and the fact that Netrin-1 inhibition leads to the decreased expression of the full-length NOTCH3 protein. It is not the first report of an interaction between Netrin-1 and the NOTCH pathway. Indeed, Netrin-1 was previously shown to form a complex with both NOTCH2 and Jagged-1, activating NOTCH signalling and subsequent glioblastoma cell invasion [68]. A similar interaction could occur in mCRC cells between Jagged-1, NOTCH3 and Netrin-1 and prevent NOTCH3 receptor internalization and/or degradation. This hypothesis is coherent with the fact that we don't see the downregulation of either NOTCH3 and JAG1 mRNA upon treatment with NP137 in our PIK315LT and ySAW190LT cell lines (Data not shown).

The fact that UNC5B deletion seems to induce an over-activation of the NOTCH3 receptor rather than induce its expression, suggests that UNC5B may have Netrin-1 independent effects on the NOTCH3 receptor and that the effect of UNC5B on NOTCH3 is not comparable to the one of a competitive inhibitor regarding Netrin-1. Overall, our study underscores the need for further investigations to fully understand the mechanisms and therapeutic implications of NOTCH3 in CRC, particularly in the context of chemotherapy response.

g. Conclusion & future directions

In summary, through our investigation of the UNC5B receptor and its ligand Netrin-1, we uncovered their significant roles in CRC phenotype modulation, stemness and self-renewal regulation, and provided evidence supporting their interest as potential therapeutic targets in metastatic CRC to improve patient outcomes.

Further research in this area could though allow us to consolidate our knowledge of dependence receptor importance for mCRC cells and to develop more effective strategies in managing CRC and overcoming challenges such as post-treatment recurrence.

This research might involve experiments on other patient-derived organoids to further understand the inter-patient heterogeneity of this pathway. We should also further confirm the therapeutic interest of targeting this pathway for the prevention of recurrence. To do so, as said earlier, a proper experiment investigating the effect of NP137 on tumour recurrence after FOLFOX treatment. Moreover, the analysis of the tumours using different techniques such as IHC or RNA sequencing at the end of treatment and at the time of recurrence would allow us to monitor CSC markers, their dynamics during relapse and the effect of NP137 on their expression.

Furthermore, the characterisation of the potential connection between the Netrin-1/UNC5B pathway and TFF3 is needed. Using recombinant TFF3 and a TFF3 inhibitor (AMPC) to revert effects observed on self-renewal and CSC apoptosis observed upon treatment with recombinant Netrin-1 and NP137.

Finally, some further investigations are needed to precise the exact relationships between the NOTCH3 receptor and NTN1/UNC5B. The experiments to be performed should include the observation of the HES1 transcriptional target protein levels upon NP137 treatment. The possible implications of the UNC5B receptor into this effect should also be assessed by performing the same experiment in CRISPR-Cas9 mediated UNC5B-invalidated organoids.

It would be interesting to investigate whether the communication between Netrin-1 and the Notch pathway involves a direct or indirect physical interaction. This could be performed by incubating organoids with His-tagged Netrin-1, followed by immunoprecipitation of the tagged Netrin-1 and mass-spectrophotometry-mediated

analysis of co-immunoprecipitated proteins to observe if this effect may be linked to direct binding of Netrin-1 to the NOTCH3 receptors or to one of its partners.

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