Author/s:
Vijayakumaran, Reshma

Title:
Exploration of novel regulators of mutant p53 in cancer cells: a role for NDFIP1

Date:
2019

Persistent Link:
https://hdl.handle.net/11343/227061

Exploration of novel regulators of mutant p53 in cancer cells: a role for NDFIP1

Reshma Vijayakumaran

ORCID ID: 0000-0002-1663-3940

Submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

April 2019

Sir Peter MacCallum Department of Oncology
&
Faculty of Medicine, Dentistry & Health Sciences,
The University of Melbourne

Abstract

Mutations in the tumour suppressor gene, *TP53* occur in more than 50% of human cancers. Mutant p53 proteins not only lose their tumour suppressive capacities, but also gain oncogenic functions, broadly referred to as gain of function (GOF). Cancer cells frequently accumulate mutant p53 and may become addicted to this protein for their survival. During development, mutant p53, like its wild-type counterpart, is inherently labile, however in cancer cells mutant p53 frequently accumulates. In part, this is due to the interrupted auto-regulatory loop with MDM2. However, MDM2 alone cannot explain the stability of mutant p53 in many cancer contexts. We therefore argued that additional factors are responsible for its stability in cancer cells. In order to identify the major players in the regulation of mutant p53, we performed a high-throughput RNAi screen through which we evaluated 18,120 genes for their effects on mutant p53 levels in two different mutant p53 expressing cell lines. Based on network analyses, pathway analyses and extensive literature mining, we selected 37 candidate genes to be validated through p53 immunoblotting. From the validated genes, we chose to investigate an E3 ligase adaptor protein, NDFIP1.

NEDD4 family interacting protein-1 can influence the ubiquitination of several substrates of NEDD4 E3 ligases and thereby alter their stability and localization. Our original findings identified that NDFIP1 may interact with mutant p53 and is capable of regulating its protein expression but not its mRNA expression. We observed reduced mutant p53 ubiquitination in NDFIP1 knockout mouse embryonic fibroblasts, indicating a facilitating role for NDFIP1 in mutant p53 ubiquitination. Importantly, NDFIP1-mediated ubiquitination did not result in increased destruction of mutant p53 in the proteasome. In contrast, upon NDFIP1 overexpression, we observed increased secretion of mutant p53 in exosomes. We found physical, spatial and biochemical interactions between NDFIP1 and mutant p53. For the first time, we demonstrated that mutant p53 could be transferred inter-cellularly by exosomes. We investigated the impact of the intake of mutant p53 by recipient cells. This analyses revealed that recipient cells exhibited higher invasive capacity and an upregulation of mutant p53 target genes; indicating conferral of mutant p53 GOFs from donor to recipient cells.

Our results highlight a previously unknown mode of mutant p53 regulation in cancer cells. Future studies will explore in greater depth the functional consequences of NDFIP1-mutant p53 interaction for the dissemination of oncogenic activities of mutant p53. Excitingly, these studies expose new vulnerabilities for therapeutic intervention and these opportunities for targeting aggressive cancers with mutant p53 will also be the focus of ongoing research.

Declaration

This is to certify that:

(i) The thesis comprises of only my original work towards the PhD except where indicated in preface sections.

(ii) Due acknowledgement has been made in the text to all other material used.

(iii) The thesis is fewer than 80,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Reshma Vijayakumaran

Preface

This work was done under the supervision of Prof. Ygal Haupt, Dr. Sue Haupt and A/Prof. Jason Howitt.

All additional and/or technical contributions are duly noted in the prefaces of chapters 3, 4 and 5.

Publications arising from this thesis/during the candidature:

1. Haupt S, Hernandez OM, **Vijayakumaran R**, Keam S, Haupt Y. The long and the short of it: the MDM4 tail so far. J Mol Cell Biol. 2019.

2. Haupt S, **Vijayakumaran R**, Miranda PJ, Burgess A, Lim E, Haupt Y. The role of MDM2 and MDM4 in breast cancer development and prevention. J Mol Cell Biol. 2017;9(1):53-61.

3. Miranda PJ, Buckley D, Raghu D, Pang JB, Takano EA, **Vijayakumaran R**, Teunisse A, Posner A, Procter T, Herold MJ, Gamell C, Marine JC, Fox SB, Jochemsen A, Haupt S, Haupt Y. MDM4 is a rational target for treating breast cancers with mutant p53. J Pathol. 2017;241(5):661-70.

4. **Vijayakumaran R**, Tan KH, Miranda PJ, Haupt S, Haupt Y. Regulation of Mutant p53 Protein Expression. Front Oncol. 2015; 5:284.

Manuscript under preparation:
“NDFIP1 promotes exosomal release of mutant p53.”

Acknowledgements

It has been four exciting years and I have a great many people to thank for all the support during the course of my PhD.

This thesis would not have been possible without the guidance and vision of my supervisors. I really appreciate and have enjoyed the extensive meetings that helped shape this project and my aptitude for research. Firstly, I would like to express my sincere gratitude to my primary supervisor, Prof. Ygal Haupt, for giving me the opportunity to work in his lab. His persistent approach to break down complex problems has been a great learning experience specially while traversing through unexpected data. I also thank him for giving me the right amount of independence in exploring the project and steering me in the right direction. I would like to thank my co-supervisor, Dr. Sue Haupt, who has been immensely helpful during the course of this project. Her suggestions and scientific rigor have driven me in perfecting both my experimental protocols and writing. I would also like to thank my co-supervisor, A/Prof. Jason Howitt for being a great supervisor and mentor, whose infectious passion for research continues to inspire me.

I thank my doctoral committee members, Prof. Kieran Harvey, A/Prof. Kaylene Simpson, Dr. Mark Devlin, Dr. Clare Fedele and Dr. Jian Kang for their valuable suggestions and support through the years. Many thanks to my mentor, Dr. Joep Vissers, for always looking out for me. His honest opinions and advice helped me navigate the ups and downs of research. Also thanks to my student coordinator, Dr. Caroline Owen and Niranga Nawagamuwa for the support and for shielding me from the administrative side of PhD.

My sincere thanks to all members of the Tumour suppression lab (both past and present) – Dr. Cristina Gamell, Dr. Simon Keam, Kah Hin, Franco, Twishi, Dinesh, Jeffreena, Tahlia, Dan, Piotr, Atara, Octavio, Arielle, Ivona, Cassie, Arjelle, Clelia, Emma, Don, James, Abi, Jess for their support, feedback and friendship. Special thanks to Kah Hin, Franco, Tahlia, Arielle, Octavio, Cristina and Simon for their help

with many aspects of my PhD project. Many thanks to Michelle Tang (Howitt Lab, Florey) for training me on exosome related protocols and for the chats during those hour long spins.

My sincere thanks to our collaborators – A/Prof. Marco Herold (WEHI), Prof. Sharad Kumar and Dr. Natalie Foot (Centre for cancer biology, UNISA) for sharing valuable reagents. I would like to thank Dr. Iva Nikolic (VCFG) for her valuable advice in generating CRISPR knockouts. I am also grateful to Sam, Lucas, Aparna, Suraya, Shaun, Swati, Maz, Susan, Jessica, Anje, Elena, Suraya, Tinaz and Aesha for imparting valuable technical advice.

I would like to thank all members of the FACS facility (Viki, Sophie, Ralph), Microscopy and Histology (Chad, Jill, Metta, Cameron), VCFG (A/Prof. Kaylene Simpson, Dan Thomas, Jennii Lu, Laura), animal facility (Lauren, Kat and Mick), media kitchen (Graham, Lara), lab managers (Jason, Andrew) and Paul Dunne for their help and support throughout my PhD.

Thanks to many researchers at the PeterMac - A/Prof. Phil Darcy, Dr. Nicole Haynes, Prof. Sarah Jane Dawson, Dr. Louise Cheng, Dr. Rita Busuttil, A/Prof. Sarah Ellis, Dr. Nicholas Clemons, Dr. Carleen Cullinane, Prof. Rob Ramsay, Dr. Keefe Chan, Dr. Francesca Froldi, Dr. Gisela Mir Arnau, Elena Takano for their advice at different phases of my PhD.

I thank the University of Melbourne and Australia India Institute for the Melbourne International Fee Remission Scholarship (MIFRS) and the Victoria India Doctoral Scholarship (VIDS).

Many thanks to friends (Shatha, Kat, Na, AJ, Shirley, Jonathan, Carole, Balaji, Fiona, Han, Michael Neeson, Hannah, Talhah, Em, Kevin, Ruby, Paz, Dani, Jeanne, Ash, Michael Yates, Kenji) at the PeterMac for their support.

I thank my wonderful housemates, Sneha and Gitansh who have since become my best friends, for being there during the best and worst of times. Our cats, Chester and Gordy have been great thesis writing buddies! Thanks to all my friends back

home especially, Pavitra, Anbu, Satya, Rehna, Raghul, Teja, Indu Jose, Sunny, Hoori, Sindhu, Ruby and many others who still keep in touch despite the time difference.

Last but not the least, I am indebted to my parents and my sister, Rohini for being an endless source of encouragement and for always supporting my decisions. Thank you! I couldn't have made this far without their unconditional love and emotional support.

Abbreviations

17AAG	17-(Allylamino)-17-demethoxygeldanamycin
ABRO1	Abraxas brother 1
ANAPC1	Anaphase-promoting complex subunit 1
APC/C	Anaphase promoting complex/Cyclosome
APP/PS1	Amyloid precursor protein/Presenilin 1
ASAH1	N-Acylsphingosine Amidohydrolase
ATG5	Autophagy related 5
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia And Rad3-Related Protein
AURKB	Aurora Kinase B
Bag1	BCL2 Associated Athanogene 1
Bag2	BCL2 Associated Athanogene 2
BiFC	Bimolecular fluorescent complementation
BMDC	Bone marrow derived cells
BRAT1	BRCA1 Associated ATM Activator 1
BRCA1/2	Breast cancer type 1 susceptibility protein
BSA	Bovine serum albumin
Bsd2p	Bypass SOD defects protein 2
CAF	Cancer associated fibroblasts
CDK	Cyclin Dependent Kinase
CERK	Ceramide kinase
CHIP	C-terminus of HSC70-interacting protein
CHK1/2	Checkpoint kinase 1/2
CHX	Cycloheximide
CKs1	CDK subunit 1
COP1	Constitutive Photomorphogenesis Protein 1 Homolog
CRISPR	Clustered regularly interspaced short palindromic repeats
ctDNA	Circulating tumour DNA
CycA	Cyclin A
CycB2	Cyclin B2
DAPI	4′,6-diamidino-2-phenylindole
DAVID	Database for Annotation, Visualization and Integrated Discovery
DBC1	Deleted in breast cancer 1
DBD	DNA binding domain
DEPC	Diethyl pyrocarbonate
DLEC1	Deleted In Lung And Esophageal Cancer Protein 1
DMEM	Dulbecco's Modified Eagle media
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DNAJA1	DnaJ Heat Shock Protein Family (Hsp40) Member A1
DNAJB1	DnaJ Heat Shock Protein Family (Hsp40) Member B1
DNAJB6	DnaJ Heat Shock Protein Family (Hsp40) Member B6
dNTP	Deoxy nucleotide
Dox	Doxycycline
DUB	Deubiquitinase

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
ERK1/2	Extracellular signal regulated kinase 1/2
ESCRT	Endosomal sorting complex required for transport
FACS	Fluorescence activated cell sorting
FBS	Feta bovine serum
GFP	Green fluorescent protein
GOF	Gain of function
H3K9	Histone 3 Lysine 9
HBV	Hepatitis B virus
HDAC6	Histone deacetylase 6
HDAC8	Histone deacetylase 8
HECT	Homologous to E6-AP carboxyl terminus
HEK293	Human embryonic kidney cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human Epidermal Growth Factor Receptor 2
hERG	Human ether-a-go-go-related gene
HI	Hypoxia-ischemia
Hop	Hsp70-Hsp90 organizing protein
HoxA5	Homeobox protein A5
HPV	Human papillomavirus
Hsc70	Heat shock cognate 71 kDa protein
HSF1	Heat shock transcription factor 1
Hsp40	Heat shock protein 40
Hsp70	Heat shock protein 70
Hsp90	Heat shock protein 90
K63	Lysine-63
KEGG	Kyoto Encyclopedia of Genes and Genomes
KO	Knockout
LOH	Loss of heterozygosity
LPP1	Lipid phosphate phosphatase 1
Lyn	Lck/Yes novel tyrosine kinase
Lys-63	Lysine 63
MAP2K4	Mitogen-Activated Protein Kinase Kinase 4
MAPK1	Mitogen-Activated Protein Kinase 1
MAVS	Mitochondrial Antiviral Signaling Protein
MDM2	Mouse double minute 2
MDM4	Mouse double minute 4
MEF	Mouse embryonic fibroblasts
miRNA	microRNA
MK2	MAP kinase-activated protein kinase 2
MLL1	Mixed lineage leukemia protein-1
MMPs	Matrix metalloproteinases
MOZ	Monocytic leukemia zinc finger protein
mRNA	messenger RNA
mTOR	Mammalian target of Rapamycin

MVB	Multivesicular body
NDFIP1	NEDD4 family interacting protein-1
NDFIP2	NEDD4 family interacting protein 2
NEDD4	Neural Precursor Cell Expressed, Developmentally Down-Regulated 4
NEDD4L	Neural Precursor Cell Expressed, Developmentally Down-Regulated 4L
NF-Y	Nuclear transcription factor Y subunit alpha
NF-κB	Nuclear Factor-κB
NGS	Next-Generation sequencing
NQO1	NAD(P)H Quinone Dehydrogenase 1
NSG	NOD-SCID IL-2RγKO
ORF	Open reading frame
OTP	On-target control
pAKT	phospho-AKT
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFRβ	Platelet Derived Growth Factor Receptor β
PEI	Polyethyleneimine
Pirh2	P53-induced RING H2 protein
PLK1	Polo like kinase 1
PML	Promyelocytic leukemia protein
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
PR	Proline rich domain
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modifications
PVDF	Polyvinylidene fluoride
R175H	Arginine 175 Histidine
Rb1	Retinoblastoma transcriptional corepressor 1
RE	Response elements
RFP	Red Fluorescent protein
Rho	Ras homologous protein
RING	Really interesting new gene domain
RIPA	Radioimmunoprecipitation assay
RNAi	RNA interference
ROCK	Rho-associated Coiled-coil Kinase
RPL26	Ribosomal protein L26
RPMI	Roswell Park Memorial Insitute
RPS27	Ribosomal protein S27
RT	Room temperature
SAHA	Suberoylanilide Hydroxamic Acid
SDS	Sodium dodceyl sulphate
Ser-15	Serine-15
SETDB1	SET domain bifurcated 1
shRNA	Short hairpin RNA
SIRT1	Sirtuin 1
SLC4A7	Solute Carrier Family 4 Member 7
SMAD	Mothers against decapentaplegic homolog 2
SMURF1/2	SMAD Specific E3 Ubiquitin Protein Ligase 1/2
Sp1	Specificity protein 1

SREBP	Sterol regulatory element-binding protein
TA	Transactivation domain
TCGA	The Cancer Genome Atlas
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor β1
TIMP1-4	Tissue inhibitor of metalloproteinase 1-4
TNBC	Triple Negative Breast Cancer
TNF-α	Tumour necrosis factor-α
TRE	Tet response element
Treg	Regulatory T cells
TRIM32	Tripartite Motif containing 2
Trk	Tropomyosin receptor kinase
Tsg101	Tumor susceptibility gene 101
Ub	Ubiquitin
ULK1	Unc-51 like autophagy activating kinase 1
USP10	Ubiquitin specific peptidase 10
USP7	Ubiquitin specific peptidase 7
UTR	Untranslated region
VDR	Vitamin D receptor
WWP2	WW domain containing E3 ubiquitin protein ligase 2
XRCC6BP1	XRCC6-Binding Protein 1

Table of Contents

List of figures

List of tables

Chapter 1

Literature review

1.1 Cancer

Cancer is a disease characterized by dysregulated cell growth associated with cell 'immortalization'. In healthy cells, multiple checkpoints are in place to prevent uncontrolled division. Disruption of these normal systems is a serious risk for cancer development. Perturbations of such regulatory systems commonly arise from DNA mutations or viral infection, which may debilitate tumour suppressors and activate oncogenes. Mutations in DNA can be caused by environmental factors (such as carcinogens and irradiation), heredity or by random errors. Cancers have different characteristics (such as cell of origin, location, tumour size, lymph node involvement, metastasis) and these have been exploited for their classification [1]. With the advancement in sequencing technologies, molecular characteristics are now being used for classification of cancer patients based on even subtle variations, which are now being identified and exploited in the clinic, in the growing field of Precision Medicine [2].

1.1.1 Breast cancer - subtypes

Breast cancer is the most common cause of cancer among women in many countries. While the risk factors for familial breast cancer can be attributed to mutations in *BRCA1/2* or *TP53* (Li-Fraumeni syndrome), other risk factors such as reproductive factors, alcohol use, obesity and physical inactivity have been identified as a cause of overall breast cancer burden (World Health Organization https://www.who.int/cancer/prevention/diagnosis-screening/breast-cancer/en/). In addition to tumour size, grade and nodal involvement, the presence of markers such as Estrogen receptor (ER), Progesterone receptor (PR) and Human Epidermal Growth Factor Receptor 2 (HER2) have been used to classify the different types of

breast cancer. Based on the expression of markers, breast cancer is subdivided into 5 types – luminal A, luminal B, HER2 overexpressing, basal and normal-like (Table 1.1). The incidence and outcomes of each of the subtypes vary, as do their response to therapy. Tumours of the basal subtype are most likely to have p53 mutations and are very aggressive [3] [4].

Subtype	IHC status	Mutation prevalence – p53
Luminal A	ER+ PR+ HER2- KI67-	13%
Luminal B	ER+ PR+ HER2- KI67+ ER+ PR+ HER2+ KI67-	40%
HER2 overexpressing	ER- PR- HER2+	71%
Basal	ER- PR- HER2-	82%
Normal-like	ER+ PR+ HER2- KI67-	33%

Table 1.1: Table showing molecular subtypes of breast cancer.

The molecular subtypes of breast cancer and the presence of Estrogen receptor (ER), Progesterone receptor (PR) and Human Epidermal growth factor receptor 2 (HER2) in each of the subtypes and the proliferation marker Ki67. This table was made based on the data from [3, 5].

1.1.2 Current therapies for breast cancer

Breast cancer survival rates are very highly dependent on early detection and intervention, and as with other cancers, it is not the primary tumour but the metastasis that causes death [6]. Chemotherapy is the first line treatment adopted for most breast cancers, however the specific drug of choice depends on the molecular subtype. The first monoclonal antibody against cancers, Trastuzumab (against HER2) specifically targets HER2 expressing cells and is an effective treatment for many patients with amplified HER2+ breast cancers. Up to 50% of these patients however exhibit resistance within the first 10 months of therapy, which has prompted intensive international searches for new effective treatments [7].

The triple negative breast cancer (TNBC) subtype falls under the basal type of breast cancers exhibiting high rate of p53 mutations (mentioned in Section 1.1.1). Cancers of this subtype do not express ER, PR or HER2 and tend to be very responsive to

standard chemotherapies early in disease development, but is prone to relapse with metastatic, drug recalcitrant disease [8]. With the advances in next-generation sequencing (NGS), the implementation of personalized cancer medicine has been very useful in tailoring cancer treatments. Recently, circulating tumour cells and DNA (ctDNA) have been employed to monitor the success of chemotherapy [9]. Despite these advances, the limited repertoire of efficacious drugs toward many of these targets remains the major restriction of therapeutic success in these breast cancers.

1.2 Tumour suppressor, p53

Early studies on cancer initiation and progression revealed two broad classes of genes – tumour suppressors and oncogenes, as important factors in uncontrolled cell proliferation. Tumour suppressor proteins typically function in regulating the cell cycle and triggering cell death (apoptosis). P53 is the second tumour suppressor to be identified and its 1979 discovery arrived eight years after the report of the prototype, *Retinoblastoma Transcriptional Corepressor 1* (*Rb1)*; a negative regulator of the cell cycle, whose inherited mutations are founders for the hereditary eye cancer, retinoblastoma [10]. Originally however, p53 was thought to be an oncogene working with SV40 virus in cell transformation, but in 1989, ten years after the discovery of the gene, the normal function of p53 in cells was identified to be suppression of cell growth [11]. The primary role of p53 is that of a transcription factor whose target genes limit cell proliferation upon various stimuli. P53 has a central DNA binding domain, an N-terminal transactivation domain, a Proline rich domain, an oligomerization domain and a C-terminal regulatory domain (Fig 1.1). The oligomerization domain facilitates p53 tetramerization which is critical for its DNA binding functions. On the other hand, the N-terminal transactivation domain aids recruitment of various chromatin modifying factors which in turn can activate gene expression. Post-translational modifications to the C-terminal regulatory domain can alter the stability and activity of p53 [12]. In cancer, various alterations in p53 domains result in loss of some or all of its tumour suppressive capacities and gain of new, oncogenic functions commonly termed as GOF[13].

1.2.1 Functions of p53 are lost in cancer

Several years of research have revealed that wild type p53 (hereafter, wild type p53 will be referred to as p53) is a stress sensing protein and DNA damage is the most studied trigger in the context of cancer. But many studies have shown that beyond radiation related DNA damage, stress conditions affecting the oxidative state of a cell such as hypoxia, metabolic stress, ribosome dysfunction, and oncogene expression can also lead to activation of p53 [14]. In a normal, non-stressed cell, p53 is kept labile, under the influence of its major E3-ligase MDM2. Upon exposure to stimuli such as radiation that directly causes DNA damage, ATM/ATR kinases phosphorylate p53 thereby protecting it from degradation. Stabilized p53 transactivates its key transcriptional targets including p21, an inhibitor of Cyclin Dependent Kinases (CDKs), which delays cell cycle progress to allow DNA repair. When DNA damage is irreparable, there is continual p53 stabilization, which results in induction of apoptotic genes goading the faulty cells to die. In addition to cell cycle arrest and apoptosis, p53 can affect many pathways depending upon the cell type and the type of stress signal. Alternatively, p53 can induce the cells to enter a senescent state, which is an important tumour suppressive mechanism [15, 16].

Besides tumour suppressive functions, p53 target genes are also involved in regulating metabolism, oxidative state of cells and restriction of stem cell state [17, 18]. In the nucleus, p53 binds to specific response elements (RE) on the DNA and recruits factors for the activation of its target genes [14]. In addition to nuclear functions as a transcription factor, p53 can influence apoptosis and autophagy in the cytoplasm [19]. P53 mediated apoptotic response, although important to curb malignant cells from dividing, is absolutely disadvantageous in neurological disorders such as Alzheimer’s and Parkinson’s and in the cases of stroke and myocardial infarctions [19, 20], where vital cell function can be lost with diabolical consequences.

1.2.2 Regulation of wild type p53 is disrupted in cancer

Basal levels of p53 are very low in cells unexposed to adverse signals. P53 protein levels spike when cells are exposed to stress related events. If the stress signal is short-lived, cells recover during which time p53 levels relapse to basal levels. The set of events in regulating p53 levels has been widely studied and multiple factors have been identified in this process. Low, basal levels of p53 protein are maintained by its constant ubiquitination which is catalyzed by MDM2, which promotes proteasomal degradation of this key substrate [21, 22]. Stress signals can activate proteins that post-translationally modify p53, protecting it from MDM2 mediated ubiquitination. P53 can also transactivate the MDM2 promoter, causing an increase in MDM2 mRNA levels, which in turn forms a negative feedback loop and brings p53 back to basal levels [23, 24]. In fact, loss of p53 can reverse the deleterious effects of MDM2 loss in mice [25].

Full length p53 protein is subjected to extensive post-translational modifications at multiple residues, involving phosphorylation, ubiquitination, sumoylation and acetylation by different proteins, which can either cause activation or repression of its function. In general, phosphorylation of the p53 DNA binding domain is an activation signal and can result in upregulation of p53 target genes. A host of phosphatases and deubiquitinases can reverse these modifications of p53, which allows for its recovery from activation, post-stress [26]. In tumour cells with p53, its function is frequently negated, either through inactivation caused by viral proteins (such as E6 of HPV in cervical cancers or HBx in HBV induced HCC) or by amplification of its negative regulators, notably MDM2 [27, 28]. However, in multiple cancers, *TP53* is mutated.

1.3 Mutant p53

TP53 mutations are the major cause of a hereditary syndrome called Li-Fraumeni syndrome (confirmed in ~70% of families), which is named after the physicians, Frederick Pei Li and Joseph F. Fraumeni, Jr. who described it for the first time [29]. Patients with this syndrome exhibit extremely high predisposition to developing cancers, predominantly breast cancers (up to 80% in females) and sarcomas (19.7%) [30-32]. Mutations in *TP53* gene are found in almost 50% of all cancers

(non-hereditary cancers). Loss of heterozygosity of *TP53* in combination with inactivating/activating mutations in the gene contribute to many cancers: brain tumours, breast, colon/rectum, esophageal, liver, lung carcinomas; sarcomas; leukemias and lymphomas [33]. The majority of mutations are missense and frequently confer aggressive properties on the tumour, which reflects negatively on patient prognosis [34]. As will be discussed in section 1.3.2, this is because of acquisition of new oncogenic (neomorphic) properties commonly referred to as gain of function (GOF) by mutant p53 (mt-p53) due to higher stability and new interactions. Tumour cells seem to be selected for specific pathogenic mutations which give them an advantage to survive in the tumour microenvironment by tailoring its metabolic and other adaptations [35].

1.3.1 Types of p53 mutants

Mutations in p53 have been documented in almost every domain, however some mutations are highly deleterious compared to others, due to their destabilizing impact and capacity to confer oncogenic activity. The most common mutations called the hotspot mutants are found to occur in the central DBD (R175, G245, R248, R249, R273, R282) (Fig 1.1) [33]. In general, mutations in p53 are grouped into – conformational mutants and contact mutants. While contact mutants (R248, R273) affect amino acids directly involved in binding to DNA, conformational mutants (R175H) are characterized by substitutions in amino acids leading to stabilized tertiary structure of the protein due to alteration in the conformation [36]. In both cases, DNA binding and therefore activation of target genes are severely compromised [37]. These mutant proteins interact with p53, but the resulting tetramer is non-functional causing a dominant negative effect in heterozygous conditions. Hence, a single hit (mutation in one of the p53 alleles) is sufficient to release cells from growth inhibition and is a classic example of dominant negative effect [36].

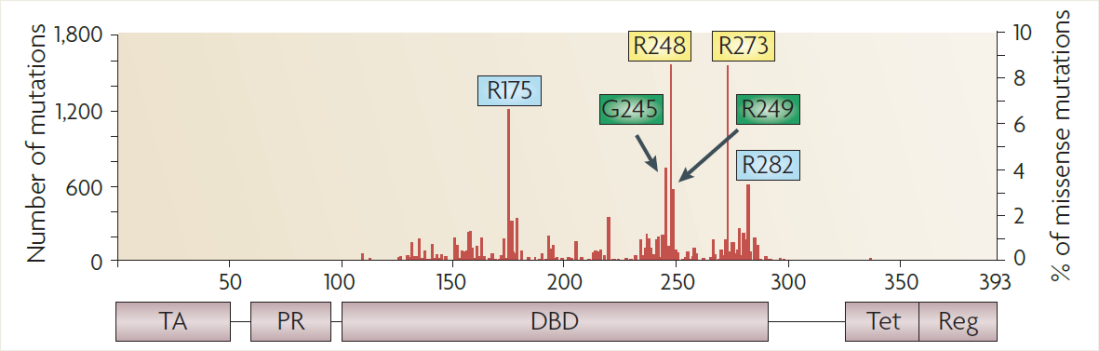


Figure 1.1: Hot-spot mutations found in the DNA binding domain of p53.
P53 has an N-terminal transactivation domain (TA), proline rich domain (PR), a central DNA binding domain (DBD) and a C-terminal tetramerization (Tet) and regulatory domain (Reg). (Image from [38]).

1.3.2 Mutant p53 dependent oncogenesis

Mt-p53 exhibits several characteristics favouring cancer growth and progression and these functions, which are independent of p53, are collectively termed as Gain of Function (GOF). GOF hypothesis was formulated from the observation of cellular changes upon overexpression of mt-p53 in a TP53 null setting or in mouse models. Also, mouse models have been particularly helpful in studying the manifestation of the disease. The loss or inactivation of p53 in cancer essentially removes a roadblock in the conversion of a normal cell to a transformed cell and exposes the chromosome of the cell to more DNA damage and therefore clones of cells with most favourable mutations divide and form tumours. Importantly however, while p53 knockout and p53 mutant (R172H mutation) mice have similar disease profiles; mt-p53 mice show increased metastasis compared with mice lacking p53 [39, 40].

Mutations in the DNA binding domain do not affect the N-terminal transcriptional activation domain and this domain can recruit DNA modifying factors. Notably, mt-p53 (especially the hotspot mutants, R273H and R175H) by partnering with other transcription factors such as NF-Y, VDR, SREBP, Sp1, Ets-2 is able to modify the transcription of their target genes [37]. This results in changes in the transcriptional landscape. Another major gain of function effect of mt-p53 is in the transformation of a tumour cell to a more aggressive and invasive phenotype. Previous studies have shown that this transformation occurs through multiple pathways. While some of this is due to mt-p53-TGFβ-SMAD pathway affecting p63 function, mt-p53 is also known

to upregulate components of the Rho/ROCK signaling pathway, enhance NF-kB activation by TNF-α and other pathways of cell invasion such as EGFR and integrin recycling [41, 42]. Cooperation of mt-p53 (R172H) with PML loss has also been observed in driving cancer progression [43].

In addition to effects on cell cycle regulation and cell migration, mt-p53 targets can increase drug resistance, angiogenesis, genomic instability, stem cell de-differerentiation, anchorage independent growth, EMT (epithelial mesenchymal transition), increased colony formation, mammary architecture disruption [44]. Studies in certain cell line models with mt-p53 knockdown have revealed an addiction to mt-p53 for continued growth. Also, knockdown of mt-p53 can reverse some of the aggressive phenotypes of malignant cell lines [45]. This addiction to mt-p53 is reminiscent of oncogene (Myc/Ras) addiction and therefore, mt-p53 has been aptly described as the guardian of the cancer cell [46, 47]. Mt-p53 can also affect the secretome and thereby modify cells in the tumour microenvironment to provide continued support and sustenance favouring tumor growth [48]. This aspect of mt-p53 GOF is discussed in detail in section 1.6.1.

It is well known that mt-p53 accumulates to high levels in cancer cell lines and tumour tissues and the oncogenic functions of mt-p53 are very much dependent on its expression levels [49, 50]. Few mechanisms have been identified through which mt-p53 stabilization occurs in cancer cells. In the next section, we discuss the known regulators of mt-p53, which play a role in either stabilizing the protein or degrading it.

1.4 Regulation of mutant p53

Mt-p53 regulation has been studied in parallel with the control mechanisms governing of p53 protein levels and many similarities in pathways and protein partners are evident. Mouse models of mt-p53 have been valuable in comparing wild type and mt-p53 regulation and it was a great surprise to find that during development, mt-p53, like its wild type counterpart exists inherently as a labile protein at low levels [39, 51]. Signals that stabilize p53 such as exposure to γ radiation, Doxorubicin, oncogenic stress (Ras/Myc) and loss of p16INK4a also

stabilize mt-p53, revealing that the modes of stabilization of mutant and wild type p53 are essentially the same [52]. However, two distinctions exist that cause variations in wild type and mt-p53 regulation: Firstly, a complete/partial lack of DNA binding ability of most p53 mutants leading to non-functional negative feedback loops. Secondly, conformational changes in mt-p53 result in new interaction partners and post-translational modifications protecting mt-p53 from degradation. The continued stabilization of mt-p53 observed in cancer cells is markedly different from p53 relapse to low levels in normal cells and this stability is found to be a consequence of degradation defects as will be explained in section 1.4.2 (Fig 1.2) (reviewed in [53, 54]).

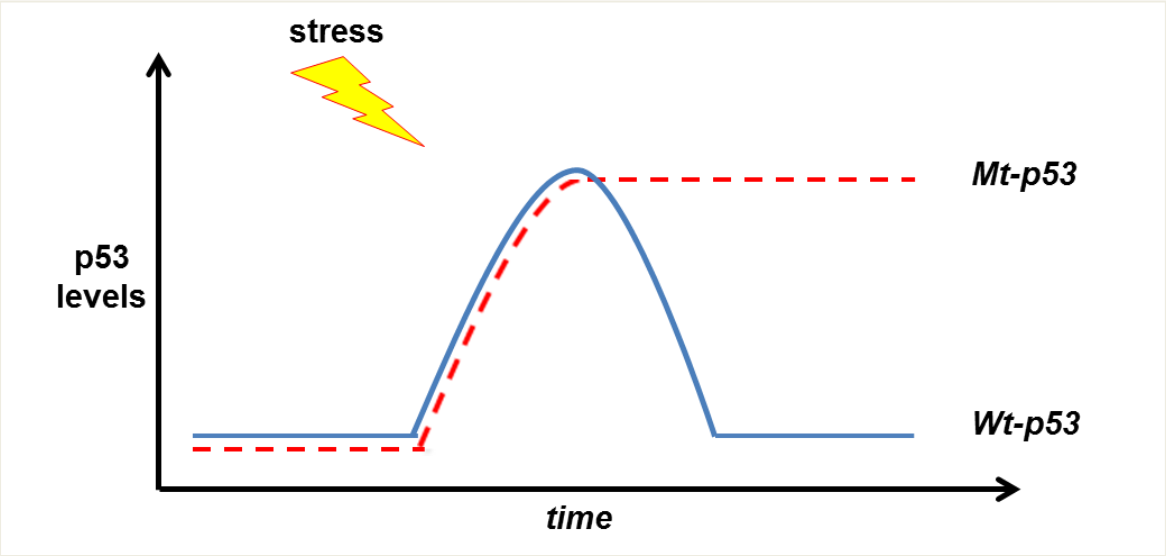


Figure 1.2: Graph depicting the levels of wild type and mt-p53 with respect to time.
Both wild type (wt-p53) and mt-p53 proteins are originally labile and are stabilized in a similar manner by the same stress stimuli. P53 protein levels reach a saturation state and then reduce to basal levels. However, mt-p53 remains stabilized and accumulates in cancer cells.

1.4.1 Transcriptional controls

The stabilization of mt-p53 that is observed in cancer cells has been mainly studied with respect to its protein level; however a number of mechanisms that operate at the promoter and RNA levels have also been observed. TGF-β1 can attenuate wild type and mt-p53 levels by controlling both transcription and translation [55]. HDAC8 can increase mt-p53 transcription through HoxA5 and so, treatment of cells with

HDAC inhibitors can reduce mt-p53 levels [56]. But these controls that act on the promoter level cannot distinguish between wild type and mutant.

Post transcription of *TP53* gene, regulation can take place at the mRNA levels by microRNAs or protein factors. While ribosomal protein, RPL26 can bind to 5' UTR of *TP53* mRNA and enhance translation, Nucleolin can adversely affect translation [57]. MicroRNAs are short RNA molecules (18-24 nucleotide long) that can reduce mRNA stability and negatively affect translation. miRNAs cannot differentiate between wild type and mt-p53, therefore miRNAs that target p53 directly can also target the mutant. In addition, miRNAs that target regulators of mt-p53 can also indirectly affect mt-p53 protein levels (reviewed in [53]). However, not much is known about the extent of control miRNAs have on mt-p53. Conversely, the effect of mt-p53 on miRNA biogenesis and processing is well documented and modulation of this pathway results in a mt-p53 specific miRNA signature which further contributes to its GOF [58-60].

1.4.2 E3 ligases

Much of the spatial and temporal regulation of wild type and mutant p53 takes place at the post-transcriptional level through ubiquitination by E3 ligases.

Ubiquitin dependent proteasomal degradation:

The covalent addition of ubiquitin polypeptide to the lysine residues of a protein is the result of a sequential process of reactions involving 3 enzymes E1, E2 and E3, the E3 enzyme being specific for substrates [61, 62]. Proteins tagged with polyubiquitin particularly Lys-48 chains are targeted for degradation by the 26S proteasome machinery [63]. Almost 19 E3 ligases are known to ubiquitinate p53, while the E3 ligases known to directly ubiquitinate mt-p53 are MDM2, Pirh2, COP1 and CHIP [64].

MDM2:

MDM2, which is the cognate E3 ubiquitin ligase of p53, can also ubiquitinate mt-p53 and cause its degradation [52]. However, mt-p53 is unable to induce MDM2 transcription and does not form a negative feedback loop [65]. Therefore, while p53

returns to low levels under the influence of MDM2, mt-p53 remains stabilized. However, upon overexpression of MDM2, mt-p53 can be ubiquitinated and targeted for degradation [21]. Interaction of MDM2 with multiple domains of p53, allows successful ubiquitination of different p53 mutants, even though the efficiency of ubiquitination is much lower when compared to the wild type (Fig. 1.3) [66-68]. In addition, a number of proteins including chaperones interact with mt-p53 and protect it from degradation and these are discussed in the forthcoming sections.

Effect of chaperones:

A large part of mt-p53 stability can be attributed to chaperones. CHIP (C-terminus of Hsc-70 interacting protein) is a U-box domain containing E3 ligase, which selectively ubiquitinates incorrectly folded/unfolded proteins that also interact with chaperones Hsp90 and/or Hsp70. Many studies have shown that mt-p53 forms stable complexes with Hsp90, which offers protection from both MDM2 and CHIP mediated ubiquitination, in contrast to p53, which is not bound or protected by this chaperone [69-71]. Inhibition of Hsp90 by 17AAG or knockdown of its upstream transcriptional activator, HSF1 results in an increase in mt-p53 ubiquitination and a further decrease in mt-p53 protein levels (Fig. 1.3) [71, 72]. Similarly, inhibition of HDAC6, a deacetylase and positive regulator of Hsp90, also results in mt-p53 degradation [73, 74]. In fact, Ganetespib and AUY-922, both inhibitors of Hsp90 have entered clinical trials for a number of cancers and have shown potency in a mt-p53 setting [47, 75].

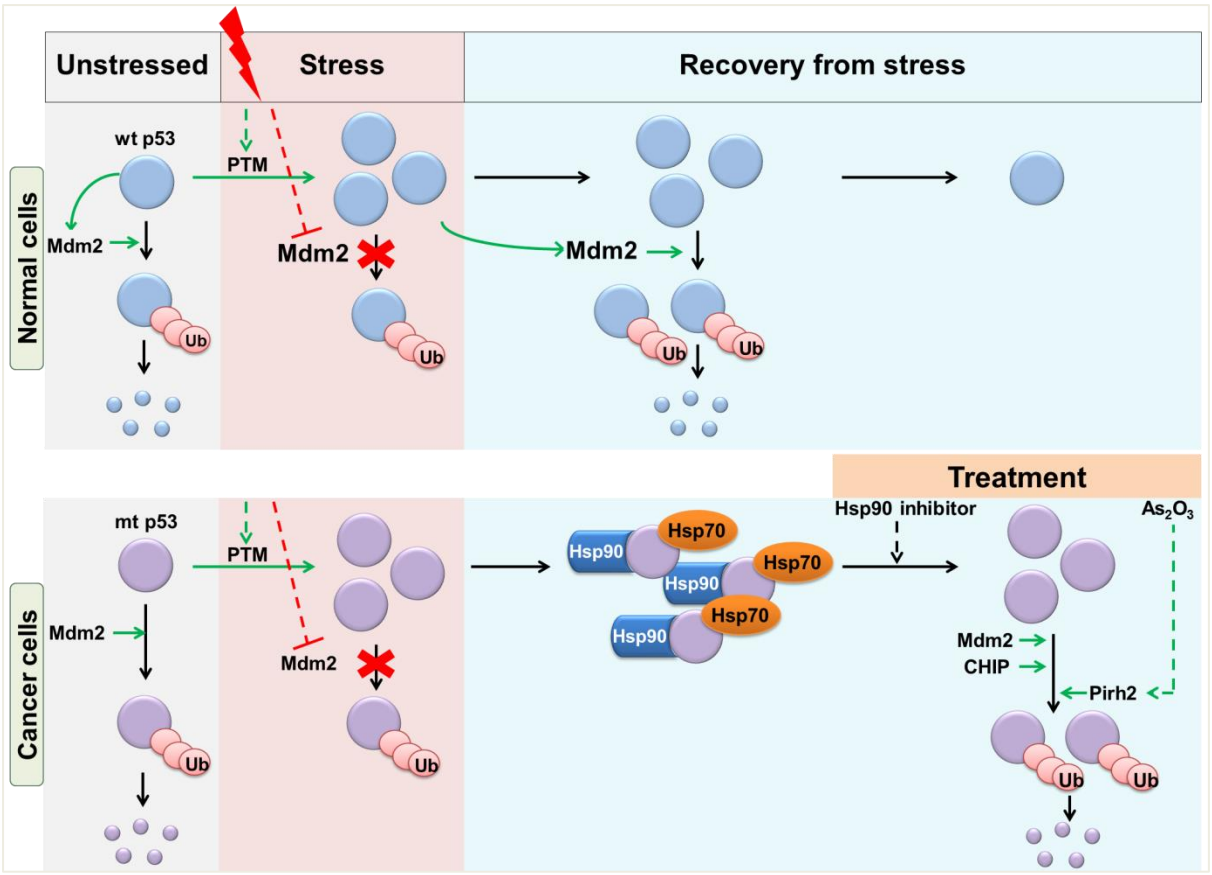


Figure 1.3: Regulation of wild type and mt-p53 stability.
Both wild type (wt-p53) and mt-p53 levels are maintained by MDM2 mediated proteasomal degradation. Under stress conditions, both wild type and mt-p53 are stabilized due to post-translational modifications (PTM). When cells recover from stress, p53 returns to low levels due to negative feedback loop with MDM2. In contrast, mt-p53 fails to transactivate MDM2 and accumulates. Mt-p53 also interacts with chaperones, Hsp90 and Hsp70, which prevent MDM2 and CHIP mediated degradation. Inhibiting Hsp90 or treating cells with Arsenic trioxide can activate MDM2/CHIP and Pirh2 mediated proteasomal degradation respectively. Image from [53].

Further, Hsp70 interacts with unfolded mt-p53 and forms aggregates which are protected from ubiquitination through CHIP and MDM2 [72]; pertinently, overexpression of Hsp70 blocks MDM2 mediated degradation of mt-p53. DNAJA1 (a member of the HSP family) also protects mt-p53 from ubiquitination through CHIP and this is reversed by treatment of cells with statins [76]. Heat shock proteins are upregulated in many cancers and there also exists a positive feed forward loop, with mt-p53 upregulating the levels of HSF1, the major transcriptional regulator of heat shock proteins, and consequently ensuring accumulation of mt-p53 [77-79]. Tracz-Gaszewska et al. (2017) identified in BRCA cohorts (from the TCGA pan-cancer dataset which includes both inherited and somatic inactivation of the BRCA1/2 pathway), a striking correlation between high levels of DNAJB1 (Hsp40) and

DNAJB6 in mt-p53-high MDM2 cases with poor survival [80]. In addition to protecting mt-p53 from degradation, these chaperones are also involved inactivation of p73 by forming a complex with both mt-p53 and p73 [81, 82].

It was also found that while p53 transiently interacts with the HSPs, p53 R175H is stabilized in a large complex containing Hsp90-Hsp40-Hop-Hsc70 and this complex is resistant to Bag-1 mediated dissociation [82]. A BRCA1/ΔNp63 target gene, S100A2 can block Hsp90 mediated stabilization of mt-p53 and particularly in breast cancers, S100A2 is downregulated [83].

Pirh2:

Pirh2 is a RING domain containing E3 ligase, which interacts with and directly ubiquitinates p53 and mt-p53 independent of MDM2 [84]. Studies have shown that treatment of cell lines with arsenic can induce Pirh2, which lowers mt-p53 (H179Y and R248W) levels [85, 86].

TRIM32 (Tripartite motif-containing protein 32) is a RING domain containing E3 ligase, which degrades mt-p53 (R273H and R175H) less efficiently when compared to wild type. Both Pirh2 and TRIM32 are p53 target genes, whose expression could be deregulated in mt-p53 expressing cancer cells due to the absence of functional p53 transcriptional activation [84, 87]. Other E3 ligases, which have been described to ubiquitinate p53, have not been studied extensively in the context of mt-p53 [88].

1.4.3 Ubiquitin independent proteasomal degradation:

Degradation of proteins occurring through the 20S proteasome does not require the target protein to be ubiquitinated and p53 is degraded by this MDM2 independent mechanism [89]. However, mt-p53 is protected from 20S proteasome mediated degradation by interaction with NQO1 with increased affinity, when compared to the wild type [90]. In many cancers, NQO1 is expressed at high levels and may contribute to mt-p53 stability, however this has not been explored [91].

1.4.4 Autophagy

In addition to ubiquitin mediated proteasomal degradation, mt-p53 is also degraded by autophagy. The role played by autophagy and the mechanisms involving the degradation of mt-p53 through the lysosomal pathway is not very well understood. However, studies have identified ubiquitination as a signal for lysosomal degradation [92].

Glucose restriction can cause an increase in mt-p53 deacetylation, and degradation through the autophagic machinery [93]. Interestingly, this degradation is dependent on E3 ligase, MDM2, but does not involve the proteasome. This indicates that pools of ubiquitinated mt-p53 exist in cells and are degraded by different mechanisms based on cellular conditions. Degradation of mt-p53 is reduced upon knockdown of autophagic genes, ULK1 and ATG5 and glucose restriction in MDA-MB-231 cells expressing R175H. Similar effects were observed upon treatment of cells with Chloroquine, which inhibits autophagy [53, 94, 95].

However, it is known that mt-p53 localized in the cytoplasm can inhibit autophagy, which could be one of the reasons for stabilization of the mutant protein (Morselli et al; 2008, Tasdemir et al; 2008, Yang and Klionsky, 2010).

1.4.5 Factors that stabilize mutant p53:

In addition to chaperone proteins (HSPs) discussed previously in Section 1.4.2, several proteins are known to regulate mt-p53 indirectly by blocking its degradation. For example: DBC1 (Deleted in Breast Cancer 1) competes with MDM2 to bind to the N terminal and DNA binding domains of mt-p53 (R280K) and prevents its degradation [96]. Bag2 protein interaction with mt-p53 (R175H, R248W and R273H) leads to its nuclear translocation and further inhibition of MDM2 mediated ubiquitination [97]. Gankyrin enhances the ability of MDM2 to ubiquitinate p53 and can target mt-p53 as well [98].

P53 is known to be activated in events of ribosomal stress, due to the interaction of free ribosomal proteins with MDM2 (reviewed in Zhou, Liao [99]). Ribosomal protein, RPS27 and RPL26 increase can stabilize mt-p53 through the same mechanism [100, 101]. RPL26 also binds to the 5'-UTR of p53 mRNA and enhances translation positively regulating both mt-p53 mRNA and protein expression [57, 101].

1.4.6 Deubiquitinases

Deubiquitinases (DUBs) are enzymes that catalyze the removal of ubiquitin moieties from proteins resulting in stabilization of the protein. Many DUBs have been found to deubiquitinate p53 and stabilize it. However, their effect on mt-p53 has not been studied. ABRO1 activates the DUB, USP7 and causes p53 deubiquitination. Although there are no reports regarding ABRO effects on mt-p53, overexpression of ABRO1 in cell lines expressing mt-p53 (HT29 and BT474 cells) increases cell growth which could be due to increased mt-p53 stability as a result of deubiquitination by USP7 [102]. USP10, which is highly expressed in cancers, can deubiquitinate mt-p53 and protects it from degradation possibly through the autophagic pathway [103]. Treatment of cells with Spautin-1, an inhibitor of USP10 and autophagy can reduce mt-p53 levels during glucose free and confluent contexts [94]. This indicates that in addition to genes mentioned in Section 1.4.4, DUBs may aid in preventing autophagic degradation of mt-p53. Further experiments are required to ascertain these speculations and the effects of other DUBs on mt-p53.

1.4.7 Post-translational modifications of mutant p53

Post-translational modifications other than ubiquitination may also play a role in increasing mt-p53 stability. ERK1/2 phosphorylates mt-p53 at Ser-15 and the phosphorylated form of mt-p53 is stable and protected from MDM2 mediated ubiquitination [104]. On the other hand, mt-p53 can be degraded upon deacetylation by SIRT1 by an unknown mechanism [105]. In hepatocellular carcinoma, SETDB1, a histone H3K9 methyltransferase interacts with and dimethylates mt-p53 (R249S) at K370, which eventually protects mt-p53 from degradation [106]. In some cases, mt-p53 stability is the result of lack of interaction with negative regulators. For example,

Calpains that cleave and deactivate p53 are unable to act on the mutant form [107]. Much remains to be done to validate whether mt-p53 undergoes the same post-translational modifications as p53 and the subsequent effects on its stability and oncogenic function.

1.5 Therapeutic targeting of mutant p53

The loss of p53 is absolutely deleterious for cancer therapy because most therapies involving radiation and many chemotherapeutic molecules (for example: Cisplatin, Doxorubicin) depend on p53 for induction of apoptosis in cancer cells. In contrast, mt-p53 expression augments drug resistance through its oncogenic pathways. Therefore, certain p53 mutations may render tumour cells resistant to therapy and harder to target [108]. Yet, over the years, many strategies for therapeutic targeting of mt-p53 expressing cancers have been developed and can be broadly classified into three major categories: (1) Compounds that reactivate mt-p53 into its wild type conformation (2) Targeting the oncogenic pathways downstream of mt-p53 (3) Targeting the expression and enabling the degradation of mt-p53 [44].

1.5.1 Reactivation of mutant p53

Structural studies on wild type and mt-p53 revealed that Zinc is absolutely critical for the correct folding of the core domain and therefore indispensable for DNA binding. Zinc treatment can convert certain p53 mutants (such as p53 R175H) into a wild type form, thereby restoring its tumour suppressive capacities and rendering cancer cells susceptible to drugs [109]. Many additional compounds have been selected for a capacity to facilitate the conversion of mt-p53 to a wild type like conformation. These compounds include: COTI-2, RITA, PEITC, Chetomin, PK7088, NSC319726, P53R3, CP-31398 and APR-246 and studies are ongoing to identify their mode of action [75, 110].

Of all the therapies, the most successful is the molecule, Prima-1^{MET} (APR-246). APR-246 is converted to MQ in cells and MQ binds to mt-p53 and restores it to wild type conformation [111-113]. In addition however, APR-246 is associated with compounding activities including targeting the glutathione and thioredoxin reductase

system, which in turn promotes ROS production [114]. APR-246 is the most advanced of these therapies and is the first to enter clinical trials (reviewed in [75, 110]).

1.5.2 Targeting regulators of mutant p53

As detailed in section 1.4, only few regulators of mt-p53 stability and their mechanism of action are known. SAHA/Vorinostat inhibits HDAC6 (a positive regulator of Hsp90) and HDAC8 (a positive regulator of p53 expression) and thereby results in reduction in mt-p53 through an MDM2 dependent mechanism as mentioned in Section 1.4.2. These have demonstrated killing efficacy toward mt-p53 expressing cells and have been shown to improve survival in mice [56, 74, 115, 116]. As previously described, statins target mt-p53 degradation through the DNAJA1-CHIP axis and can reduce cell growth [76]. Gambogic acid, Capsaicin and YK-3-231 are additional molecules that affect mt-p53 stability and target it for degradation [75].

1.5.3 Targeting oncogenic pathways of mutant p53

As discussed in section 1.3.2, mt-p53 can activate a number of oncogenic pathways. By targeting these oncogenic pathways, various strategies of cancer cell survival can be terminated. Small molecule inhibitor, RETRA-1 can inhibit the interaction between mt-p53 and tumour suppressor, p73. Treatment with RETRA-1 results in the activation of p73 target genes and tumour suppression. Drugs that target the mevalonate pathway, epigenetic factors (such as MLL1, MOZ) and mTOR signaling have also demonstrated ability to block mt-p53 GOF mediated tumor proliferation pathways [76, 117, 118]. Additionally, ROCK inhibitor (Dasatinib), EGFR inhibitors, and also drugs that block PDGFRβ, are able to inhibit mt-p53 driven invasive and metastatic pathways (reviewed in [75]).

Investigators have also explored targeting other critical factors for survival of mt-p53 tumours and p53 null tumours such as Wee1, PLK1, MK2, CHK1/2 [75]. Targeting mt-p53 at the transcriptional and at the mRNA level does not result in an appreciable decrease in the protein levels [55, 57]. Therefore, mt-p53 must be targeted at both transcriptional and protein levels to be able to bring about significant decrease in its oncogenic effects.

1.6 Tumour microenvironment

Tumour microenvironment refers to the collection of cells in the vicinity of the tumour, secreted factors and extracellular matrix, which have the capacity to influence the growth of the primary tumour, vascularization, invasion and metastasis of the primary benign tumour. Cancer associated fibroblasts (CAFs) form a major group of cells associated with the tumour and they release growth promoting factors, modify the ECM to favour tumour growth. The ECM provides a scaffold for tumour cell adhesion and factors secreted by both CAFs and malignant cells can remodel ECM components to give way for cell proliferation, metastasis and angiogenesis. Specifically, both tumour cells and CAFs can secrete matrix metalloproteinases (MMPs) and lysyl oxidases, which drive ECM remodeling [119]. Also, hypoxic conditions in the tumour interior can result in the release of angiogenic factors that influence endothelial cells to branch out and cause the development of new blood vessels ensuring supply of oxygen and nutrients to the cancer cells [120]. Another major subset of cells within the tumour are immune related cells such as T and B cells, and macrophages. Different T and B cell populations are found infiltrating tumour areas and many studies have observed a correlation between particular types of immune cells and patient prognosis [121]. Recent studies have shown that tumour microenvironment can be modified in an effort to curb tumour growth and metastasis [122]. Studies have identified the roles played by both wild type and mt-p53 in bringing about changes in the tumour microenvironment.

1.6.1 Mutant p53 influences tumour microenvironment

Wild type and mt-p53 have opposing roles in shaping the tumour microenvironment [123]. By repressing TIMP1-4 (tissue inhibitors of metalloproteinases), mt-p53 can increase the secretion of MMPs which play a major role in ECM remodeling. Also, mt-p53 can upregulate the expression of various inflammatory cytokines through the NF-κB pathway. There is also a correlation between secreted proteins/biomarkers such as PSA (Prostate specific antigen) with mt-p53 in predicting tumour metastasis [124]. In addition to increased angiogenic factors, chemokines, lactate, mt-p53 also alters the extracellular vesicle/ exosome contents [48, 125, 126]. In contrast, p53

inhibits the development of an inflammatory tumour microenvironment, which adversely affects tumour growth [127].

1.7 Exosomes

Cells release hormones, factors, chemokines, RNA, miRNAs in extracellular vesicles. Initially, it was suggested that extracellular vesicles are a mode of cellular waste disposal, however far more complex roles are now unraveling [128]. Exosomes refer to a specific set of extracellular vesicles of the size range 40-100nm and are released by the fusion of multivesicular bodies to the plasma membrane. Exosome secretion is a tightly controlled process and is distinct from microvesicle secretion, which are formed by budding of the plasma membrane [129]. Exosomes of varying features and composition are secreted by most cells and are therefore found in various body fluids. [130].

1.7.1 Exosome biogenesis

The endosomal sorting pathway is closely related to the exosome biogenesis pathway. Multivesicular bodies (MVBs) are formed due to invagination of plasma membrane and fusion with endosomes or invagination of the endosomal membrane and these multivesicular bodies can either fuse with lysosome in which case the protein contents are degraded, or fuse with the plasma membrane, where the intraluminal vesicles are released as bilipid layered exosomes. Studies have revealed the presence of two pools of multivesicular bodies differing in cholesterol content which have different destinations – lysosomal degradation or fusion with the plasma membrane (Fig 1.4) [131, 132].

Both membrane proteins and cytosolic proteins can be trafficked to multivesicular bodies by controlled sorting through either of the following two mechanisms: Endosomal sorting complex required for transport (ESCRT) dependent and the ESCRT independent pathway. ESCRTs are the major set of proteins identified based on an RNAi screen as being important for multiple steps of MVB (multivesicular body) biogenesis [133]. The ESCRT independent pathway is

controlled by sphingolipids and proteins of the tetraspannin family [129]. Many studies have identified the importance of ubiquitination (especially Lys-63 linkage) of protein cargoes for targeting to the membrane of multiple vesicular bodies [134].

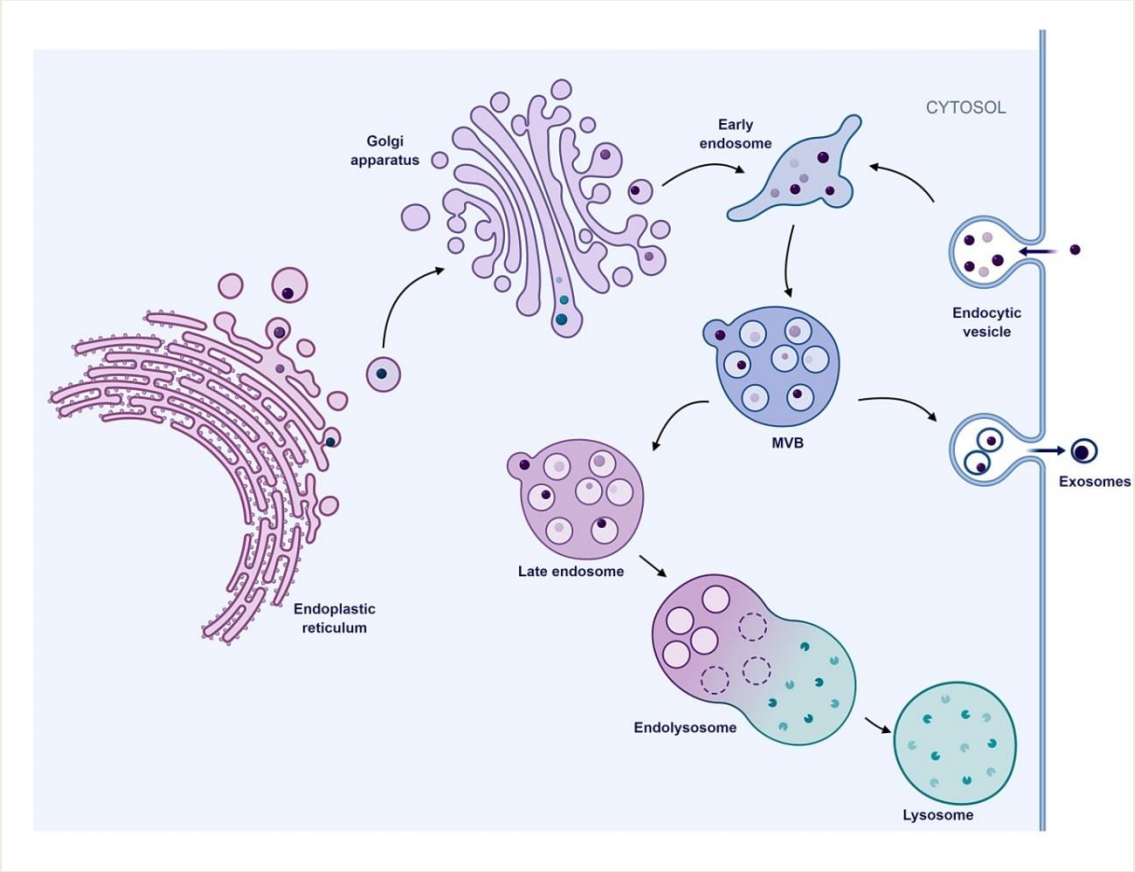


Figure 1.4: Mechanism of exosome biogenesis.
Ubiquitinated proteins targeted for exosomes are first sorted into multivesicular bodies, which are formed by invagination of the plasma membrane and fusion with early endosomes. ESCRT components play a major role in multivesicular body formation. Protein contents of multivesicular bodies can be degraded upon fusion with the lysosome. Proteins in the intraluminal vesicles can also be released as exosomes by fusion of a multivesicular body to the plasma membrane. (This image was made on Biorender based on [131])

1.7.2 Exosomes in cancer

Tumour cells actively communicate with other tumour cells and cells of the microenvironment through exosomes (Fig 1.5). Exosomes released by tumour cells and can initiate signaling at the plasma membrane of recipient cells by specific receptor binding or through its contents upon internalization [129]. Exosomal RNA transferred between cells were found to be functional in the recipient cells: miRNAs acting on targets and mRNAs being translated to proteins [135]. Exosomes from

cancer cells also carry oncoproteins (EGFR, K-Ras, c-Met), which can act as biomarkers [136]. Tumour derived exosomes are not only involved in ECM remodeling but modulating recipient cells to favour tumour growth and invasion [137].

It was also found that prostate cancer cells release large vesicles (1-10μm), prostasomes, which are also named “large oncosomes” due to their cancer specific origin [138, 139]. While putting forth the ‘seed and soil theory’ of cancer metastasis, Stephen Paget alluded to the fact that some organs are predisposed for secondary cancer and from recent studies, it is now clear that exosomes from primary tumours play a significant role in preparing pre-metastatic niches in distant organs that then offer a conducive ‘soil’ for the growth of ‘seeded’ metastases [140]. Recently, it was identified that mt-p53 in colon cancer cell lines can increase the levels of miR-1246 in exosomes. Uptake of this exosome cargo by tumour associated macrophages, in turn causes them to release immunosuppressive signals [126]. Novo et al., identified a GOF pathway of mt-p53 in modulating ECM by increasing the levels of exosomal podocalyxin and migration of p53 null cells [125]. These findings reveal that mt-p53 affects cells in the tumour microenvironment by manipulating the immune landscape and migratory capacities through exosomes.

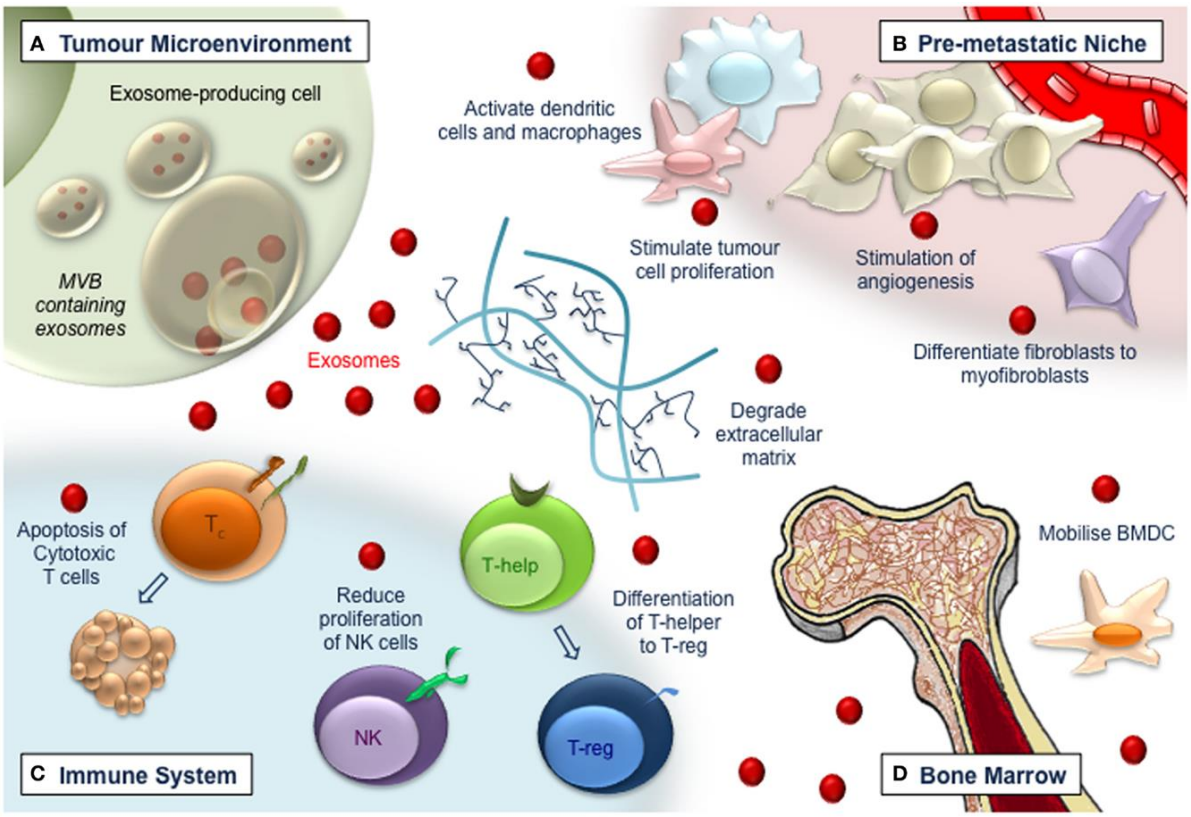


Figure 1.5: Functions of exosomes in cancer.
Contents of tumour derived exosomes (A) can influence multiple cell types to favour tumour progression and metastasis. Exosomes can influence secondary sites of metastasis by preparing an environment conducive for cancer cells to establish (B). Exosomes can stimulate tumour cell proliferation, angiogenesis and can affect the ECM and immune cells, which include T cells and Natural killer cells (NK) (C). Bone marrow derived cells (BMDC), which are crucial for generation of suitable microenvironment for tumour are influenced by exosomes (D) (Image from [141]).

1.8 NEDD4 family interacting protein-1 (NDFIP1)

NEDD4 family of E3 ligases are a part of the ubiquitin ligase family which differ from the HECT domain E3 ligase family by the characteristic presence of WW domains and a C2 domain. Through ubiquitination of substrates, NEDD4 family controls several processes such as viral budding, endocytosis and protein sorting [142]. NDFIP1 was initially identified in a far-western screen as an interactor of the WW domain of NEDD4 [143]. It was subsequently demonstrated to bind to other members of the NEDD4 family, hence the name. NDFIP2 was later identified based on sequence homology. These family members are present on different chromosomes - NDFIP1 in Chr5 and NDFIP2 in Chr13 [144]. Both NDFIP1 and

NDFIP2 are evolutionary conserved in both yeast and Drosophila as a single homolog, Bsd2p and dNdfip1 respectively [145, 146].

1.8.1 NDFIP1 – structure

NDFIP1 is a 26kDa protein containing 221 amino acids. NDFIP1 has 3 PPxY motifs and 3 conserved transmembrane domains towards the C terminus and a C2 domain, which is a Ca or lipid binding domain (Fig 1.6) [147].

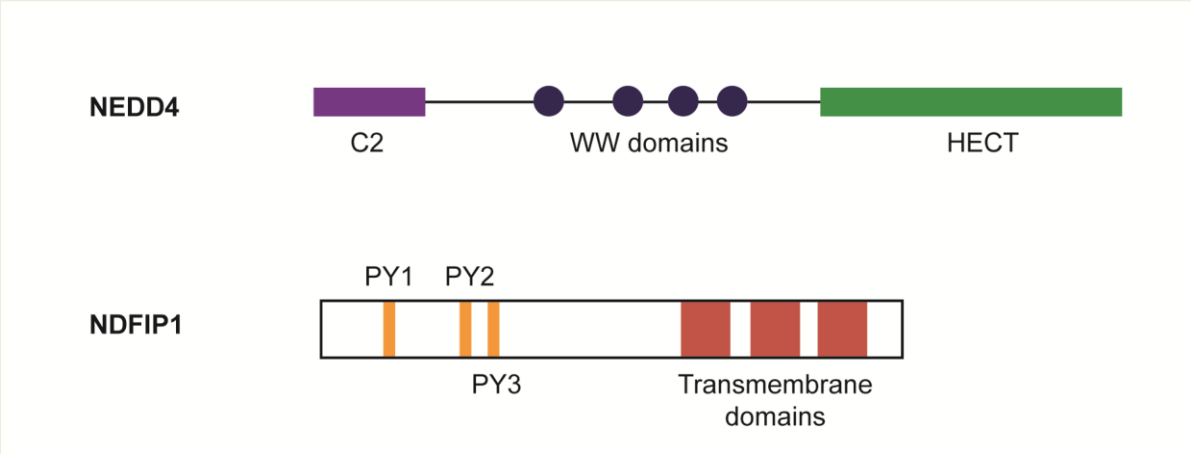


Figure 1.6: Domain organization of NEDD4 family of E3 ligases and NDFIP1.
NEDD4 members contain an N-terminal C2 domain, 2-4 WW domains and a C-terminal catalytic HECT domain. NDFIP1 has 3 PY domains and 3 transmembrane regions in the C-terminus. Images based on [142, 148]

NEDD4 E3 ligases are in a constant state of inhibition as the catalytic HECT domain is bound by its own WW domains. The mechanism of E3 ligase activation by NDFIP1 requires the binding of PPxY motifs of NDFIP1 to the WW domains of NEDD4 E3 ligases releasing the HECT domain from auto-inhibition (Fig 1.7) [148, 149]. In addition to NEDD4, it was found that NDFIP1 can also bind to and activate other members of the NEDD4 family: including NEDD4L, WWP2, ITCH. It is also known that NEDD4 itself is an E3 ligase of NDFIP1 and can ubiquitinate and control its stability [147].

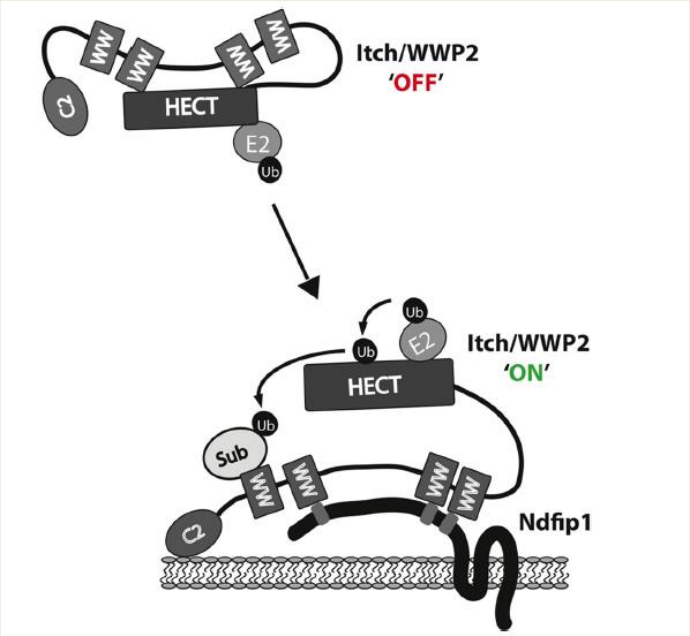


Figure 1.7: Release of E3 ligase ITCH from auto-inhibition by NDFIP1.
HECT domain of E3 ligase, ITCH is inhibited by the binding of WW domains resulting in an inactivated state. Upon the binding of the NDFIP1 PY domains to the WW domains of Itch, the HECT domain becomes activated and releases the other WW domain to bind the substrate. (Image from [148]).

NDFIP1 is found to be distinctly localized in the Golgi apparatus, endosomal vesicles and perinuclear region [147, 150]. Ectopic expression of NDFIP1 can disrupt Golgi and result in mislocalization indicating that it is an integral protein of the Golgi [147]. Generally, the N-terminus of NDFIP1 is located in the cytosol, while its C- terminus is situated on the intra-luminal side of the endocytic membrane [150].

1.8.2 NDFIP1- known functions

The consequences of NDFIP1 mediated ubiquitination of substrates largely depend on the cell type and the nature of the stress signals that the cells encounter. NDFIP1 upregulation upon brain injury/cerebral ischemia leads to a protective effect on cortical neurons, preventing them from dying. In this context, NDFIP1 upregulation upon ischemia drives PTEN nuclear import and subsequently maintains pAKT levels in the cytoplasm aiding cell survival. Also, NDFIP1 deletion increases neuron death after HI (hypoxia-ischemia) reduces the number of pAkt-positive neurons [151]. In

contrast, in mouse embryonic fibroblasts and the neuroblastoma cell line, SH-SY5Y, deletion of NDFIP1 can enhance cell proliferation due to lack of nuclear PTEN [152].

In 2014, Hammond et al identified structural imperfections and atrophic characteristics in cortical and hippocampal neurons in NDFIP1 conditional KO mice suggesting a major role of NDFIP1 in the development of parts of the brain cortex [153]. NDFIP1$^{-/-}$ mice suffer from skin lesions owing to increased inflammation of skin and lung and die at approximately 14 weeks of age. This phenotype is very similar to Itchy mice characterized by lack of ITCH E3 ligases. Indeed, higher inflammation and increased T cell activation is due to repressed ITCH activity in the absence of NDFIP1 and consequent JunB protein stability [154]. Studies on NDFIP1$^{-/-}$ mice show that in addition to T-cell activation, NDFIP1 is indispensable for T_{reg} cell differentiation resulting in increased inflammation [155-157]. Overall, these studies provide important insights into the role of NDFIP1 in the neurological and immunological spheres.

Interestingly, knockdown of NDFIP1 in cervical cancer cell line, HeLa causes cell proliferation. NDFIP1 knockdown can also increase levels of Src, Lyn, EGFR, however the mechanism is not completely known. Both NDFIP1 and NDFIP2 can be phosphorylated through the EGFR pathway, however the effect of phosphorylation is not known [158]. Beck et al., 2015 showed that knockdown of NDFIP1 reduces cytokine induced apoptotic death indicating a pro-apoptotic role for NDFIP1 in pancreatic beta cells [159].

NDFIP1 can mediate the ubiquitination of several substrates, such as DMT1, hERG, Jun, MAVS, Aquaporin2, Trk receptor through NEDD4 E3 ligases and target these proteins for proteasome mediated degradation [160-164]. NDFIP1 mediated ubiquitination can also cause nuclear trafficking as in the case of PTEN and BRAT1 [150, 165]. From studies on Drosophila Ndfip1, it is clear that NDFIP1 can promote Notch ubiquitination through lysosomes, while the ubiquitination of Notch is possibly through Nedd4 and Su(dx)(the Drosophila ortholog for ITCH and WWP1/2) [166, 167].

In contrast to its role in directing proteins for destruction through the lysosomes, a significant function of NDFIP1 is the trafficking of proteins (non-destructively) into exosomes. It was found that NDFIP1 overexpression can increase protein ubiquitination in cells and concurrently, the protein content of exosomes. NDFIP1 overexpression also results in trafficking of NEDD4, NEDD4L, ITCH and PTEN to exosomes [168]. Exosomal PTEN is taken up by recipient cells and reduces the levels of p-AKT [169]. The presence of WW domains or interaction with WW domains seem to be important for NDFIP1 mediated trafficking of proteins to exosomes [170].

1.8.3 NDFIP1 in disease

NDFIP1 has long been implicated in disorders of the brain. NDFIP1 expression was found to be upregulated in samples with traumatic brain injury [151] (as introduced in Section 1.8.2). Lower expression of NDFIP1 is found in APP/PS1 mouse brain (expressing mutated amyloid precursor protein and mutant human presenilin 1), which also exhibits Alzheimer's disease pathogenesis. Low levels of NDFIP1 leads to a lack of proteasomal degradation of DMT1 (iron transporter) and a subsequent imbalance in iron levels [171]. Indeed, NDFIP1 is elevated in Parkinson's brains and the resulting iron accumulation is due to DMT1 levels [172]. Studies on the relation between NDFIP1 and T-cell activation/tolerance have also revealed that NDFIP1 may play a role in auto-immune disorders [156] [173].

Little is known about NDFIP1 in the context of cancer and the available reports are quite conflicting. NDFIP1 levels are found to be regulated by micro-RNAs – particularly miR-155 and miR-338-5p in uveal melanoma and glioblastoma respectively. Increase in apoptosis after miR-338-5p overexpression and irradiation is attributed to NDFIP1 downregulation in the case of glioblastoma. MiR-338-5p is significantly downregulated in glioblastoma samples and the targeting of NDFIP1 by miR-338-5p alludes to an oncogenic role for NDFIP1 in glioblastoma [174]. However, in uveal melanoma, overexpression of miR-155 mimic can downregulate NDFIP1 and thereby increase cell proliferation pointing to a growth inhibitory tumour-suppressive role for NDFIP1 [175]. Regulation of PTEN localization by NDFIP1 also

indicates that NDFIP1 could play an important role in lung endocrine tumours [176]. Knockout of miR17-92 can upregulate NDFIP1 resulting in nuclear localization of PTEN causing apoptosis of a subtype of motor neurons called the lateral motor column motor neurons (LMC-MN) [177]. This indicates a potential growth suppressive role for NDFIP1 in this particular subtype of cells. A bioinformatic study in 2007 revealed that NDFIP1 levels negatively correlate with miR-181a, which is elevated in a sub-class of AML [178]. Loss of expression of NDFIP1 (5q31.3 to 5q33.1) is also observed in a subset of colorectal carcinomas (Staub et al., 2006). However, there is no information available on NDFIP1 levels in tumour derived exosomes.

1.8.4 NEDD4 E3 ligases and p53

Of all the E3 ligases of the NEDD4 family, WWP1 is the only ligase that directly binds to p53 and facilitates its ubiquitination. Ubiquitination by WWP1 results in cytoplasmic localization of p53 and reduced transcriptional activity [179]. There exists a feedback loop between p53 and WWP1, such that increased expression of p53 reduces WWP1 levels [180]. In contrast, NEDD4 ubiquitinates MDM2 (K63 type ubiquitin chains) and stabilizes it. Interestingly, another report suggests that knockdown of NEDD4 can result in increased basal p53 levels [181]. It was also found that in embryonic stem cells, NEDD4 is involved in conversion of mt-p53 conformation to wild type and maintaining its activity [182]. Another NEDD4 E3 ligase, SMURF1/2 is known to stabilize MDM2 by inhibiting its autoubiquitination and it also enhances interaction of MDM2 and MDMX [183]. In spite of the known association of these E3 ligases with p53 directly and/or indirectly, the influence of these ligases on mt-p53 has not been explored.

1.9 Aims of the project

Mt-p53 stability is a serious problem in the clinic due to the aggressive nature of the associated tumours and poor prognosis. Current knowledge about regulators of the mutated form of the major tumour suppressor p53 is inadequate. Lack of mt-p53 regulation in cancers can only be partially explained by the limited capacity of

MDM2/CHIP and the effect of chaperones. It is possible that mt-p53 is destabilized by proteins whose functions remain to be exposed. We hypothesize that there are still unknown regulators of mt-p53 and that understanding their mechanisms of action will offer potential to open up new approaches to overcome the oncogenic functions of mt-p53. This defines a considerable gap in our knowledge and such candidates may have important implications for understanding cancer mechanisms and predicting anti-cancer therapies. Therefore, to identify novel regulators of mt-p53, a high-throughput RNAi screen was performed in our lab. My project focusses on –

- Analysis of results of the high-throughput RNAi screen and validation of selected candidates.
- Identification of NDFIP1 as a candidate regulator and deciphering the mechanism of mt-p53 regulation through NDFIP1.
- Establishment of functional assays to study NDFIP1 mediated oncogenic effects of mt-p53.

Chapter 2
Materials and methods

2.1 Bioinformatic analyses

For pathway enrichment analysis of tertiary screen hits, we used DAVID gene functional classification tool and KEGG database as reference. The test gene list was compared to a background list of the human genome to identify enriched pathways. The gene count threshold for pathway enrichment was set to 2 and enriched pathways with a p value>0.05 were considered significant [184, 185]. Classification based on protein class was done using PANTHER, which utilizes GO (gene ontology) terms to segregate proteins (Protein classes with p values greater than 0.05 were considered significant) [186]. Network analysis of the candidates of the tertiary screen was carried out using GeneGo's METACORE tool (https://portal.genego.com/). Venn diagram to analyze overlap of candidates across 4 cell lines was generated using Venny2.0 software [187]. Proportional venn diagram of the validated hits was made using BioVenn [188]. Heat map was made using MORPHEUS (https://software.broadinstitute.org/morpheus).

2.2 Cell culture and reagents

MDA-MB-468, MDA-MB-231, AU565, JH-EsoAd1, H1299 were maintained in RPMI (GIBCO) supplemented with 10% fetal bovine serum and 0.2% Penicillin-streptomycin (GIBCO). LN18 and HEK293T cells were maintained in DMEM supplemented with 10% FBS and 0.2% Penicillin-streptomycin. All cell lines were maintained in 10cm/15cm dishes below confluence and subcultured based on growth rate.

For preparation of mouse embryonic fibroblasts (MEFs), E12.5-13.5 embryos were used. After removal of organs and other entrails, the emryos are cut into smaller pieces with a blade and passed through 18G needle 4 times. These are spread on gelatinized dishes. MEFs were maintained in DMEM supplemented with 10% heat-

inactivated FBS, 10mM non-essential amino acids, 2mM L-Glutamine, 1mM Sodium pyruvate, 10mM HEPES (GIBCO) and 50mM β-mercaptoethanol (Calbiochem).

Table 2.1: List of cell lines used

Cell line	Organism	Origin/Cancer type	*TP53* status	*TP53* mutation
MDA-MB-468	Human	Breast adenocarcinoma (TNBC)	Mt	R273H
JH-EsoAd1	Human	Esophageal adenocarcinoma	Mt	G266E
MDA-MB-231	Human	Breast adenocarcinoma (TNBC)	Mt	R280K
AU565	Human	Breast adenocarcinoma (luminal)	Mt	R175H
MCF7	Human	Breast adenocarcinoma (luminal)	Wt	-
H1299	Human	Non-small cell lung carcinoma	Null	-
LN18	Human	Glioblastoma	Wt/Mt	wt/C238S
Cos	Monkey	Kidney	Wt	-

2.3 Plasmids and cloning

2.3.1 Gene knockdown strategy

Lentiviral constructs for knockdown of NDFIP1 were constructed by cloning oligos into FH1T-cherry at BsmBI/XhoI (NEB) sites as described in [189]. Oligos for NDFIP1 shRNA construct were designed based on Open Biosystems clone V2LHS_99041 [161].

Table 2.2: Oligos for NDFIP1 shRNA

shNDFIP1#1_F	TCCCGCTGTTCAAGTTAATCTAGAATTCAAGAGATTCTAGATTAACTTGAACAGCTTTTTC
shNDFIP1#1_R	TCGAGAAAAAGCTGTTCAAGTTAATCTAGAATCTCTTGAATTCTAGATTAACTTGAACAGC
Scrambled_F	TCCCGTTAATTATTGGTAACCGACATTCAAGAGATGTCGGTTACCAATAATTAACTTTTTC
Scrambled_R	TCGAGAAAAAGTTAATTATTGGTAACCGACATCTCTTGAATGTCGGTTACCAATAATTAAC

2.3.2 Overexpression strategy

Doxycycline inducible NDFIP1 overexpression plasmids were constructed by cloning human NDFIP1 (transcript ID: NM_030571.4) into pTRE at the restriction sites BglII/EcoRI and then recloned with the Tet-responsive element (TRE) into lentiviral vector, FUV1-cherry (kindly provided by A/Prof. Marco Herold (Walter and Eliza Hall

Institute of Medical Research)). NDFIP1 ORF was amplified from a human NDFIP1 expressing vector using primers listed in Table 2.3 and then digested with the restriction enzymes, BglII and EcoRI (Promega). The vector pTRE was digested with the restriction enzymes, BamHI and EcoRI (Promega). Making use of compatible BglII-BamHI cohesive ends, the digested plasmid and the PCR product were ligated. The TRE element and NDFIP1 ORF were then released from the vector through PacI (NEB) digestion and then subcloned into FUV1-cherry vector. Positive clones were sequenced using the primers listed in Table 2.3 to check for orientation and fidelity of amplification.

Table 2.3: Primers for NDFIP1 overexpression construct

Primers for NDFIP1 PCR			
	Sequence 5'->3'	Restriction site	T_a ºC
NDFIP1 Forward primer	GAGATCAGATCTCCACCATGGCGTTGGCGT	BglII	60
NDFIP1 Reverse primer	TCAGACGAATTCTTACTTGTCATCGTCGTCCTTGT	EcoRI	
Primer for sequencing			
	Sequence 5'->3'		
FUV seq R	GCTGTCCTGAGCGTCCG		

Lentivirus for knockdown and overexpression of NDFIP1 were made as discussed in the sections 2.4 and 2.5. Cancer cell lines transduced with appropriate lentivirus were sorted using the method described in section 2.6. For determining the optimal Doxycycline (Sigma) concentration for efficient knockdown/overexpression, cells were cultured in 6 well plates with different concentrations of Doxycycline and NDFIP1 levels were determined by immunoblotting at 48 or 72 hrs for overexpression or knockdown respectively.

2.3.3 Site directed mutagenesis

Mutations in the construct, VN-p53 were introduced using Q5 site directed mutagenesis kit (NEB E0554S) as per manufacturer's instructions. Primers are listed in Table 2.4.

Table 2.4: Primers for site directed mutagenesis

Primer	Sequence 5'->3'	T_a ºC
VN-p53R273Hfwd	TTTGAGGTGCATGTTTGTGCC	64
VN-p53R273Hrev	GCTGTTCCGTCCCAGTAG	
VN-p53R175Hfwd	GTTGTGAGGCACTGCCCCCAC	66
VN-p53R175Hrev	CTCCGTCATGTGCTGTGAC	

2.4 Transfection

Reverse transfection of MDA-MB-468 and JH-EsoAd1 cells (60,000 cells/well in a 12 well plate) were performed with 25nM siRNA pools (Dharmacon) using the following protocol. Briefly, the transfection reagent (Dharmafect4) was diluted in Opti-MEM (0.733uL/well for MDA-MB-468 and 1.08uL/well for JH-EsoAd1), incubated for 5 min following which the appropriate amount of siRNA was added to the mix. This was incubated for 20 min at RT for complex formation to occur after which the appropriate number of cells were added and seeded onto 12 well plates. Media was replaced after 24 hrs and the cells were harvested for extraction of protein after 72 hrs of transfection.

Transient transfections were performed in H1299, HEK293T and LN18 (seeding densities of $1.5X10^6$ for H1299 and $1.75X10^6$ for HEK293T and LN18) using polyethylenimine (PEI, Sigma). The DNA:PEI ratio (in terms of µg) used is as follows - 1:4.3 for HEK293T and 1:1 for H1299 and LN18. The appropriate amount of PEI was diluted in media (without FBS or antibiotic) after which DNA was added to the mix and incubated for 5 mins at RT. The DNA:PEI mixture was then added to cells and incubated for 2 hrs for H1299 and 6 hrs for HEK293T and LN18 after which the transfection media was replaced.

2.5 Lentivirus generation and infection

Lentiviral packaging plasmids (pMDLg/PRRE, pRSV-Rev, pMD2.G) were generously provided by A/Prof. Marco Herold. To generate lentivirus, HEK293T cells were transfected with shRNA vectors (10 µg) and 2 µg each of the packaging plasmids using polyethylenimine (PEI) as transfection reagent. The supernatant containing virus was collected 30, 45, 52, 58 and 70 hours post-transfection and concentrated using Amicon Ultra 15 centrifugal filter unit (UFC901024, Millipore). Cells were infected with concentrated virus in the presence of 8 µg/ml polybrene for 6 hours. The transduced cells were then sorted based on the appropriate fluorescent marker by flow cytometry.

2.6 Cell sorting

Viral transduced cells were trypsinized and resuspended in FACS (Fluorescence-activated cell sorting) buffer ($PBS^{-/-}$ with 2% FBS and 0.5mM EDTA (Sigma)) at a concentration of $2x10^7$ cells/ml. The cell suspension was filtered using a 35µm nylon mesh (Corning, VIC, Australia) to exclude clumps and then sorted using BD FACS Fusion5 (BD Biosciences) based on the expression of GFP/cherry.

2.7 CRISPR based gene knockout

Cas9 vector (FUCas9cherry) and FgH1tUTG_hup53_Ex5 (plasmid with Doxycycline inducible guide RNA expression targeting exon 5 of human p53) were kindly provided by A/Prof. Marco Herold. In order to knockout p53 from LN18 cell line, cells were transiently transfected with the 5µg each of the above plasmids and transfection reagent, PEI as discussed in Section 2.5 and Doxycycline (20ng/ml) was added 6 hrs after transfection. At 48 hrs post transfection, clones positive for both GFP and cherry were sorted as single cells into a 96 well plate using FACS sorting. We selected clones with successful knockout of p53 using p53 immunoblotting.

2.8 Bimolecular Fluorescence Complementation assay (BiFC assay)

Cells were seeded on coverslips 16-18hrs before transfection. $H1299p53^{-/-}$ cells were transfected with 0.5µg of VN-p53 or mutants, 0.5µg of VC-NDFIP1 and 0.25µg of Rab5-RFP using polyethylenimine (PEI). Rab5-RFP was used as a transfection control. After 12-16hrs, the coverslips were washed once with PBS and then fixed with 100% ice-cold methanol (Merck) for 10min at -20ºC. The coverslips were then incubated with DAPI to stain nucleus for 1hour at RT, following which they were mounted on slides using Fluorsafe (Calbiochem). $Venus^+$ and $Rab5^+$ cells were counted manually using Olympus Bx51 microscope and images were captured using Leica Sp5 confocal microscope. The number of $Venus^+$ cells normalized to $Rab5^+$ cells was calculated to determine the proportion of cells with positive BiFC signal.

2.9 RNA extraction

Cell pellets were resuspended in Trizol (ThermoFisher), followed by Chloroform (Sigma-Aldrich) and then centrifuged at 12,000 g for 15 min. The aqueous phase containing RNA was transferred to a fresh tube and then precipitated using

isopropanol (Merck) and was further washed with 70% ethanol. The RNA pellet was air-dried and dissolved in Diethyl pyrocarbonate DEPC-treated water (ThermoFisher). RNA concentration measured at 260-280nm using NanoDrop 2000 spectrophotometer (ThermoFisher) and a A260/280 ratio of 2 was considered pure RNA. For extracting RNA from $<10^5$ cells, Qiagen mRNAeasy column was used.

2.10 Quantitative real-time PCR

One µg of extracted RNA was used for cDNA synthesis using 0.5µg Random primers, 0.5mM dNTP and 2U of M-MLV reverse transcriptase (Promega) as per manufacturer's instructions (RT, 10 min; 40^0C, 50 min) The resulting cDNA was amplified with 2µM forward and reverse primers and Fast SYBRGreen (ThermoFisher) in Applied Biosystems StepOne Real-Time PCR system (ThermoFisher). The PCR run is as follows – initial denaturation step of 95^0C, 20s, 1X followed by 40 cycles of 95^0C, 3s and 60^0C, 30s. Primers used are listed in Table 2.5. The primers sequences of *NDFIP1* (Primerbank ID 188595693c2), *MAPK1* (Primerbank ID: 75709179c1) were taken from Primer Bank database [190-192]. The primers for *TP53* were made in-house and the primers for *CycB2*, *CycA* were taken from [193]. The primers for *AURKB* were made using the NCBI primer designing tool [194]. Relative gene expression was calculated using $2^{-\Delta\Delta CT}$ method as described in Livak and Schmittgen et al., 2001 [195]

Table 2.5: qPCR primer sequences

Primer	Sequence (5' -> 3')
hNDFIP1	F: TTTGTGGGTCGGGATGATTTTG R: AAATGGCCCCATACCTTCCTG
hTP53	F: CCTGTGCAGCTGTGGGTTGATTC R: ACCATCGCTATCTGAGCAGC
hCycB2	F: GGCTGGTACAAGTCCACTCC R: GAAGCCAAGAGCAGAGCAGT
hCycA	F: AGCAGCCTGCAAACTGCAAAGTTG R: TGGTGGGTTGAGGAGAGAAACAC
hAURKB	F: GCGTGGCAGATTCAGTTGTT R: GACAAGTGCAGATGGGGTGA
hMAPK1	F: TACACCAACCTCTCGTACATCG R: CATGTCTGAAGCGCAGTAAGATT
hRPL37a	F: GCCAGCACGCCAAGTACAC R: CCCCACAGCTCGTCTCTTCA

2.11 Cell lysis for immunoblotting

Cell pellets were resuspended in Radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl (Sigma), 150mM NaCl, 1% NP40, 0.1% SDS, 2mM EDTA (Sigma)) supplemented with 5µg/ml Aprotinin and 1mM PMSF (Phenylmethylsulfonyl fluoride) followed by incubation in ice for 20 min. The lysates were then centrifuged at 12,000 g for 15 min at 4ºC to remove debris. Protein concentration was determined using BCA assay based on a standard curve of known BSA concentrations (Pierce BCA protein assay kit, ThermoFisher). (BCA assay (Bicinchoninic acid assay works on the principle of absorbance by Bicinchoninic Cu^{+} produced as a result of reaction between peptide bods and Cu^{2+} ions.) Protein sample buffer 4.5X (225mM Tris pH 6.8, 9% SDS, 22.5% β-mercaptoethanol, 45% glycerol, bromophenol blue (Sigma)) was added to 50-80µg of lysate to make up a final concentration of 1X. The lysates were boiled at 95ºC for 10 min and loaded onto an SDS PAGE.

2.12 Immunoblotting

Cell lysates were separated on SDS-PAGE (containing 0.38M Tris pH 8.5, 0.1% SDS, 0.05% Ammonium persulfate (Bio-rad) and 0.08% Tetramethylethylenediamine (TEMED, Merck) with 12% Acrylamide (Acrylamide/Bis-acrylamide 37.5:1, Amresco, Astral Scientific) along with a molecular weight protein standard (Precision Plus Protein Standard Dual Colour, Bio-Rad). Proteins were separated over a charge gradient using a running buffer (25 mM Tris-base, 250 mM Glycine (Sigma), 0.1% SDS) then transfered to 0.45 µm PVDF (Polyvinylidene fluoride) membrane (Immobilon-P Membrane, Merck) in transfer buffer (25 mM Tris-base, 192 mM Glycine, 0.01% SDS, 5% Methanol) at 20V for 2 hours. The membrane was then blocked with 5% skim milk in PBS-T ($PBS^{-/-}$ containing 0.05% Tween-20 (Sigma)) for 1 hour at RT. This was followed by incubation with primary antibody (conditions vary depending on the antibody and is listed in Table), washing with PBS-T and incubating with secondary antibody for 1 hour at RT. The membrane was then washed with PBS-T and PBS and the chemiluminescent intensity was detected using Western Lightning Plus-ECL (Perkin-Elmer) and visualized on ChemiDoc MP (Bio-Rad) using ImageLab software (version 4.1). The antibodies used for immunoblotting are listed in Table 2.6.

Table 2.6: List of antibodies used

Antibody	Clone name	Host	Supplier	Dilution for immunoblotting	Dilution for immunofluorescence
p53	DO-1	Mouse	in-house	1:20	1:4
p53	1801	Mouse	in-house	1:20	1:4
NDFIP1	polyclonal	Sheep	R&D Biosystems	1:500	
Hsp60	H-300	Rabbit	Santa-cruz	1:1000	
Tsg101	polyclonal	Rabbit	Sigma	1:1000	
H2B	Lg2-2	Mouse	in-house	1:60	
Anti-mouse HRP	polyclonal	Goat	Invitrogen	1:3000	
Anti-rabbit HRP	polyclonal	Goat	Invitrogen	1:3000	
Anti-sheep HRP	polyclonal	Donkey	R&D Biosystems	1:3000	
Anti-mouse Alexa Fluor488	polyclonal	Goat	Invitrogen		1:800

2.13 Isolation of extracellular vesicles

Cell culture supernatant was centrifuged for 3700 xg for 10 min at 4ºC to remove cell debris. The supernatant was then transferred to ultracentrifuge tubes and subsequently centrifuged at 20,000 xg for 15 min at 4ºC (Beckmann ultracentrifuge, rotor Type 70Ti) to remove apoptotic bodies. The supernatant was then transferred to a fresh ultracentrifuge tube and centrifuged at 100,000 xg for 70min at 4ºC to pellet the extracellular vesicles. The pellet was resuspended in 10ml PBS and then centrifuged at 100,000 xg for 60min at 4ºC (Beckmann ultracentrifuge, rotor SW41). For western blotting, the pellet was resuspended in RIPA buffer and boiled for 5min with Laemmli Buffer. For uptake experiments, the pellet was resuspended in 20mM HEPES.

2.14 Immunofluorescence

Immunofluorescence was performed on cells ($1.25X10^4$) originally plated on coverslips (Menzel Glazer). Cells were washed once with PBS and then fixed with ice-cold 100% methanol for 10 min at -20^0C. The coverslips were then washed with $PBS^{-/-}$ and blocked with 3% BSA (Bovogen, VIC, Australia) for half an hour at room temperature. These were then incubated with primary antibody (diluted in 1% BSA/$PBS^{-/-}$) overnight at 4^0C in a humid chamber and then washed thrice with PBS. After staining with the primary antibody, the coverslips were incubated with the

appropriate secondary antibody (diluted in 1% BSA/PBS$^{-/-}$) and DAPI (ThermoFisher, diluted 1:5000) for 1 hour at room temperature in a humid chamber and then mounted on glass slides (1mm Superfrost Plus microscope slides, ThermoFisher) with Fluorsafe mounting medium (Calbiochem). Stained cells were imaged either using Olympus BX-51 (Olympus, VIC, Australia) and software, Spot one (version 5.0.27; Diagnostics Instruments Inc., MI, USA) or Nikon C2 confocal system and NIS software 3.20.02. The antibodies used are listed in Table 2.5.

2.15 Invasion assay

Transwell assays were performed in 24 well inserts (6.5mm, 8µm membrane, Costar) for cell invasion. Matrigel coating of transwell inserts was done as per manufacturer's instructions. In brief, matrigel (GFR- growth factor reduced, from In vitro technologies) was diluted to 200µg/ml with growth media and added to transwell inserts and incubated at 37^{0}C for 2 hrs before adding the cells.

H1299 cells were incubated with exosomes in serum free media for 24 hrs after which the cells were trypsinized and counted. Then, 30,000 cells were plated on matrigel coated inserts in serum free media. Media with 20% serum was added to the bottom chamber and the cells were allowed to migrate for 24 hrs. At the end of the assay, cells on the upper chamber were removed with a cotton swab and the invaded cells were fixed in 4% paraformaldehyde (Alfa Aesar) and stained with DAPI (ThermoFisher, diluted 1:5000) for 1 hour at room temperature. The transwell filters were mounted on glass slides (1mm Superfrost Plus microscope slides, ThermoFisher) with Fluorsafe mounting medium (Calbiochem) and coverslip (22X50 No.1, Labserv). Images of 6 fields (per filter on 10X) were taken using Zeiss Axiovert microscope and software, Spot one (version 5.0.27; Diagnostics Instruments Inc., MI, USA). Cell count of images was done using Fiji [196].

2.16 Statistical analysis

Data is represented as mean ± standard deviation (SD). Statistical analysis was carried out using GraphPad Prism 7.02 (GraphPad Software, La Jolla California USA). Unpaired non-parametric student's t-test was performed for comparison of two independent groups and the determined significance was represented as * for p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001 or **** p-value < 0.0001.

Chapter 3

Analysis and validation of high-throughput RNAi screen results

3.1 Preface

The aim of this chapter is to analyze the results of a high-throughput RNAi screen designed to identify mt-p53 regulators, to rationally select candidates for evaluation and to validate their potency toward mt-p53.

The RNAi screen and initial bioinformatic analysis was performed by Kah Hin Tan and Franco Caramia, respectively. Immunoblotting represented in figures 3.5-3.7 was performed once (n=1).

3.2 Introduction

Mt-p53 accumulation is a major risk for cancer development due to its acquired oncogenic activities that are most critically associated with metastatic disease that lacks effective clinical therapeutic options (detailed in Chapter 1, Section 1.3.2). Studies in mice models reveal that mt-p53 is initially a labile protein, which is stabilized in response to stress signals. The consequent accumulation of mt-p53 is in part due to the absence of MDM2-dependent negative regulation or is effected through interaction with chaperones, which protect mt-p53 from proteasomal degradation [197] [52, 54]. Targeting protein factors and chaperones that protect mt-p53 from degradation can lower mt-p53 levels in certain cancers and this approach has some success in reducing tumour growth [115] [74]. We hypothesize that in addition to the known E3 ligases: MDM2, CHIP and Pirh2, mt-p53 stability can be affected by other molecules. Support for this concept is starting to emerge from a number of recent studies as we previously reviewed [53]. The first step to understanding the regulation of mt-p53 accumulation is to identify key factors that can stabilize or destabilize mt-p53. To this end, we performed a high throughput RNAi screen in cancer cell lines expressing mt-p53 and measured changes in mt-p53 expression through high content imaging.

The workflow of the genome wide RNAi screen in cell lines expressing mt-p53 is illustrated in Fig 3.1A. The cancer cell lines for the screen were chosen based on their mt-p53 expression levels: triple negative breast cancer cell line, MDA-MB-468 (p53 mutation R273H) and esophageal adenocarcinoma cell line, JH-EsoAd1 (p53 mutation G266E) with high and low levels of mt-p53 respectively with the aim to capture the complete range of genes upregulating and downregulating mt-p53. The cell lines were reverse transfected in a 384 well plate containing siRNA duplexes against every protein-coding gene of the human genome (Dharmacon SMARTpool library) followed by immunostaining with anti-p53 antibody and quantifying mt-p53 levels through high content imaging (Fig 3.1B). Quantification of p53 intensity was done on multiple fields and normalized to that of non-targeting control, OTP (On-target control). All cell lines used in the screen express mt-p53 with gain of function properties.

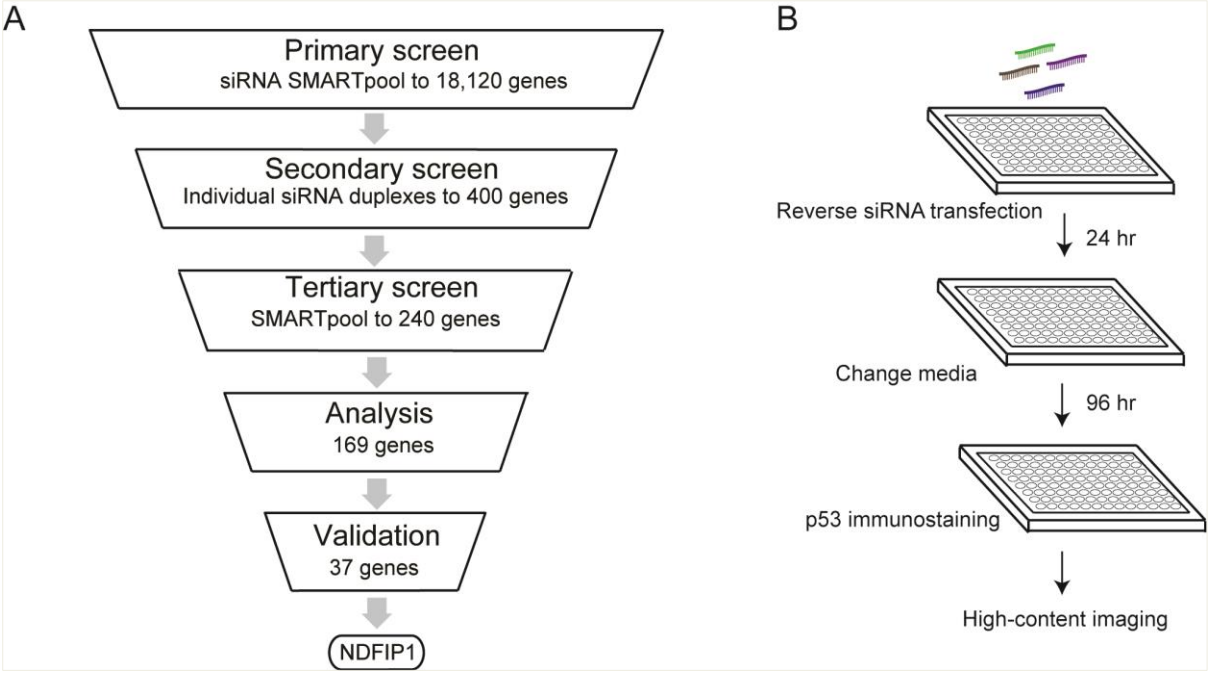


Figure 3.1: Identification of NDFIP1 as a potential mt-p53 regulator.
A. A flowchart illustrating the RNAi screening strategy. The number of genes tested in each screen is listed. **B.** Experimental design of the RNAi screen showing reverse transfection of cells in 384 well plates followed by high-content imaging.

In brief, the primary screen was performed using SMARTpools against 18,120 genes in MDA-MB-468 and JH-EsoAd1 and the top 400 genes were chosen based on Z-score (-2>Z score>2) for testing in the secondary screen (for calculation of Z-score

see Materials and Methods). This section of the screen involved the rigorous testing of 400 genes using each of the 4 siRNA duplexes individually (Deconvolution screen) and 240 genes were found to replicate the results of the primary screen in at least 2 out of 4 duplexes. Positive hits from the secondary screen (240 genes) were re-examined in a tertiary screen using the original SMARTpools in two additional cell lines – MDA-MB-231 (R280K) and AU565 (R175H) with high and low mt-p53 levels respectively.

In this chapter, we discuss the results of the tertiary RNAi screen performed on the 4 cell lines and in particular, we explain in detail the process of selection of candidate genes that are mostly likely to influence mt-p53. Furthermore, we also discuss the validation process used, the known functions of validated candidates and our gene of interest.

3.3 Analysis of the high-throughput RNAi screen

3.3.1 Important parameters in the analysis of tertiary screen results

The tertiary screen tested the effects of 240 genes on the expression levels of mt-p53 in four cell lines - MDA-MB-468, JH-EsoAd1, MDA-MB-231, AU565, two of them originally used in the primary and secondary screen and the latter two included for additional validation. In order to identify genes with the most significant impact on p53 expression, the tertiary screen results were filtered based on a threshold of 10% difference from the control (OTP), which translates to >1.1 for high mt-p53 intensity and <0.9 for low mt-p53 intensity. The threshold was decided based on the range of p53 intensities observed in the tertiary screen. Upon comparison of mt-p53 intensity scores, we observed that 150 genes scored a value higher than 1.1 and 19 genes scored a value lower than 0.9 in at least one cell line in the tertiary screen and these were considered for detailed analysis. The remaining unconfirmed genes failed to show phenotypes equivalent to the primary screen. Comparison of p53 intensities reveal that ~88% (150/169) genes identified from the tertiary screen lead to increase in mt-p53 expression levels when targeted by siRNA. Only a small proportion of genes (19/169) were found to be favouring mt-p53 stability (Fig 3.2B). We hypothesize that a possible reason for this contrast could be because of the difficulty in observing decreases in mt-p53 levels owing to its high stability and also the fact that stabilization of mt-p53 could be due to multiple factors. There is 19% overlap

between the candidates (32/169) identified across the 4 cell lines (Fig 3.2C). Candidates common to all the 4 cell lines are shown in a representative heat map (Fig 3.2A). If a knockdown of a gene results in decrease in mt-p53, that gene is an attractive target for inhibition in the context of cancer. On the other hand, if knockdown of a gene results in increased mt-p53 levels, that gene represents a regulatory checkpoint in maintaining mt-p53 levels.

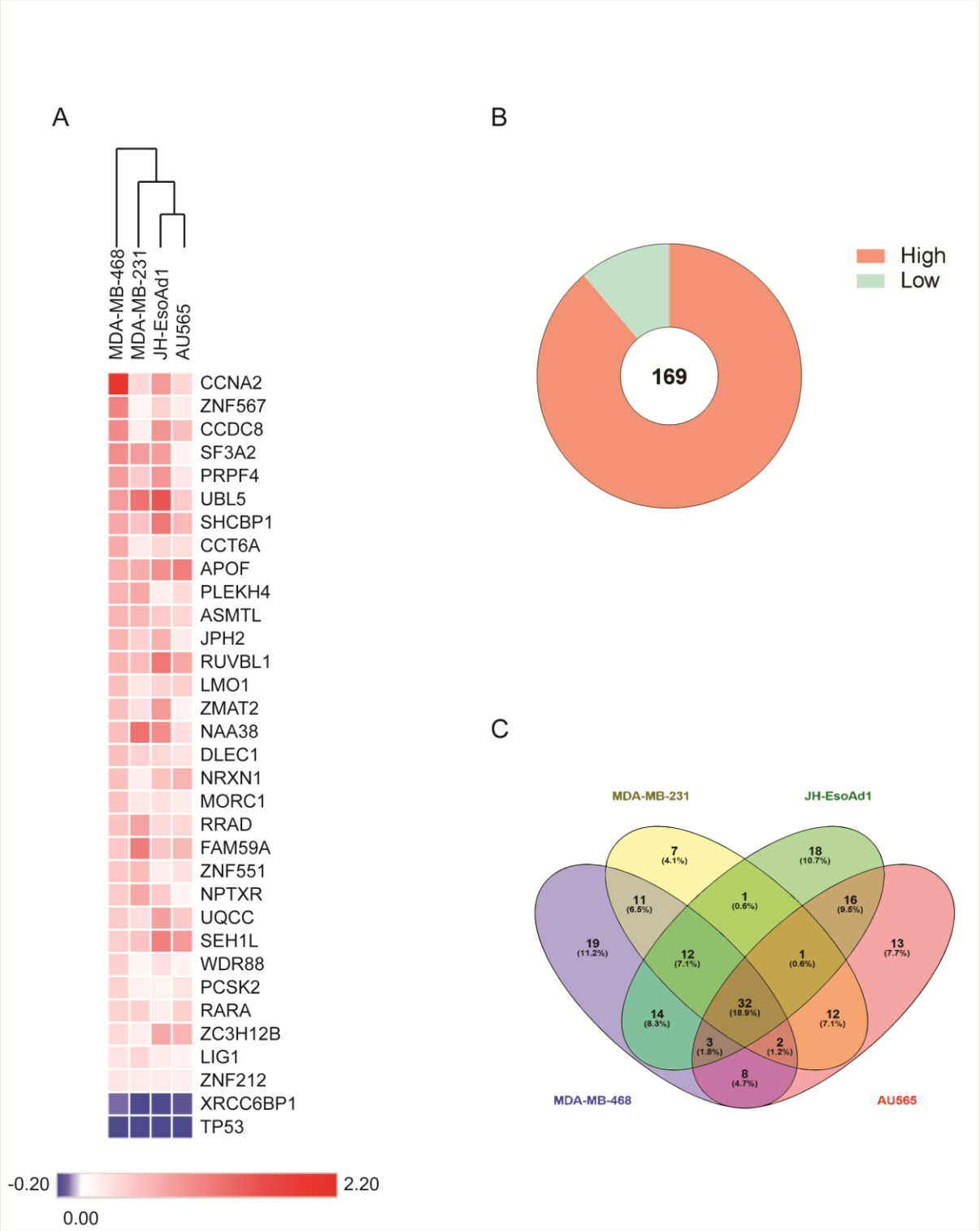


Figure 3.2: Analysis of candidates from the tertiary screen.
A. Heat map of common candidates. p53 intensities were normalized to OTP (intensity=0). XRCC6BP1 is the only candidate across all cell lines to aid mt-p53 stability. **B.** Pie chart illustrating the proportion of candidates showing high vs low mt-p53. **C.** Analysis of overlap of candidates across the 4 cell lines used in the tertiary screen.

The following criteria were taken into account when prioritizing the selection of genes for further consideration.

(1) One important factor for selecting proteins was the DAPI score which indicates relative toxicity. Low DAPI score would indicate that knockdown of that gene is toxic to the cells and that the gene is essential for cellular function. Therefore, we chose a cutoff of 0.3 for DAPI score and a candidate was selected for further analyses only if the DAPI score was greater than 0.3.

(2) The candidate genes were classified into categories in an attempt to identify the pathways which are most crucial for mt-p53 stability.

(3) Network analysis was carried out using GeneGo's METACORE to enrich for proteins closest to p53 in the networks.

(4) The association of the candidate gene with cancer and whether the protein is 'druggable' were also considered to be important criteria. In fact, 122 out of 169 candidates are found to be related to cancer in terms of expression level patterns, epigenetic modifications or mutations.

(5) It was also considered whether the candidate gene was already known to affect p53. As most of the databases rely on information on pathways with respect to p53, network analyses revealed candidates associated with the wild-type form of p53.

3.3.2 Enrichment analysis of genes

In order to gain insight into the screen results, we subjected the hits through pathway and network analyses in addition to extensive data mining. Enrichment analysis of candidate genes identified in the tertiary screen, performed based on multiple

databases (KEGG, Reactome and Biocarta) in DAVID bioinformatics tool [184, 185] gave similar results and the top 10 pathways enriched based on KEGG database are listed in Fig 3.3A [198]. The top pathways are related to genes of the spliceosome, RNA transport and degradation. Proteins of the splicing machinery have been implicated in regulation of p53 levels and we believe that a similar pathway could be in place for mt-p53 regulation [199-201].

Of particular interest are three enzymes (LPP1, ASAH1 and CERK) of the pathway for sphingolipid metabolism, which may play a role in regulating mt-p53 levels. In fact, many enzymes of this pathway are deregulated in cancers and key metabolites of this pathway are linked to p53. Bioinformatic analyses of NCI-60 gene datasets identified CERK as a possible synthetic lethal target in combination with p53 [201]. Both p53 and metabolites of the sphingolipid pathways act during events of cellular stress and in fact, studies indicate that the metabolites function both upstream (direct binding of Ceramide to p53) and downstream (p53 dependent increase in Ceramide) [202, 203]. However, all reports are in the context of p53 and have not been investigated in the context of mt-p53. The genes from the tertiary screen represented in the top 10 pathways are listed in Table 3.1.

To understand the representation of the types of genes in our gene list, we performed functional clustering using PANTHER [186]. The genes were classified into categories based on their protein class. By making use of gene ontology terms, it was clear that “nucleic acid binding proteins” is the most represented protein class among the candidates (Fig 3.3B). This is not surprising as there are both transcription factors and RNA binding proteins in our list of candidates. This raises the question as to whether mt-p53 is directly regulated at the transcriptional and mRNA levels in these cells or indirectly through other regulators.

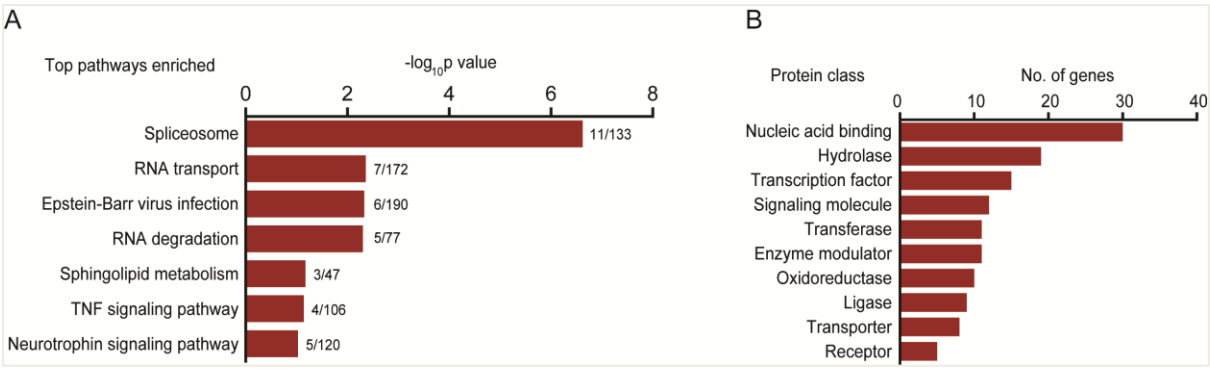


Figure 3.3: Enrichment analysis of candidates from the tertiary screen.
A. An overview of the enriched pathways identified based on KEGG database. The number of hits identified in the screen to the total number of genes in the pathway is also listed at the end of each bar. **B.** Top 10 protein classes identified from the functional classification of 169 genes from the screen using PANTHER.

Table 3.1: List of candidate genes in the top 10 KEGG pathways

Gene ID	Gene symbol	Gene Name
hsa03040:Spliceosome		
51503	CWC15	CWC15 spliceosome associated protein homolog
9416	DDX23	DEAD-box helicase 23
51729	WBP11	WW domain binding protein 11
10523	CHERP	Calcium homeostasis endoplasmic reticulum protein
55119	PRPF38B	Pre-mRNA processing factor 38B
9128	PRPF4	Pre-mRNA processing factor 4
8175	SF3A2	Splicing factor 3a subunit 2
153527	ZMAT2	Zinc finger matrin-type 2
5683	PSMA2	Proteasome subunit alpha 2
23658	LSM5	LSM5 homolog, U6 small nuclear RNA and mRNA degradation associated
57819	LSM2	LSM2 homolog, U6 small nuclear RNA and mRNA degradation associated
51691	LSM8	LSM8 homolog, U6 small nuclear RNA associated
hsa03013:RNA transport		
81929	SEH1L	SEH1 like nucleoporin
8894	EIF2S2	Eukaryotic translation initiation factor 2 subunit beta
8661	EIF3A	Eukaryotic translation initiation factor 3 subunit A
2332	FMR1	Fragile X mental retardation 1
4927	NUP88	Nucleoporin 88
1977	EIF4E	Eukaryotic translation initiation factor 4E
1981	EIF4G1	Eukaryotic translation initiation factor 4 gamma 1
hsa05169:Epstein-Barr virus infection		
5290	PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
5713	PSMD7	Proteasome 26S subunit, non-ATPase 7
6416	MAP2K4	Mitogen-activated protein kinase kinase 4
8737	RIPK1	Receptor interacting serine/threonine kinase 1
890	CCNA2	Cyclin A2
3105	HLA-A	Major histocompatibility complex, class I, A
hsa03018:RNA degradation		
4849	CNOT3	CCR4-NOT transcription complex subunit 3
57819	LSM2	LSM2 homolog, U6 small nuclear RNA and mRNA degradation associated

23658	LSM5	LSM5 homolog, U6 small nuclear RNA and mRNA degradation associated
51691	LSM8	LSM8 homolog, U6 small nuclear RNA associated
3313	HSPA9	Heat shock protein family A (Hsp70) member 9
hsa00600:Sphingolipid metabolism		
427	ASAH1	N-acylsphingosine amidohydrolase 1
64781	CERK	Ceramide kinase
8611	PLPP1	Phospholipid phosphatase 1
hsa04668:TNF signaling pathway		
6416	MAP2K4	Mitogen-activated protein kinase kinase 4
4217	MAP3K5	Mitogen-activated protein kinase kinase kinase 5
5290	PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
8737	RIPK1	Receptor interacting serine/threonine kinase 1
hsa04722:Neurotrophin signaling pathway		
5598	MAPK7	Mitogen-activated protein kinase 7
4214	MAP3K1	Mitogen-activated protein kinase kinase kinase 1
4217	MAP3K5	Mitogen-activated protein kinase kinase kinase 5
5290	PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha

3.3.3 Network analysis

Network analysis was carried out to identify related genes and protein interaction networks through GeneGo's METACORE software, which is based on available literature (https://portal.genego.com/). Shortest path algorithm was used to perform network analysis. This algorithm not only identifies relationships between genes in our list based on available literature but also uncovers links with additional genes in the database, which directly affect our genes of interest. We included *TP53* in our gene list in a bid to identify candidates that are already known to interact with p53. The genes connected to these hubs were identified to interact with p53 directly or indirectly through any of the following mechanisms – transcriptional regulation, binding, influence on expression, ubiquitination. The directionality of the interaction and the outcome (activation/inhibition) were also acquired for most of these hits. The networks are based on available knowledge (which mostly pertains to the wild type form of p53) and the nodes in this network are those genes, which are already known to associate with p53. This analysis was of utmost importance considering the fact that there are common pathways regulating both wild type and mt-p53. From the network, we found that 47 genes (out of 169) are related to p53 (Fig 3.4). When the directionality of the interaction is considered, we found that only 11 genes are known to affect p53 levels indicating that 93% of the genes discovered from the screen are novel.

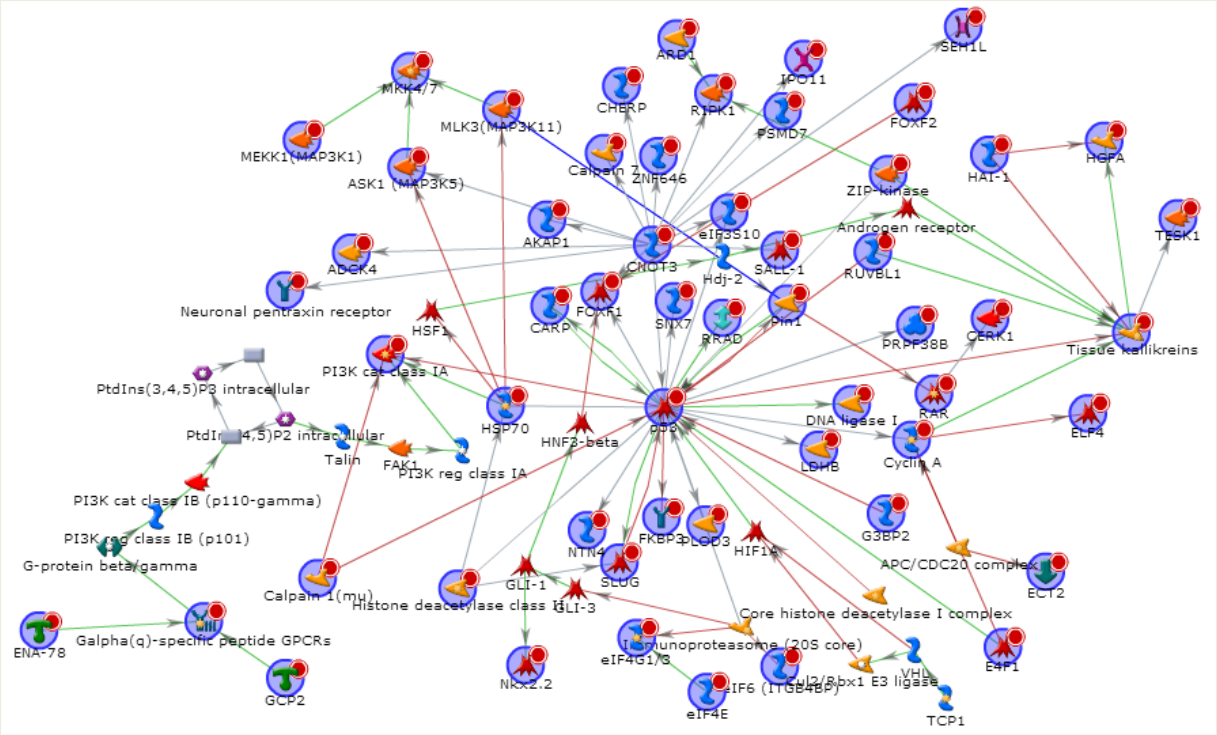


Figure 3.4: Network of related candidate genes of the tertiary screen.
The green arrows indicate activation and the red arrows indicate inhibition. More details on the symbols have been included in the appendix.

3.3.4 Potential drug targets uncovered from the RNAi screen

We also investigated whether the candidates from the RNAi screen are potential drug targets. Specifically, we identified inhibitors against 22% (37 out of 169) of the candidates through extensive data mining of various databases such as GeneCards and NCBI gene. These inhibitors can directly or indirectly affect the activity or level of the candidates. Table 3.2 summarizes the information obtained based on available literature.

Table 3.2: List of druggable candidates

	Gene symbol	Gene name	Inhibitor	p53 intensity (tertiary screen)	Cell line (primary-tertiary)
1	ANAPC1	Anaphase-promoting complex subunit 1;ANAPC1;ortholog	proTAME	High	468-468, JH-JH
2	ANKRD1	Ankyrin repeat domain-containing protein 1;ANKRD1;ortholog	Doxorubicin	High	468-468, JH-JH
3	ASAH1	Acid ceramidase;ASAH1;ortholog	Carmofur(variant of 5-fluorouracil)	High	468-468, JH-JH
4	CAPN1	Calpain-1 catalytic subunit;CAPN1;ortholog	MDL-28170	High	468-468, JH-565
5	CERK	Ceramide kinase;CERK;ortholog	NVP-231	High	468-468, JH-565
6	COPS6	COP9 signalosome complex subunit 6;COPS6;ortholog	Doxycycline	High	468-468, JH-JH
7	COPS8	COP9 signalosome complex subunit 8;COPS8;ortholog	Doxycycline	High	468-468, JH-JH
8	CRBN	Protein cereblon;CRBN;ortholog	Pomalidomide	High	JH-565
9	DHFR	Dihydrofolate reductase;DHFR;ortholog	Methotrexate	High	468-231, JH-565
10	DHRS4	Dehydrogenase/reductase SDR family member 4;DHRS4;ortholog	Finasteride	High	468-468, 468-231, JH-JH
11	EIF2S2	Eukaryotic translation initiation factor 2 subunit 2;EIF2S2;ortholog	Rapamycin	High	468-468, 468-231, JH-JH
12	EIF3A	Eukaryotic translation initiation factor 3 subunit A;EIF3A;ortholog	Rapamycin	High	JH-JH
13	EIF4E	Eukaryotic translation initiation factor 4E;EIF4E;ortholog	LY2275796, Rapamycin	High	468-231
14	EIF4G1	Eukaryotic translation initiation factor 4 gamma 1;EIF4G1;ortholog	Rapamycin	High	JH-JH
15	FKBP3	Peptidyl-prolyl cis-trans isomerase FKBP3;FKBP3;ortholog	Rapamycin	High	468-231, JH-565
16	HSPA9	Stress-70 protein, mitochondrial;HSPA9;ortholog	MKT077	High	JH-JH
17	KDM2B	Lysine-specific demethylase 2B;KDM2B;ortholog	ML324	High	468-468
18	LDHB	L-lactate dehydrogenase B chain;LDHB;ortholog	Sodium oxamate	High	468-231, JH-565
19	MAP2K4	Dual specificity mitogen-activated protein kinase kinase 4;MAP2K4;ortholog	Dabrafenib, Trametinib	High	468-468, 468-231

20	MAP3K1	Mitogen-activated protein kinase kinase kinase 1;MAP3K1;ortholog	E6201	High	all
21	MAP3K11	Mitogen-activated protein kinase kinase kinase 11	CEP1347, URMC-099	High	468-468, 468-231
22	MAP3K5	Mitogen-activated protein kinase kinase kinase 5;MAP3K5;ortholog	GS 4997, AGI 1067	High	468-231, JH-JH
23	MAPK7	Mitogen-activated protein kinase 7;MAPK7;ortholog	XMD 8-92	Low	468-468, 468-231
24	MMEL1	Membrane metallo-endopeptidase-like 1;MMEL1;ortholog	Phosphoramidon	High	468-468
25	NUP88	Nuclear pore complex protein Nup88;NUP88;ortholog	Deguelin	High	468-468, JH-JH
26	PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform;PIK3CA;ortholog	Copanlisib and many inhibitors	High	468-468, JH-JH
27	PLOD3	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 3;PLOD3;ortholog	Minoxidil	High	JH-565
28	PPAP2A	Phospholipid phosphatase 1;PLPP1;ortholog	XY-14	High	JH-JH
29	RARA	Retinoic acid receptor alpha;RARA;ortholog	all trans retinoic acid (ATRA)	High	all
30	SLC4A7	Sodium bicarbonate cotransporter 3;SLC4A7;ortholog	EIPA (Ethylisopropyl ameloride)	High	JH-JH, JH-565
31	USP5	Ubiquitin carboxyl-terminal hydrolase 5;USP5;ortholog	DUB inhibitor WP1130	High	468-468
32	DAPK3	Death-associated protein kinase 3;DAPK3;ortholog	TC-DAPK-6	Low	JH-565
33	RIPK1	Receptor-interacting serine/threonine-protein kinase 1;RIPK1;ortholog	Necrostatin	Low	468-231
34	TNK2	Activated CDC42 kinase 1;TNK2;ortholog	Dasatinib(tyr kinase inhibitor)	Low	JH-565
35	HDAC9	Histone deacetylase 9;HDAC9;ortholog	TMP-269, TFMO	Low	JH-JH
36	HK3	Hexokinase-3;HK3;ortholog	Lonidamine	Low	JH-565

3.4 Validation of putative regulators of mutant p53

The analyses of hits from the tertiary screen yielded 37 putative candidates selected largely on the basis of novelty and p53 intensity. The next step was to validate the candidates using a method alternative to immunostaining, used in the screen. Therefore, we employed immunoblotting as our validation method to check the effect of the selected candidates on mt-p53 levels. We achieved gene knockdown by transiently transfecting siRNA against the selected candidates in MDA-MB-468 and JH-EsoAd1 and harvesting the cells after 72 hrs and immunoblotting for mt-p53. siRNA against p53 was used as a positive control to confirm successful transfection. The intensity of p53 expression was normalized to that of Hsp60 and was plotted in a graph and compared to that of non-targeting control (OTP) (Fig 3.5-3.7). Candidates with relative p53 intensity greater than 1.2 or less than 0.9 were considered to be validated.

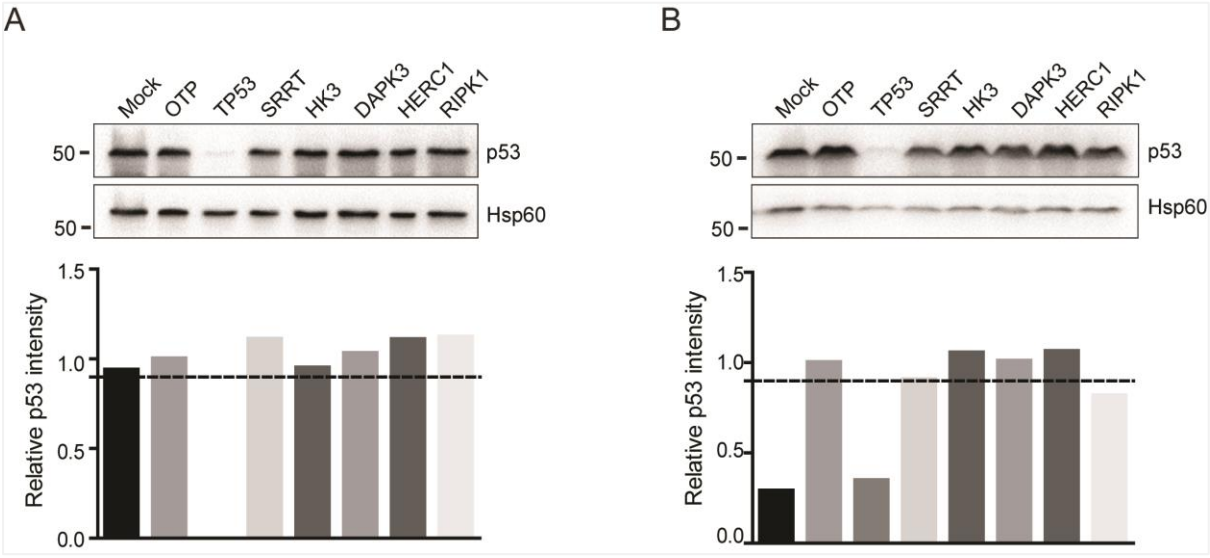


Figure 3.5: Validation blots in MDA-MB-468 (A) and JH-EsoAd1 (B).
5 candidates (SRRT, HK3, DAPK3, HERC1 and RIPK1) were selected out of 19 candidates (showing low mt-p53 upon knockdown). The validation blots and associated graph presenting p53 intensity quantitation is shown in the figure.

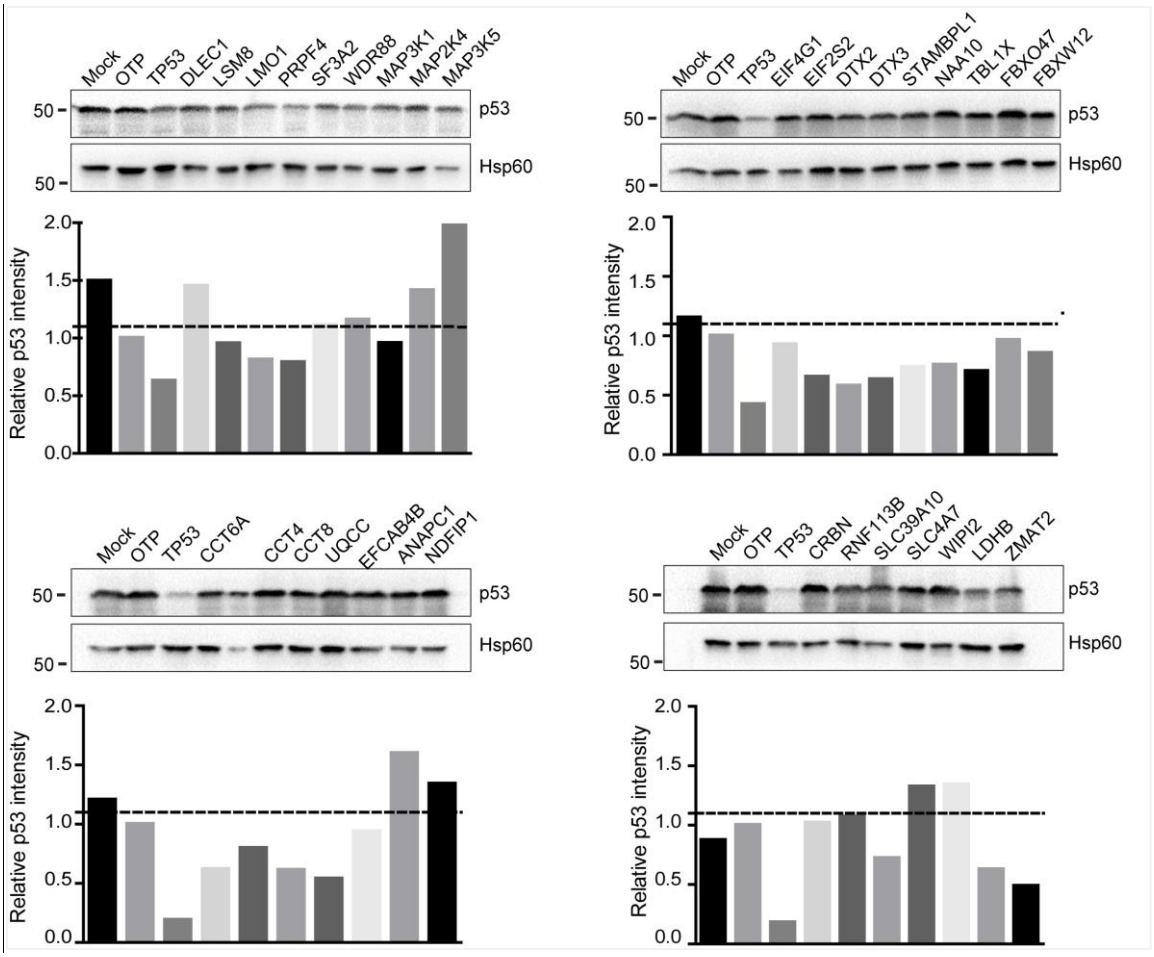


Figure 3.6: Validation blots of candidates in MDA-MB-468 (A-D).
32 candidates selected for validation out of 150 candidates showing high mt-p53 phenotype. The western blots and the associated graphs showing p53 intensity is presented in the figure.

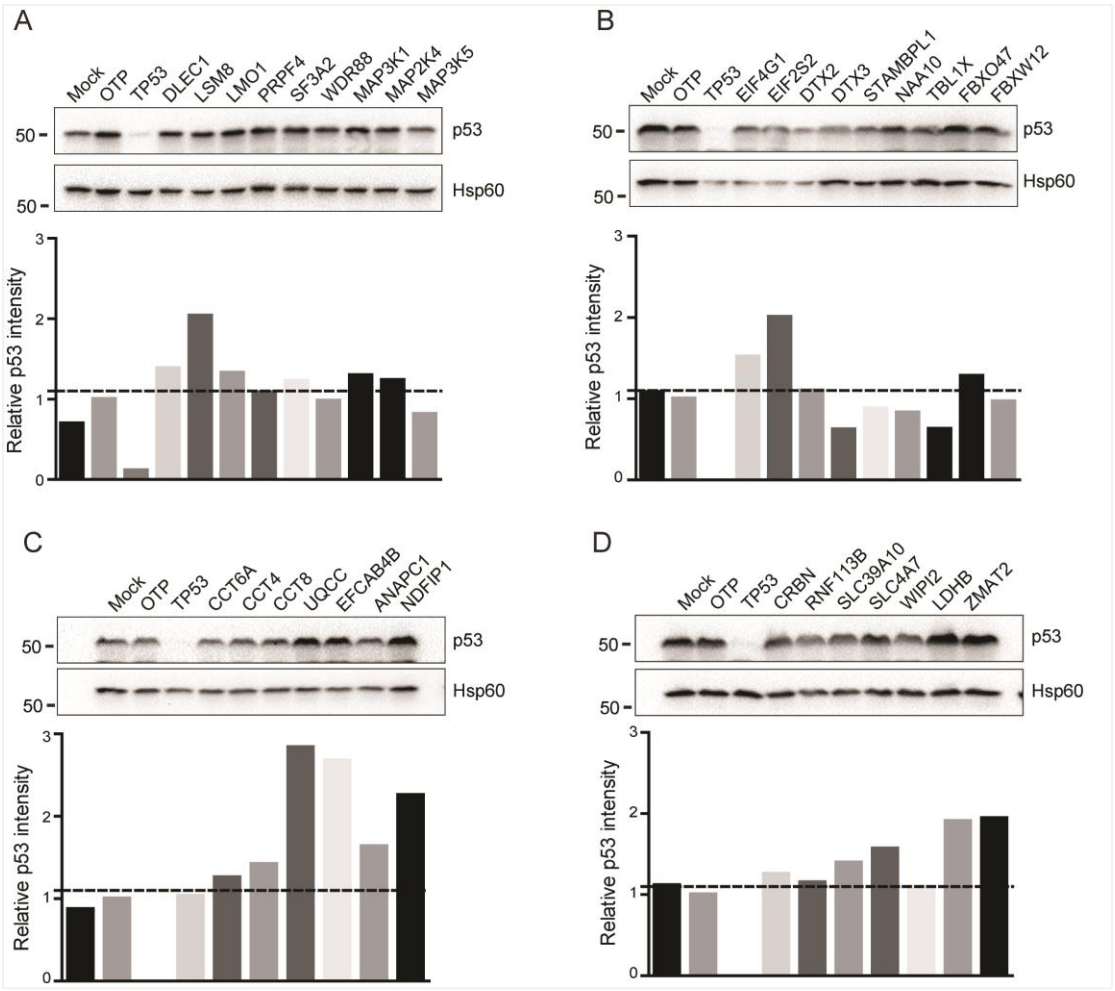


Figure 3.7: Validation blots in JH-EsoAd1 (A-D).
32 candidates selected for validation out of 150 candidates showing high mt-p53 phenotype. The western blots and the associated graphs showing p53 intensity is presented in the figure.

3.4.1 Validation results

Mt-p53 protein levels were compared to OTP (non-targeting control) and we observed that 5 candidates validated true in both cell lines and in total 23 candidates out of 37 validated in at least one cell line. The list of all candidates considered for validation and their validation scores are listed in table 3.3. The 5 common candidates are ANAPC1, DLEC1, MAP2K4, SLC4A7 and NDFIP1 and their known functions are briefly discussed in the following section.

Table 3.3: List of candidates selected for validation and validation scores

	Gene symbol	Gene name	Function	p53 intensity (screen)	Cell line (primary-tertiary)*	p53 intensity (WB)	
						JH	468
1	**DLEC1**	Deleted in lung and esophageal cancer 1	Transcription factor	High	All(Hi)	Yes(1.384)	Yes(1.452)
2	**LSM8**	Lsm8 homolog	RNA related	High	All(Hi)	Yes(2.039)	No(0.953)
3	**LMO1**	LIM domain only 1 (rhombotin 1)	Transcription factor	High	All(Hi)	Yes(1.325)	No(0.812)
4	**PRPF4**	PRP4 pre-mRNA processing factor 4 homolog (yeast)	RNA related	High	All(Hi)	No (1.083)	No(0.79)
5	**SF3A2**	Splicing factor 3a, subunit 2, 66kDa	Splicing	High	All(Hi)	Yes(1.22)	No (1.113)
6	**WDR88**	WD repeat domain 88	Splicing	High	All(Hi)	No(0.979)	No(1.158)
7	**MAP3K1**	Mitogen-activated protein kinase kinase kinase 1	Kinase	High	468-231, 468-468	Yes(1.296)	No(0.955)
8	**MAP2K4**	Mitogen-activated protein kinase kinase 4	Kinase	High	468-231, 468-468	Yes(1.235)	Yes(1.414)
9	**MAP3K5**	Mitogen-activated protein kinase kinase kinase 5	Kinase	High	468-231, JH-JH	No(0.813)	Yes(1.975)
10	**EIF4G1**	Eukaryotic translation initiation factor 4 gamma, 1	Translation initiation	High	JH-JH	Yes(1.515)	No(0.927)
11	**EIF2S2**	Eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa	Translation initiation	High	468-231, 468-468, JH-JH	Yes(2.000)	No(0.653)
12	**DTX2**	Deltex homolog 2 (Drosophila)	E3 ligase	High	468-231, JH-565	No (1.101)	No(0.579)
13	**DTX3**	Deltex homolog 3 (Drosophila)	E3 ligase	High	468-468	No(0.62)	No(0.633)
14	**STAMBPL1**	STAM binding protein-like 1	DUB	High	JH-JH	No(0.878)	No(0.735)
15	**NAA10**	N(alpha)-acetyltransferase 10, NatA catalytic subunit	Coregulator of transcription	High	468-231, JH-565	No(0.828)	No(0.756)
16	**TBL1X**	Transducin (beta)-like 1X-linked	E3 ligase/F box proteins	High	468-231, 468-468	No(0.627)	No(0.702)

17	**FBXO47**	F-box protein 47	E3 ligase/F box proteins	High	468-468, JH-565	Yes(1.279)	No(0.964)
18	**FBXW12**	F-box and WD repeat domain containing 12	E3 ligase/F box proteins	High	JH-565	No(0.967)	No(0.853)
19	**CCT6A**	Chaperonin containing TCP1, subunit 6A (zeta 1)	Chaperones	High	All	No (1.03)	No(0.619)
20	**CCT4**	Chaperonin containing TCP1, subunit 4 (delta)	Chaperones	High	JH-JH	Yes(1.25)	No(0.798)
21	**CCT8**	Chaperonin containing TCP1, subunit 8 (theta)	Chaperones	High	468-231, 468-468, JH-JH	Yes(1.419)	No(0.612)
22	**UQCC**	Ubiquinol-cytochrome c reductase complex chaperone	Chaperones	High	All	Yes(2.83)	No(0.539)
23	**EFCAB4B**	EF-hand calcium binding domain 4B	Signaling molecule	High	468-468, JH-JH, JH-565	Yes(2.67)	No(0.939)
24	**ANAPC1**	Anaphase promoting complex subunit 1	cell cycle protein	High	468-468, JH-JH	Yes(1.636)	Yes(1.59)
25	**NDFIP1**	NEDD4 family interacting protein 1	protein stability	High	JH-JH, JH-565	Yes(2.257)	Yes(1.34)
26	**CRBN**	Cereblon	protein stability	High	JH-565	Yes(1.253)	No (1.018)
27	**RNF113B**	Ring finger protein 113B	E3 ligase	High	468-468	No (1.15)	No (1.07)
28	**SLC39A10**	Solute carrier family 39 (zinc transporter), member 10	Transporters	High	JH-JH	Yes(1.39)	No(0.72)
29	**SLC4A7**	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	Transporters	High	JH-JH, JH-565	Yes(1.56)	Yes(1.32)
30	**WIPI2**	WD repeat domain, phosphoinositide interacting 2	Signaling molecule	High	JH-565	No(1.09)	Yes(1.34)
31	**LDHB**	Lactate dehydrogenase B	Metabolism	High	468-231, JH-565	Yes(1.9)	No(0.62)
32	**ZMAT2**	Zinc finger, matrin-type 2	Transcription factor	High	All	Yes(1.94)	No(0.48)
33	**SRRT**	Serrate RNA effector molecule homolog (Arabidopsis)	miRNA/RNA	Low	JH-565	No(0.90)	No(1.1)
34	**HK3**	Hexokinase 3 (white cell)	Metabolism	Low	JH-566	No(1.05)	No(0.94)
35	**DAPK3**	Death-associated protein kinase 3	kinase	Low	JH-567	No(1)	No(1.03)

36	**HERC1**	Hect (homologous to the E6-AP (UBE3A) carboxyl terminus) domain and RCC1 (CHC1)-like domain (RLD) 1	E3 ligase	Low	JH-JH	No(1.06)	No(1.1)
37	**RIPK1**	Receptor (TNFRSF)-interacting serine-threonine kinase 1	kinase	Low	468-231	Yes(0.81)	No(1.1)

*Cell line (primary-tertiary) refers to the cell lines in which the gene was validated in the primary vs the tertiary screen.

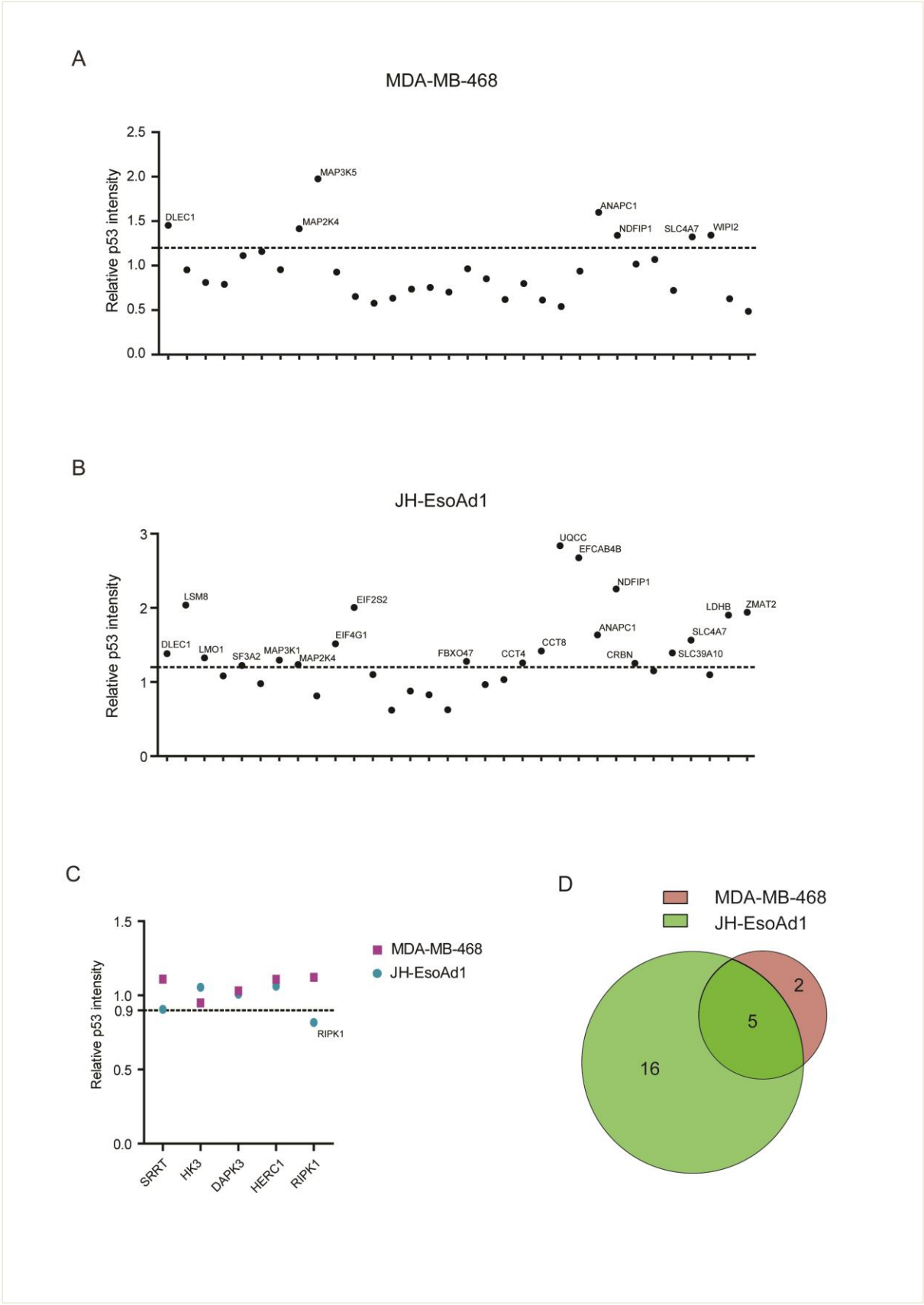


Figure 3.8: Validation results.
Graph showing all candidates (with high mt-p53 phenotype in the screen) and their p53 intensity scores in MDA-MB-468 (**A**) and JH-EsoAd1 (**B**). (**C)** Candidates (showing low mt-

p53 phenotype in the tertiary screen) represented in the graph with their validation scores. (**D**) Venn diagram depicting the overlap between the validated candidates among the two cell lines, MDA-MB-468 and JH-EsoAd1.

ANAPC1 (Anaphase promoting complex 1) is the largest subunit of the anaphase promoting complex/ cyclosome (APC/C), which functions as an E3 ubiquitin ligase [204]. By degrading securin and cyclin B, APC/C complex allows the sister chromatids to separate and aids the cells in metaphase to anaphase transition [205]. The association between APC/C and p53, while striking, is also very conflicting. While siRNA against ANAPC1 can increase p53 levels [206], knockdown of a scaffold subunit of APC/C, APC2 results in accumulation of MDM2 and attenuation of p53 [206, 207]. It is also suggested that mt-p53 acts upstream of APC complex by upregulating CKs1, which activates APC [208]. It remains to be found if there are any functions of ANAPC1 independent of the APC complex. ANAPC1 is mutated, methylated in multiple cancers due to its function in maintaining chromosomal stability [209, 210].

DLEC1 (Deleted in lung and oesophageal cancer 1) is a tumour suppressor gene, which is silenced by methylation in many cancers [211, 212]. It is also interesting to note that p53 binds to DLEC1 promoter and upregulates it [212].

MAP2K4 is an essential part of the JNK signaling pathway. The protein is found to be mutated and non-functional in many cancer types and it is known that inactivation of MAP2K4 can drive the aggressiveness of K-Ras and mt-p53 tumors. [213]

SLC4A7 is a transmembrane protein of the sodium bicarbonate cotransporter family. Literature mining of p53 target genes, transcriptome data and bioinformatic analyses of p53 response elements by Garritano et al., 2013 identified SLC4A7 to be upregulated in the presence of mt-p53 [214]. SLC4A7 has also been identified as a tumour suppressor as disruption of this gene in mice can delay breast cancer development [215].

NDFIP1 belongs to a conserved group of proteins that act as activators of NEDD4 family of E3 ligases [147, 216]. NDFIP1 interacts with WW domains of E3 ligases through its proline rich (PY) domain, which releases them from auto-inhibition [148]. Protein substrates ubiquitinated by the effect of NDFIP1 can have different fates – degradation (DMT1), nuclear transport (BRAT1) or exosomal release (PTEN) [161, 165, 217]. This gene has not been linked to wild type or mt-p53 and therefore, we sought to explore the mechanism of NDFIP1 mediated mt-p53 regulation using cancer cell line models.

3.5 Discussion

In this chapter, novel mt-p53 regulators that were identified in our genome-wide RNAi screen were profiled for known functions, using bioinformatic pathway analyses tools and extensive text mining. Intriguingly, the top pathways identified from our screen to regulate mt-p53 are RNA related: the Spliceosome and RNA degradation.

Pertinently, the spliceosome has been demonstrated to influence p53 levels. Specifically, targeting the spliceosome or mutation of its components have been demonstrated to trigger p53 accumulation. This is linked to reduction in the levels of negative regulators of p53, including MDM2 and MDM4 [199-201]. This p53:spliceosome connection provides an exciting basis for investigating a comparable link to mt-p53, as predicted by its accumulation in response to RNAi knockdown of spliceosomal components. This also reveals that there are similarities between wild type and mt-p53 regulation and therefore studying the mechanisms in cancer in detail would open up new avenues to specifically target mt-p53 expressing cancers.

It is important to ascertain whether the effect of knockdown of RNA related factors affects mt-p53 directly at the RNA level or instead at its protein levels, for example by indirect mechanisms where regulators of mt-p53 are the direct targets. Further studies can explore the actual mechanism of mt-p53 stability and identify possible drug targets.

The other pathway of interest is the Sphingolipid biosynthesis pathway. The probability of 3 enzymes involved in the same biosynthetic pathway to show the same p53 phenotype is unlikely to be coincidental. Two of the enzymes, ASAH1 and CERK convert ceramide to sphingosine and ceramide kinase respectively and considering the association between ceramide and p53, it is apparent that this metabolic pathway and its metabolites play a role in mt-p53 regulation in cancer cells [218]. This is interesting because there is a high degree of crosstalk between the sphingolipid pathway and the mevalonate pathway [219]. Also, mt-p53 and mevalonate pathway exist in a feed forward loop, with metabolites in the pathway playing a role in mt-p53 regulation [220]. It is possible that both the sphingolipid and the mevalonate pathway are working together in maintaining mt-p53 stability. Perturbations in these pathways indicate that metabolic landscape of cancer cells is altered by mt-p53 and the metabolites in turn bolster mt-p53 accumulation resulting in a positive loop favouring tumour progression. Given the growing interest in targeting metabolic pathways and the successful reduction in mt-p53 levels by statins, we will be testing the effect of targeting the sphingolipid pathway [76].

Mt-p53 stability was considered when defining end points of the knockdown experiments in the screen. Immunostaining of mt-p53 was done at 96hrs after siRNA knockdown to measure changes affecting mt-p53 stability.

Interestingly, we did not observe any preferential gene overlap between the cells lines, grouped based on the expression levels of mt-p53 – MDA-MB-231 with MDA-MB-465 or JH-EsoAd1 with AU565, As expected, the number of candidates identified to decrease mt-p53 upon knockdown were much higher in JH-EsoAd1 and AU565. The lower limit of the cut-offs for validation tests were set to 0.9 to reflect the cut-offs used in the tertiary screen and the upper limit was raised to 1.2 to increase stringency. The validation experiments must be performed in more cell lines in the future to confirm the results of the screen. Results from the validation test of candidates by immunoblotting complement the RNAi screen results and led us to the selection of 5 candidates with the potential to regulate mt-p53 with a greater degree of certainty. Of all the 5 candidates discussed in section 3.2.1, NDFIP1 is a novel candidate previously unrelated to p53. As an adaptor protein, NDFIP1 binds and activates NEDD4 E3 ligases resulting in ubiquitination of various substrate proteins,

which have proven roles in a number of cellular functions like cell survival, iron accumulation, inflammation [146]. We predicted that this adaptor protein could affect mt-p53 stability and it is probable that this association is vital in cancer cells. To test this prediction, we generated several cell line models and constructs and performed mechanistic studies, which yielded interesting insights into this pathway. In light of the fact that the aspect of mt-p53 regulation with respect to NEDD4 family of E3 ligases is underexplored, we decided to investigate this mechanism methodically and the observed results are described in detail in the next chapter.

Chapter 4

Deciphering the mechanism of regulation of mutant p53 by NDFIP1

4.1 Preface

The aim of this chapter is to establish the mechanism of mt-p53 regulation that is exerted by NDFIP1, as reflected in the RNAi discovery screen.

I thank Prof. Sharad Kumar (Centre for Cancer Biology, University of South Australia) for the kind gift of the construct, pcDNA3-NDFIP1-Flag used in this study. The qRT-PCR experiment involving JH-EsoAd1-shNDFIP1 cell line was performed by Arielle Kogan, Research Assistant in our lab. The BiFC experiments in Cos cell lines and the exosome experiment represented in figure 4.8B were performed in A/Prof. Jason Howitt's lab, The Florey Institute of Neuroscience and Mental Health. Confocal images represented in figures 4.2B, 4.3 and 4.5A were taken by Chad Johnson (formerly at Centre for Advanced Histology and Microscopy (CAHM), Peter MacCallum Cancer Centre). Confocal images represented in figures 4.4B, C, D were taken by Dr. Cristina Gamell, former postdoctoral fellow in our lab. I acknowledge Tahlia Procter, former member of the tumour suppression lab for assistance in the generation of MCF7-shNDFIP1 cell line. I acknowledge Dr. Simon Keam, a postdoctoral fellow in our lab for his assistance in the preparation of figures 4.1A, B, 4.2A, B, 4.4A, B, D, C and 4.9.

All experiments otherwise mentioned below were done thrice. The experiment represented in figure 4.1 was performed once (n=1). The experiments represented in figures 4.2C, 4.5B, 4.6A, 4.7 were performed twice (n=2).

4.2 Introduction

The NEDD4 E3 ligase family comprises of closely related ligases, all of which contain a C-terminal catalytic HECT domain. The NEDD4 ligase subset differ

markedly from other ligases of this 'HECT family' due to the presence of a C2 domain and multiple WW domains, which are important for lipid and substrate binding respectively. NEDD4 E3 ligases become catalytically active when their WW domain binds to the adaptor protein NDFIP1 through its PPXY motif (Fig 1.6) [142].

Since mt-p53 is largely regulated at the post-transcriptional level, it is of great interest to explore the possibility that its levels can be influenced by an adaptor protein that mediates engagement with relevant E3 ligases. The primary consequence of ubiquitination is degradation through the proteasomal machinery. However other functional changes are also observed [221]. In particular, mt-p53 is known to be ubiquitinated at multiple sites by multiple E3 ligases of the RING, HECT and U-box type, resulting predominantly in degradation and in certain instances in stabilization, modification of transcriptional activity and nuclear export (reviewed in [88, 222]).

We hypothesize that NDFIP1 is involved in ubiquitination of mt-p53 by activating one of the members of the NEDD4 E3 ligase family. However it is also possible that NDFIP1 acts indirectly through one of the known regulators of mt-p53. The aim of this chapter is to validate NDFIP1 as a regulator of mt-p53 levels and to elucidate its mode of action in cancer cells. In this thesis, the human (NDFIP1) and the mouse version (Ndfip1) of the candidate gene will be referred to as NDFIP1 without distinction.

4.3 Results

4.3.1 NDFIP1 knockdown affects mt-p53 at the post-transcriptional level

In order to corroborate the results of the RNAi screen, we knocked down NDFIP1 using siRNA in JH-EsoAd1 and AU565 and checked the expression levels of mt-p53 at the protein level through western blotting. Both AU565 and JH-EsoAd1 have missense mutations in p53 and express mutant forms with gain of function properties. As we had observed in the screen, mt-p53 protein levels increased upon NDFIP1 knockdown in both AU565 and JH-EsoAd1cells (Fig 4.1A, B). These findings confirmed that cellular mt-p53 protein levels were negatively regulated by NDFIP1. This raised the question whether NDFIP1 was directly controlling mt-p53 at the protein level and/or at the RNA level? We made use of both siRNA and shRNA

mediated knockdown of NDFIP1 in JH-EsoAd1 and AU565. Transfections using siRNA were performed as described in section 2.4. Cell lines with inducible NDFIP1 knockdown were created for ease of control of NDFIP1 expression and to avoid sub-optimal siRNA transfection. Optimization of Doxycyclin concentrations were performed as described in section 2.3.2.

To determine whether NDFIP1 affects p53 protein or mRNA levels, the levels of p53 mRNA upon NDFIP1 knockdown in AU565 cells were then checked, but no significant increase in mt-p53 expression was observed (Fig 4.1C). We also transduced JH-EsoAd1 cells to stably express a Doxycycline (Dox) inducible NDFIP1 shRNA construct and examined mt-p53 mRNA levels upon Doxycycline treatment (Fig 4.1D). As in the case of AU565, we did not observe any modulation in mt-p53 mRNA levels in response in NDFIP1 reduction. This result substantiates our hypothesis that NDFIP1 regulation of mt-p53 is at the post-transcriptional level.

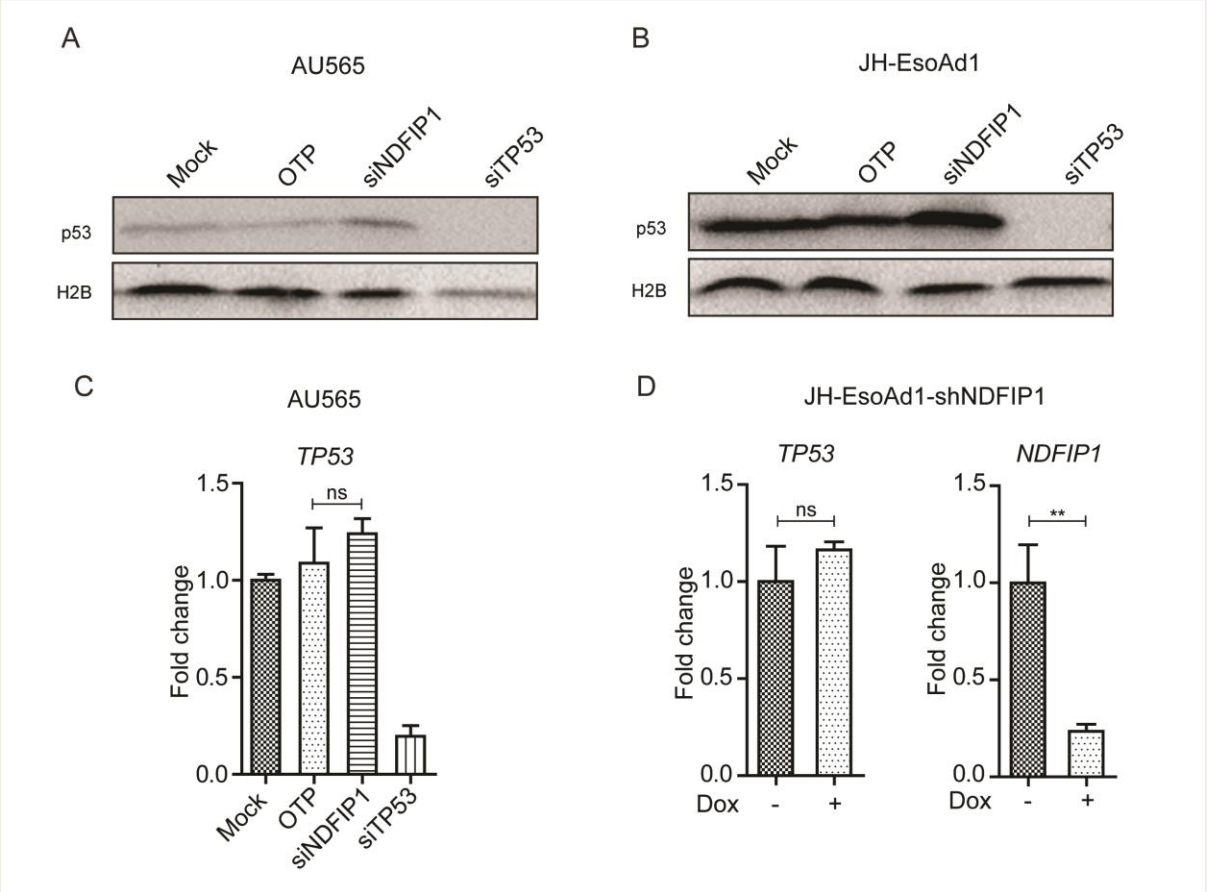


Figure 4.1: NDFIP1 knockdown increases mt-p53 protein levels.
Western blot analysis of AU565 **(A)** and JH-EsoAd1 **(B)** cells transfected with NDFIP1 siRNA and negative control (OTP). Increase in mt-p53 protein levels is observed upon

NDFIP1 knockdown. **C.** NDFIP1 levels were targeted in AU565 cells by siRNA and p53 mRNA levels were checked using quantitative RT-PCR. The mRNA expression levels were normalized to the housekeeping gene, *hRPL37a* and expressed relative to Mock or no Doxycycline. **D**. JH-EsoAd1-shNDFIP1 cells were treated with 10ng/ml Doxycycline for 3 days and the *NDFIP1* and *TP53* mRNA levels were analyzed using quantitative real time-PCR.

4.3.2 NDFIP1 interacts with mutant p53

We were interested to test whether NDFIP1 dependent regulation of cellular mt-p53 levels could be mediated through direct physical interaction between the two proteins. NDFIP1 has been identified as a transmembrane protein, which is predominantly observed in specific sorting organelles, such as the Golgi body and the endosomal compartments [147, 168]. On the other hand, localization of mt-p53 has not been reported in endosomes, even though p53 is known to shuttle between the nucleus and cytoplasm [223].

In order to study both interaction and localization of NDFIP1 and mt-p53, we employed a fluorescent technique called the bimolecular fluorescence complementation (BiFC) assay. This assay relies on the capacity of two non-fluorescent peptide fragments to enter close proximity and configure into a fluorescent protein. If two putative protein partners are chemically linked to these two complementary non-fluorescent peptides, direct binding of the proteins will be visualized through the fluorescence emitted upon engagement of their tags [224]. We utilized mt-p53 tagged with N-terminal Venus domain (VN^{1-173}) and NDFIP1 tagged with C-terminal Venus domain ($VC^{155-238}$) for BiFC assay (Fig 4.2A). Specifically, we created plasmid constructs expressing two common hotspot mutants of p53 R273H and R175H tagged to VN and transiently transfected these with VC tagged NDFIP1 to perform BiFC assay. These hot spot mutants were selected for our current study as these mutations are highly common and pathogenic. These mutants were also expressed in the cell lines used in the screen – R273H in MDA-MB-468 and R175H in AU565. We chose Cos cells, which due to its large size is ideal for visualizing BIFC signal. In addition to Cos, we also utilized H1299, a non-small cell lung cancer which is null for p53. We observed interaction and colocalization of mt-p53 and NDFIP1 in H1299 and Cos cells, indicating that the two proteins physically bind to each other (Fig 4.2 B,C). Endosomal markers, Rab5,

Rab7, Rab9 and Rab11 tagged with red fluorescent protein (RFP) were co-transfected with the BiFC constructs to identify whether the interaction occurs in the early or the late compartments of the endocytic pathway [225].

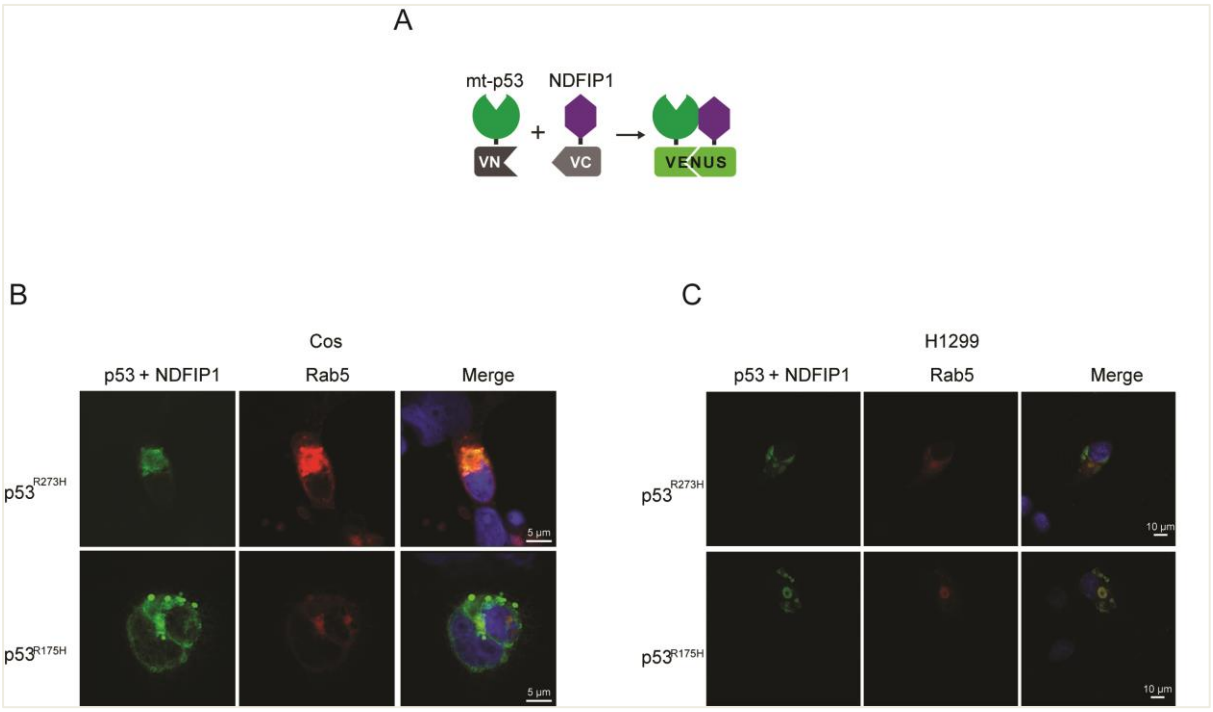


Figure 4.2: NDFIP1 interacts with mt-p53.
A. Schematic representation of the BiFC assay showing VN-tagged mt-p53 and VC-tagged NDFIP1. Co-expression of Rab5 with BiFC constructs in Cos **(B)** and H1299 cells **(C).** Presence of functional Venus protein (Green) indicates interaction between NDFIP1 and mt-p53 (R273H and R175H). Nuclei are stained with DAPI (Blue) and early endosome marker protein, Rab5 is tagged with RFP.

Interaction between NDFIP1 and mt-p53 was predominantly observed within early endosomes (as indicated by co-localization with protein Rab5) and also in recycling endosomes (identified through co-staining with Rab11) (Fig 4.2 and 4.3). However, the BiFC signal was not observed to be colocalized with Rab7 and Rab9, which indicates that mt-p53 in association with NDFIP1 is not trafficked into the late endosomes or lysosomes (Fig 4.3B,C) [225]. Firstly, identification of mt-p53 in endosomes is a novel observation from these studies and predicts a previously unreported mode of p53 transportation in the cell. Secondly, as NDFIP1 is frequently found within endosomal compartments, it is valid to speculate that localization of mt-p53 to the endosome is promoted through its association with NDFIP1 [168, 170].

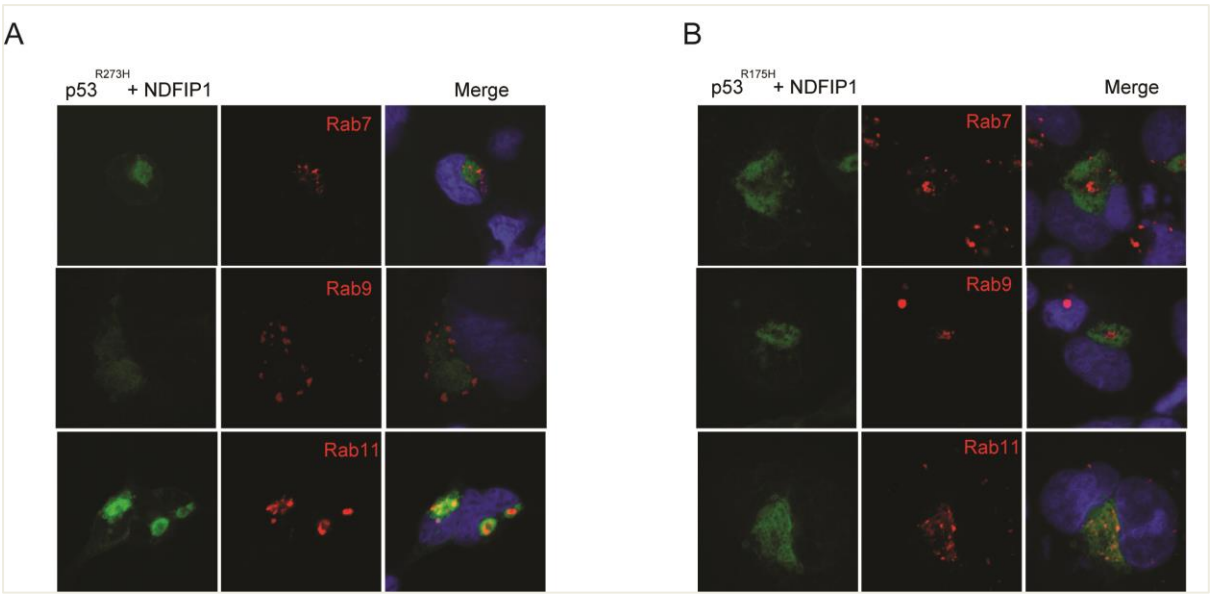


Figure 4.3: NDFIP1-mt-p53 interaction occurs in early endosomes.
Co-transfection of Rab-RFP with BiFC constructs VC-NDFIP1 and VN-p53R273H **(A)** or VN-R175H in Cos cells **(B)**. NDFIP1-mt-p53 BiFC signal is colocalized with Rab11 and not colocalized with Rab7 and Rab9 indicating that the interaction takes place in early and recycling endosomes but is not observed in late endosomes.

4.3.3 NDFIP1 is important for mutant p53 ubiquitination

As an adaptor protein, NDFIP1 can drive ubiquitination of multiple substrates of the NEDD4 family of E3 ligases. By extrapolation, we were interested to check whether p53 ubiquitination could be influenced by NDFIP1 [148, 150]. We performed a BiFC assay using mt-p53 and Ubiquitin, in an attempt to track and quantitate ubiquitinated mt-p53 (Fig 4.4A). To assess the possibility of tracking ubiquitinated mt-p53, we transfected Cos cells with VN-mtp53 and VC-Ubiquitin and observed fluorescent signal indicating successful tracking of p53-Ub (Fig 4.4B).

To directly visualize this interaction, we adopted primary cell systems: mouse embryonic fibroblasts (MEFs) of NDFIP1 WT and KO origin and co-transfected into them VN^{1-173}-mt-p53 and $VC^{155-238}$-Ubiquitin, with Rab5-RFP as a transfection control [165]. We noticed that there were significantly more $NDFIP1^{WT}$ MEFs with strong ubiquitinated mt-p53 signal, compared with the $NDFIP1^{KO}$ MEFs counterparts, suggesting that NDFIP1 enhances mt-p53 ubiquitination (Fig 4.4C). A representative BiFC image and the associated graph showing BiFC signal is shown in Fig 4.4C, D.

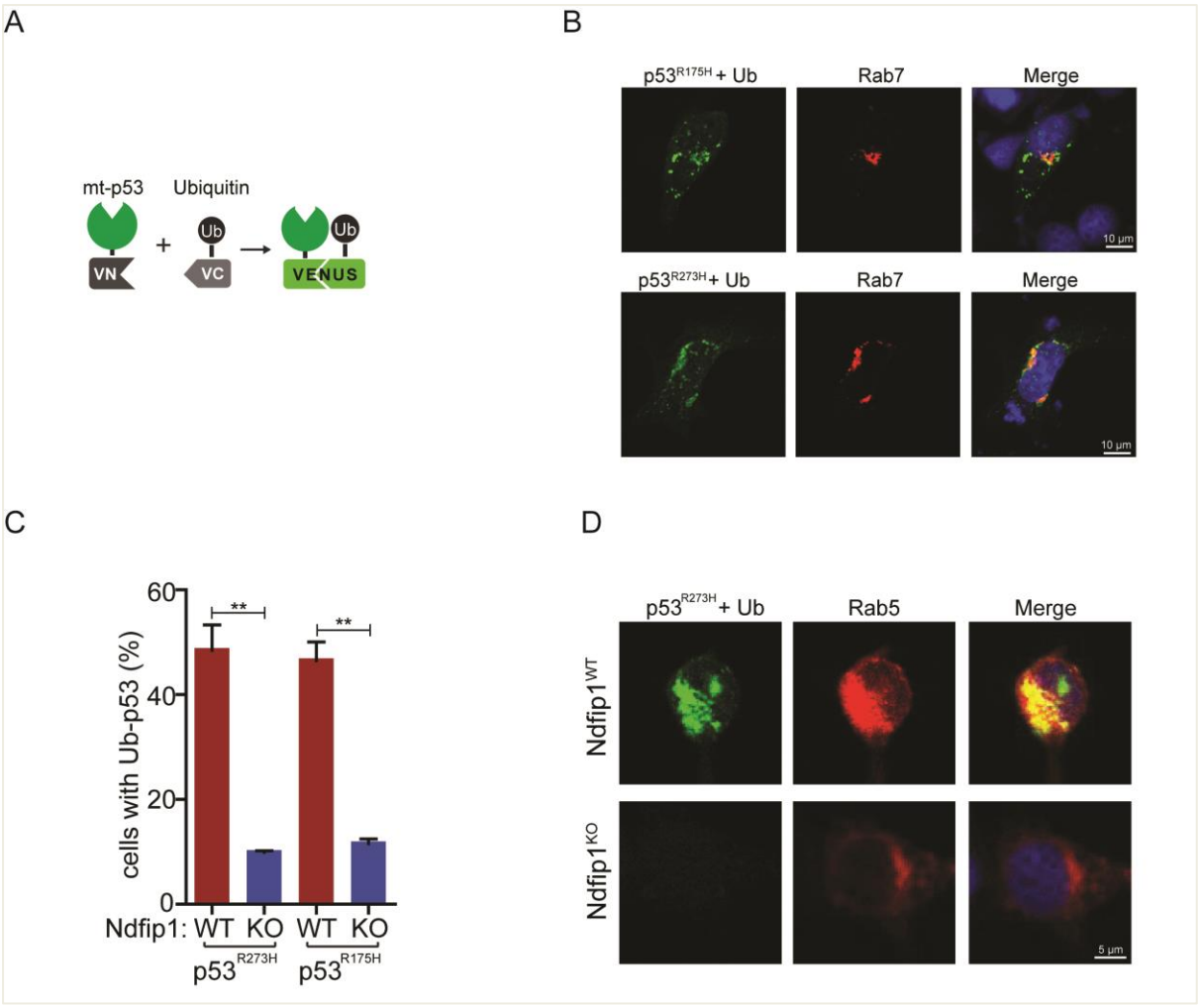


Figure 4.4: NDFIP1 promotes mt-p53 ubiquitination.
A. Constructs, VN-mtp53$^{R273H/R175H}$ and VC-Ubiquitin used to detect ubiquitinated mt-p53 are depicted in this figure. **B.** Cos cells were transfected with Rab7, VN-mt-p53 and VC-Ub and the BiFC image shows that ubiquitinated mt-p53 can be tracked in cells. **C.** NDFIP1^{WT} and NDFIP^{KO} MEFs were transfected with the BiFC constructs and Rab5-RFP and the number of Venus+ cells detected were normalized to the number of RFP+ cells to calculate %Ub-p53 in these cells. The graph represents the number of cells with ubiquitinated mt-p53 and there is significant reduction in ubiquitination of mt-p53 in the absence of NDFIP1. **D.** A representative BiFC image showing ubiquitinated mt-p53 in NDFIP1^{WT} MEFs.

4.3.4 NDFIP1 also promotes p53 ubiquitination

We were interested to explore whether this newly discovered NDFIP1 interaction is exclusive to mt-p53 or whether engagement with p53 also occurs. To this end, we tested interaction of p53 with NDFIP1 through BiFC assay as described in section 4.2. BiFC signal representing p53 and NDFIP1 was observed in both H1299 and Cos cells as with mt-p53 (Fig 4.5 A,B). The NDFIP1-p53 interaction was also found to be colocalized with Rab5 and Rab11 mirroring the results of NDFIP1-mt-p53 BiFC assay (Fig 4.5A).

We next performed p53-Ubiquitin BiFC assay in NDFIP1 WT and KO MEFs. The number of NDFIP1 KO cells showing BiFC signal was very low when compared to that of NDFIP1 WT cells revealing the NDFIP1 is also capable of promoting p53 ubiquitination (Fig 4.5C). As both mouse and human NDFIP1 have a homology of 97%, the results of the BiFC assay extend to the human version of NDFIP1. Taken together, these results indicate that NDFIP1 promotes this ubiquitination pathway independent of p53 mutation status. It also predicts that NDFIP1 has a role in normal cells with respect to p53, however exploring the consequence of this interaction and ubiquitination is beyond the scope of this thesis.

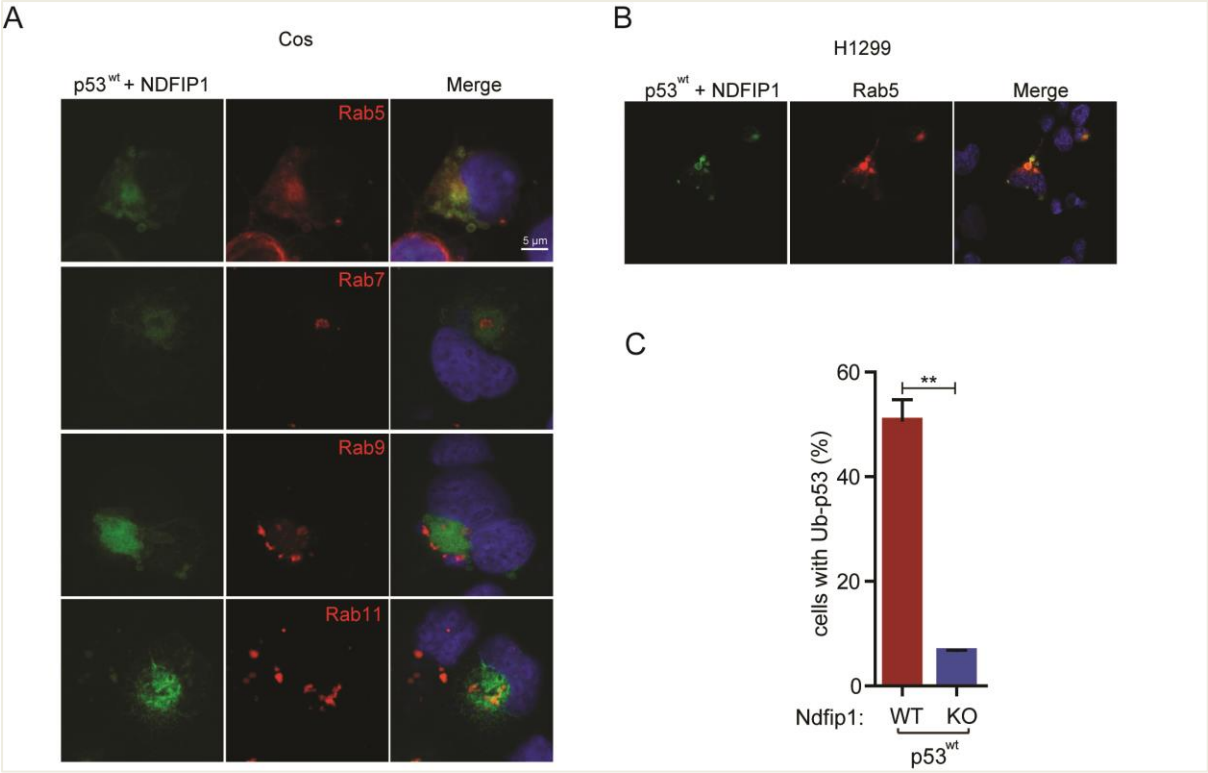


Figure 4.5: NDFIP1 interacts with p53 and enhances its ubiquitination.
BiFC assay showing interaction of NDFIP1 and p53 in Cos cells **(A)** and H1299 cells **(B). C.** Graph representing the percentage of cells with ubiquitinated p53 in NDFIP1 WT vs KO MEFs. Significant decrease in ubiquitination of p53 is observed in NDFIP1 KO cells.

4.3.5 Half-life of p53 is not affected by NDFIP1

Ubiquitination of proteins can direct their degradation through the 26S proteasomal pathway. Results in the previous section, which demonstrated that NDFIP1 facilitates p53 ubiquitination, predict that this effect is catalyzed by one of the E3 ligases of the NEDD4 family. Following the observation from our screen that NDFIP1 influences mutant p53 stability (Chapter 3, section 3.2.1) and findings from section 4.1, we

questioned whether degradation through the proteasome would be the outcome of p53 ubiquitination mediated by NDFIP1. We chose to test this hypothesis initially in a wild type p53 setting, as the wild type protein has a shorter half-life than mt-p53 (between 5-20 min for wild type protein versus hours for the mutant). We proposed to compare p53 degradation in the presence or absence of NDFIP1. Conversely, in a mt-p53 expressing cell line, we proposed to overexpress NDFIP1 and measure the rate of mt-p53 degradation. If our hypothesis that NDFIP1 can cause mt-p53 degradation were to be true, we predicted that we would be able to observe accelerated mt-p53 degradation differentially in the presence of NDFIP1.

Accordingly, we first, tested this possibility in MCF7 human breast cancer adenocarcinoma cell line expressing p53 and transduced with a Doxycycline-inducible short-hairpin RNA against NDFIP1 (shNDFIP1). Specifically, we measured the half-life of p53 in MCF7 cells in the presence of NDFIP1 and following its knockdown. Upon NDFIP1 knockdown and cycloheximide treatment, we followed p53 levels over 60 min. Contrary to our prediction; we did not observe any delay in p53 degradation in response to NDFIP1 knockdown (Fig 4.6B). We repeated a similar experiment in the mt-p53 line, JH-EsoAd1 that had been used in the screen, but modified with the introduction of a Doxycycline-inducible NDFIP1-overexpression system. Also in this case, JH-EsoAd1-NDFIP1 cells induced for NDFIP1 overexpression and subjected to cycloheximide chase assay did not manifest a significant change in mt-p53 half-life (Fig 4.6A). Together these results rule out degradation as a consequence of NDFIP1-p53 interaction and indicate that other mechanisms must be responsible for the altered levels of cellular mt-p53 that were identified in the screen.

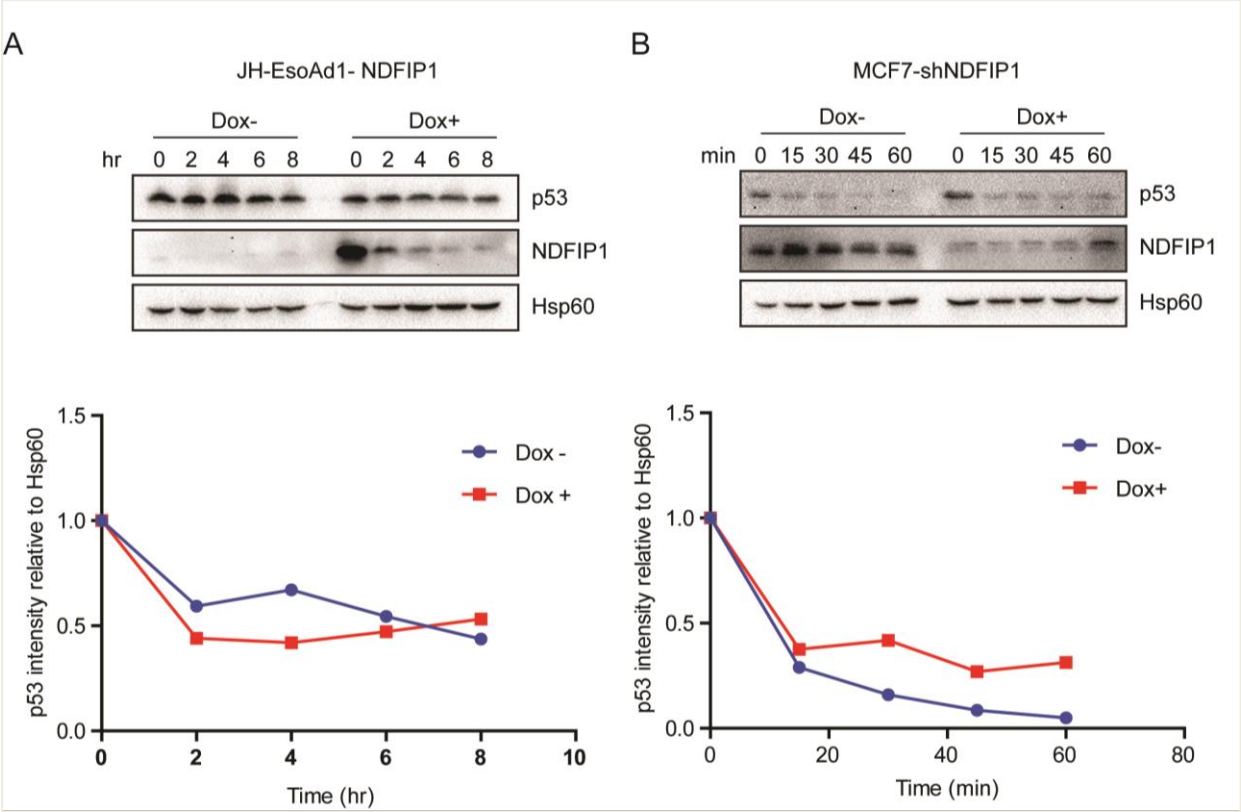


Figure 4.6: NDFIP1 does not have a major effect on p53 protein half-life in cancer cells.
A. JH-EsoAd1 cells with Doxycycline inducible NDFIP1 overexpression were subjected to CHX assay after 48hrs of Doxycycline (80ng/ml) induction and the mt-p53 levels were tracked over time. Graph shows densitometric analysis of p53 expression relative to Hsp60 and indicates no change in half-life upon NDFIP1 overexpression. **B.** MCF7-shNDFIP1 cells were created by transducing Doxycycline-inducible NDFIP1 knockdown construct in the cells. These cells were subjected to CHX assay after 72hrs of Doxycycline (10ng/ml) induction and the p53 expression was tracked over time. Graph shows densitometric analysis of p53 expression relative to Hsp60 and indicates no change in half-life upon NDFIP1 overexpression.

4.3.6 NDFIP1 overexpression results in exosomal release of p53

In addition to its identity as an integral membrane protein of the Golgi apparatus and sorting vesicles, NDFIP1 recruits several proteins to extracellular vesicles [168]. In order to check if mt-p53 is similarly channeled by NDFIP1 into exosomes, culture supernatants were collected using ultracentrifugation, from cell lines with mt-p53 and overexpressing NDFIP1 (Fig 4.7A). We transiently co-transfected either H1299 (a p53-null non-small cell lung cancer cell line) or HEK293 cells with NDFIP1 and p53R273H mutant and observed an increase in mt-p53 protein in their corresponding exosomes (Fig. 4.7B, C). Lower NDFIP1 expression observed in co-transfection of

NDFIP1 and p53-R273H could be due to the strength of promoter of these expression constructs and this was avoided by repeating the experiment in cell lines with stable overexpression of NDFIP1. Similarly, we observed an increase in p53 levels in the exosome fraction of JH-EsoAd1-NDFIP1 cells, indicating that NDFIP1 promotes the export of mt-p53 through vesicles (Fig 4.7D). In H1299 cells with stable constitutive expression of p53R273H mutant, endogenous p53 was identified in exosomes, presumably due to endogenous NDFIP1. Pertinently, levels of mt-p53 in the exosomes increased in response to NDFIP1 overexpression (Fig 4.7E). Additional experiments in HEK293 cells revealed that in response to NDFIP1 overexpression, p53R175H and wild type p53 respectively could also be promoted into exosomes. Together these novel findings indicate that NDFIP1 is able to recruit either wild type or mt-p53 into extracellular vesicles/exosomes.

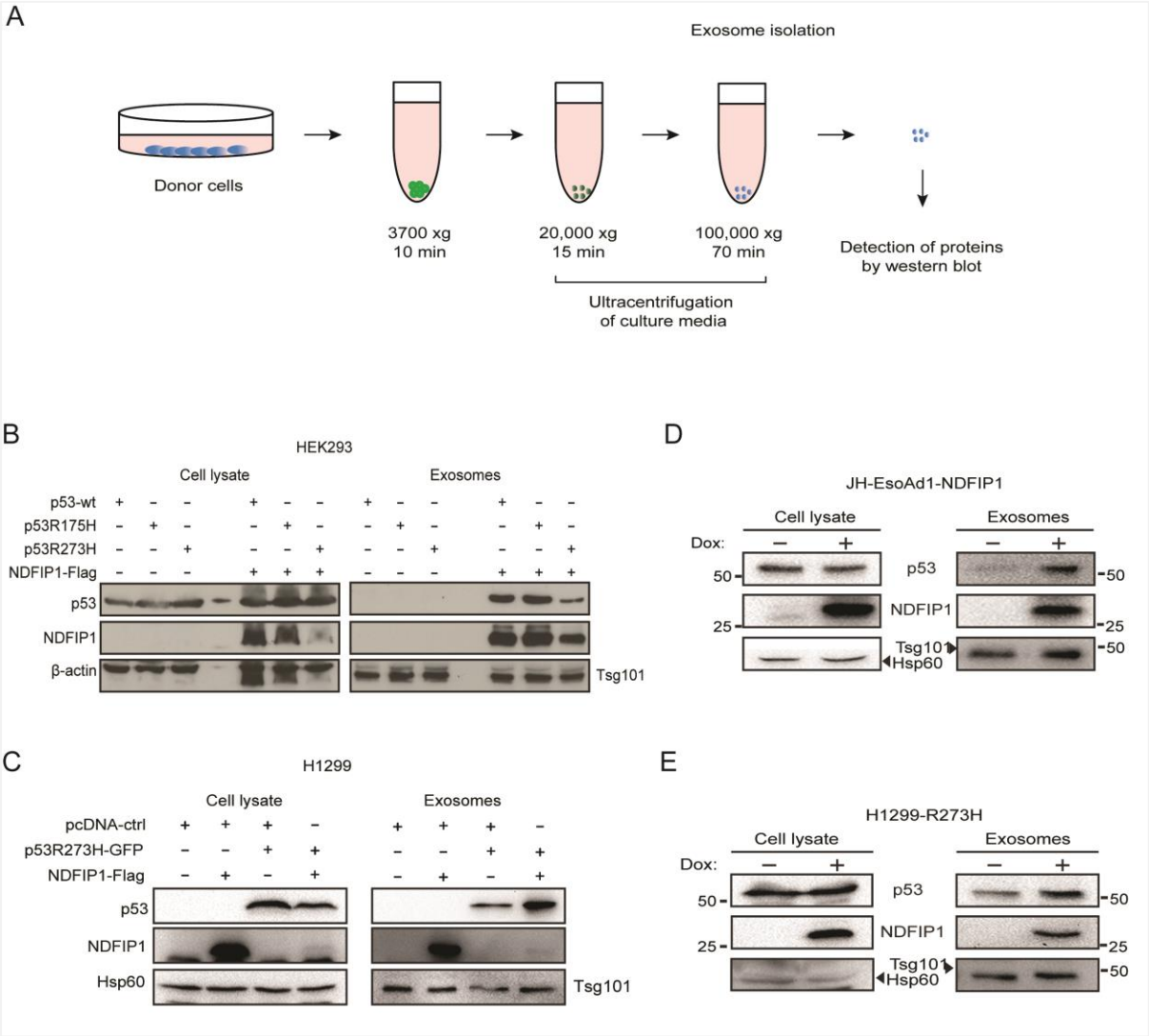


Figure 4.7: NDFIP1 overexpression results in release of mutant p53 in exosomes.
A. Experimental setup to isolate extracellular vesicles: Cell culture supernatant is centrifuged at 10,000rpm to remove cell debris and then at 35,000rpm to collect exosomes in the pellet. The pellet is then lysed and the proteins are detected by western blotting. Transient transfection of p53 with NDFIP1 in HEK293 **(B)** and H1299 **(C)** results in increased mt-p53 in the exosome fraction**.** Induction of NDFIP1 using Doxycycline in JH-EsoAD1-NDFIP1 **(D)** and H1299-R273H **(E)** also increases mt-p53 levels in exosomes.

4.4 Discussion

Mt-p53 accumulation in cancers predicts that it is being inadequately regulated at the protein level. Mt-p53 stability can be partly attributed to chaperones, deubiquitinases and lack of negative feedback loops [53]. The identified function of NDFIP1 as an E3 ligase activator and its impact on mt-p53 levels in our screen encouraged us to pursue its relationship with p53. We validated the results of the RNAi screen by confirming that NDFIP1 expression has a subtle inverse effect on mt-p53 protein levels in cells. However, there is no concomitant change in p53 mRNA levels, which substantiates that NDFIP1 mediated regulation of mt-p53 is post-transcriptional and aligns with the known function of NDFIP1 as an E3 ligase modulator.

Previous studies have reported that direct binding of NDFIP1 to its target substrates is required for effective substrate-ubiquitination by the respective E3 ligases [148, 161, 226]. In fact, our results reveal that direct physical interaction between NDFIP1 and mt-p53 takes place in the cytoplasm and based on colocalization studies with specific endosomal markers, it is clear that the spatial location of NDFIP1-mtp53 complexes is observed in early (Rab5) and recycling (Rab11) endosomes and not late endosomes (Rab7) or lysosomes (Rab9) [227, 228]. There is no previous reports of p53 localization in endosomes and it is possible that interaction with NDFIP1 (most often located in golgi and endosomes) is pivotal for the recruitment of mt-p53 in early endosomes [147, 216, 223]. Absence of NDFIP1-mtp53 in the late endosome (LE) and lysosome effectively rules out the probability of p53 degradation through the endolysosomal system.

Several studies have linked ubiquitination of proteins to trafficking through early endosomes, to multivesicular bodies, which upon fusion with the plasma membrane release from cells, bi-lipid vesicles as exosomes [229]. It is established that NDFIP1-

NEDD4 mediated ubiquitination is important to target proteins to multivesicular bodies in concert with the ESCRT protein complexes (Endoplasmic sorting complex required for transport), which are indispensable for almost all steps of exosome biogenesis [168]. Also, Rab11 (with which NDFIP1-mt-p53 interaction is colocalized) promotes fusion of multivesicular bodies to plasma membrane to trigger the release of exosomes [230]. These previous reports, together with our results from NDFIP1 overexpression cell systems are consistent with a model in which NDFIP1 enhances mt-p53 ubiquitination and subsequent trafficking into the endocytic pathway and release as exosomes (Fig 4.8).

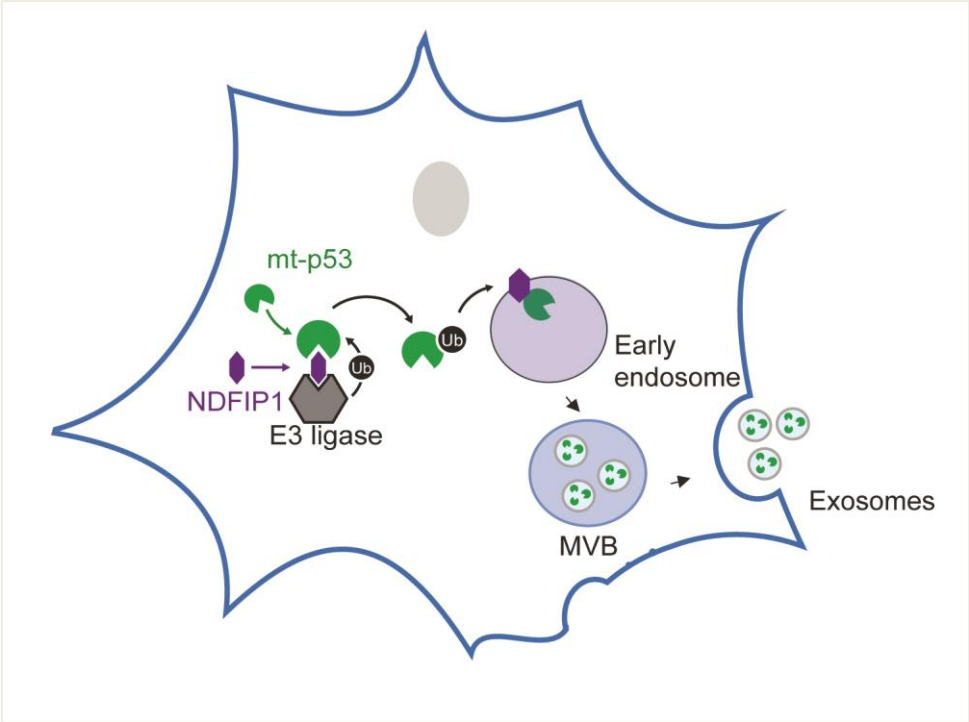


Figure 4.8: Model depicting the predicted mechanism of packaging of mt-p53 into exosomes.

Our results also show that the type of mutation (R273H and R175H) does not influence or hinder interaction with NDFIP1, p53 ubiquitination, or subsequent recruitment into exosomes. Future experiments involving BiFC constructs of p53 and Ubiquitin in human cancer cell lines with knockout of NDFIP1 gene in addition to in vivo ubiquitination assays need to be performed to validate the results from the BiFC assays. Although there are numerous reports on the effects of both wild type and mt-p53 on the endocytic pathway, there is much less information on mt-p53 protein itself in exosomes [125, 231, 232]. In 2009, Lee et al discovered that in a K-Ras mutant cell line, Snail expression results in exocytosis of p53. The released p53 however is

degraded upon proteinase treatment indicating that p53 is not protected in a vesicle [233]. This also implies the involvement of a completely different pathway compared to our findings in the absence of K-Ras mutation. A recent study revealed that overexpression of p53 could cause export of the protein into exosomes [234], however no underlying mechanism was identified. Our novel findings indicate that the packaging of p53 into exosomes is promoted by NDFIP1-mediated ubiquitination.

The only member of the NEDD4 E3 ligase family which is known to ubiquitinate p53 is WWP1 and this ubiquitination results in cytoplasmic localization of p53 and increased stability [180]. This suggests that WWP1 could be the missing link in the NDFIP1-mt-p53 pathway; however it is important to note that NDFIP1 mediated ubiquitination of mt-p53 does not alter its stability. Also, WWP1 has been studied only in the context of the wild type protein and the findings may not extend to accumulating p53 mutants in a cancer setting. Furthermore, ubiquitination of substrates through the NEDD4 family can involve both homo and heterodimerization of E3 ligases and so this does not rule out the possibility that multiple NEDD4 E3 ligases interchangeably or cooperatively ubiquitinate p53 [164, 235, 236]. A significant part of mt-p53 stability and GOF in cancer can be attributed to differences in binding efficiencies of wild type and mt-p53 to protein partners and therefore biochemical experiments need to be performed to test NDFIP1-p53 interaction efficiencies in normal and cancer conditions [37]. NDFIP1 mediated mt-p53 ubiquitination and the consequences are highly unlike the effects of MDM2 in maintaining steady state levels of p53 and therefore the conditions that trigger release of exosomes must be different and the process strictly regulated.

In this chapter, we examined the mechanism of regulation of mt-p53 by NDFIP1 through a number of breast, esophageal, lung cancer cell line models and primary MEFs. We conclude that NDFIP1 affects mt-p53 post-transcriptionally and enhances its ubiquitination. NDFIP1 also interacts with mt-p53 and the complexes are found in specific compartments of the endosomal pathway. Cancer cells with NDFIP1 overexpression show negligible change in mt-p53 levels in the cell lysate but increased mt-p53 in exosomes indicating the presence of a novel NDFIP1 mediated pathway for export of oncogenic mt-p53 from cancer cells into the tumour microenvironment.

Chapter 5

Establishing novel functional assays for studying exosome mediated mt-p53 gain of function

5.1 Preface

The aim of this chapter is test for uptake of exosomal mt-p53 by tumour cell lines and to design functional assays to determine the impact of its uptake.

Analyses of TCGA data for generation of survival curves represented in Fig 5.5A, B were performed by Franco Caramia, a PhD student of our lab. I acknowledge Dr. Simon Keam, a postdoctoral fellow in our lab for his assistance in the preparation of figures 5.3, 5.5A&B, 5.6. All experiments otherwise mentioned below were done thrice. The experiment represented in figure 5.3, 5.4D were done twice (n=2). The experiments represented in figure 5.4C were done once (n=1).

5.2 Introduction

Missense p53 mutants are highly stable and partner with proteins in a manner that is distinct from p53. Further, p53 mutants influence pathways that drive a cancer phenotype that is more aggressive than those associated with p53 null tumours. The extent and diversity of the gain of function (GOF) effects have been discussed in detail in Chapter 1, Section 1.2. Accumulation of mt-p53 is absolutely critical for effectiveness of oncogenic functions of mt-p53 and therefore its depletion can reduce cell proliferation, tumor malignancy.

In the previous chapter, we discussed a novel pathway involving NDFIP1 driven recruitment of mt-p53 into exosomes. Exosomes are a mode of intercellular communication and several studies have shown that tumour derived exosomes are central to promoting tumour establishment and metastasis. Specifically, signaling between cells of the tumor microenvironment such as endothelial cells, stromal cells, and immune cells, and cells at distant metastatic sites have been shown to involve exosomes [237]. Internalization of exosomes by cells is dependent on

ligand/receptor engagement between exosomes and recipient cells. Signaling mediated by exosomes is primarily driven by the contents. Multiple studies have revealed that the protein contents of exosomes can function in the recipient cells as well. In fact, PTEN transfer between cells by NDFIP1 is known to reduce AKT phosphorylation in the recipient cells [169]. Even in normal physiological conditions, exosomes are important for intercellular communication and for coordination of various functions including protein clearance and immunity. On the other hand, cancer cells use exosomes such that it favours their growth, survival and propagation [238]. We hypothesize that tumour cells expressing mt-p53 protein package the oncogenic protein in to exosomes through NDFIP1 and the exosomal mt-p53 contributes to aggressiveness of these tumours over p53 deficient or wild type p53 expressing tumours.

A recent study revealed that overexpression of p53 can result in packaging of the protein into exosomes and these exosomes were found to have an anti-proliferative effect on recipient cells, which was attributed to p53 function [234]. In this chapter, we describe the outcome of NDFIP1 mediated trafficking of mt-p53 into exosomes. We hypothesize that exosomes are internalized by recipient cells and mt-p53 from these exosomes enhances the oncogenic properties of recipient cells.

5.3 Methods

5.3.1 Cell line models created to study NDFIP1-mutant p53 pathway in cancer

In the previous chapters, we had extensively worked on the cell lines which harbor pathogenic mutations of p53 – MDA-MB-468 (R273H), JH-EsoAd1 (G266E). However, to study exosomal mt-p53 effects, it was important to generate a set of compatible donor and recipient cell lines. The recipient cells need to be devoid of p53 to examine uptake of exosomes and functional effects.

As donor cell lines, we made use of isogenic non-small cell lung cancer cell lines generated in our lab - H1299 parental (H1299par), which is p53 deficient and H1299R273H, which has stable overexpression of mt-p53 (R273H). We transduced H1299par and H1299R273H with a construct for Dox inducible NDFIP1 expression

so that we have isogenic lines, which upon Dox treatment, overexpresses NDFIP1 and releases exosomes with no p53 and mt-p53 (R273H) respectively.

The parental cell line H1299 lacking p53 is an ideal cell line for transfection of wild type or mt-p53 and loading of the protein into exosomes. The parental glioblastoma LN18 cells are heterozygous for p53, with a wild type allele and C238S mt allele and therefore would have a heterogenous population of p53 protein and are not well suited for this study [239, 240]. Therefore, we stably knocked out p53 from LN18 using CRISPR/Cas9 system and then performed single cell selection to select for p53 knockouts. The clone LN18p53KO can be used as both a donor (by transfecting NDFIP1 and mt-p53) and an exosome acceptor. We also have osteosarcoma cell line, Saos2, which can be used as an exosome acceptor due to the lack of p53 [241]. Table 5.1 lists all the cell lines generated to study the effects of exosomal mt-p53.

Table 5.1: List of cell lines generated/used in the study of NDFIP1-mutant p53 pathway

<table>
<tr><th colspan="4">Exosome donor cell lines</th></tr>
<tr><th>Cell line</th><th>Cancer cell line type</th><th>p53/NDFIP1 status</th><th>Experiment to prepare exosomes with mt-p53</th></tr>
<tr><td>H1299par</td><td>Non-small cell lung carcinoma</td><td>p53 deficient</td><td rowspan="3">Transient transfection of NDFIP1 and mt-p53</td></tr>
<tr><td>LN18par</td><td>Glioblastoma</td><td>p53wt/C238S</td></tr>
<tr><td>LN18p53KO</td><td>Glioblastoma</td><td>CRISPR knockout of p53</td></tr>
<tr><td>H1299par-NDFIP1</td><td rowspan="2">Non-small cell lung carcinoma</td><td>p53 deficient and Dox inducible overexpression of NDFIP1-flag</td><td rowspan="2">Dox induction to increase NDFIP1 expression</td></tr>
<tr><td>H1299R273H-NDFIP1</td><td>Stable expression of mt-p53R273H and Dox inducible overexpression of NDFIP1-flag</td></tr>
<tr><th colspan="4">Exosome recipient cell lines</th></tr>
</table>

H1299par	Non-small cell lung carcinoma	p53 deficient	
LN18p53KO	Glioblastoma		
Saos2	Osteosarcoma		

5.3.2 Experimental design for assessing uptake of exosomes

The "donor" exosomes were collected from H1299par cells (~ $4X10^6$) that were transiently transfected with NDFIP1 and p53R273H to promote mt-p53 loading into exosomes. Exosomes isolated by ultracentrifugation from the cell culture supernatant of the donor cells were then introduced to the "recipient" H1299 cells ($1.25X10^4$ cells seeded on 24-well plates) that had been plated on coverslips one day prior to exosome isolation. Specifically, donor exosomes were incubated with the acceptor cells in serum-free media for 24 hours, after which the cells were fixed with 100% methanol and stained for p53.

5.3.3 Experimental design to study the functional effects of exosomal mutant-p53 in recipient cells

The experimental setup is similar to the one described in section 5.3.2 and is shown in detail in Fig 5.1. H1299 donor cells were seeded on 10cm dishes and then transiently transfected with control constructs, NDFIP1 only, p53R273H only and NDFIP1+p53R273H using PEI. The transfection media was removed after 1.5hrs and then replenished with 12ml of complete RPMI. Exosomes were isolated from these donor cells 24hr after transfection using ultracentrifugation. Exosomes from each experimental arm were added to recipient cells (also H1299par, $0.03X10^6$ cells in a 12-well) in presence of reduced serum (2%FBS). Exosomes were isolated again at the 48hr time point from the donor cells and added to the recipient cells. One hr after the second exosome feeding, the recipient cells were harvested for functional assays – qRT-PCR and Invasion assay.

Total RNA was isolated from the recipient cells and qRT-PCR for select mt-p53 target genes were performed. For invasion assays, recipient cells exposed to exosomes were trypsinized and plated on transwells coated with matrigel and allowed to invade across a serum gradient. After 24hrs, the membrane was fixed and stained with DAPI and the number of cells that successfully crossed the membrane were counted using Fiji.

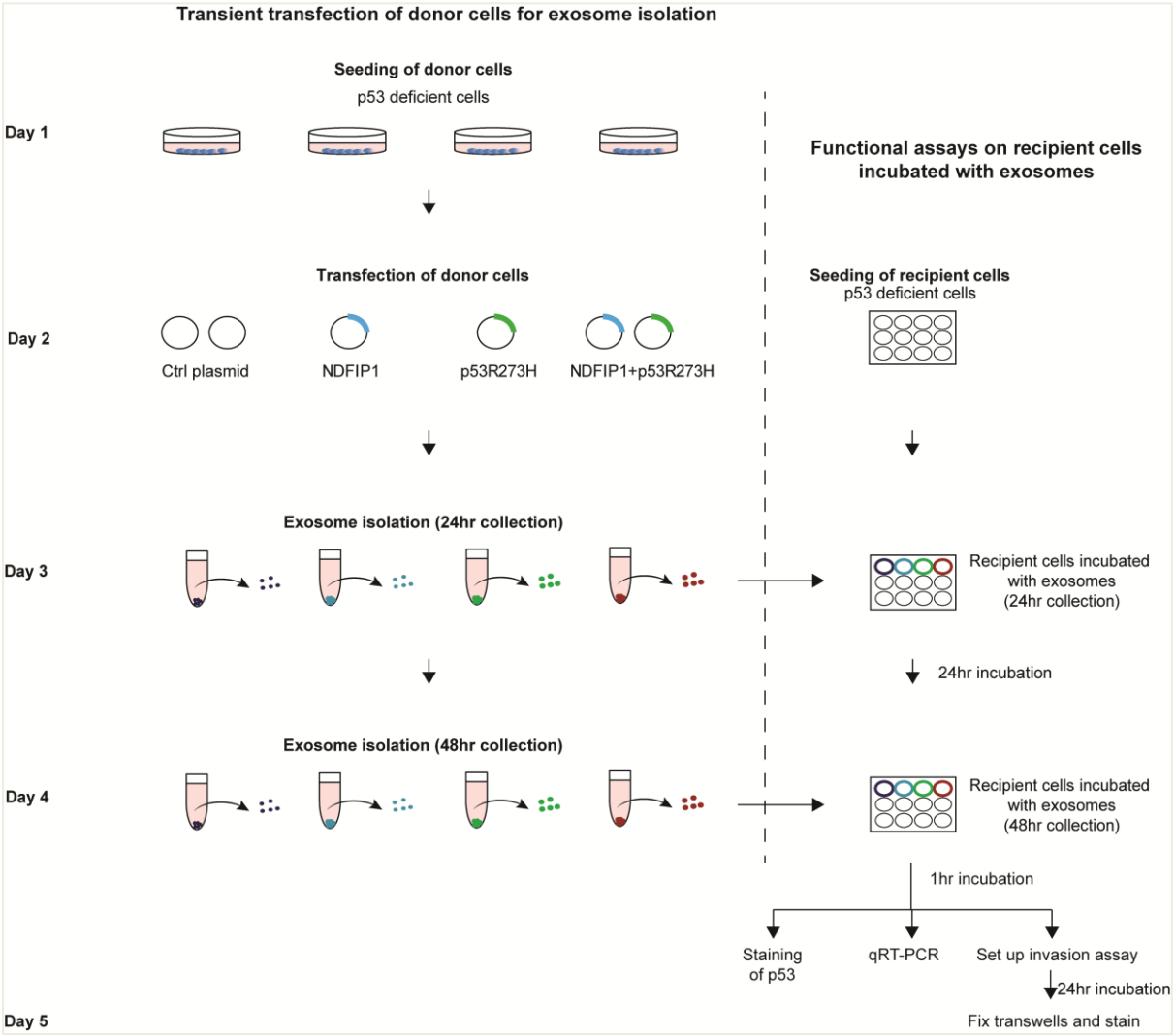


Figure 5.1: Experimental design to study the functional effects of exosomal mutant p53 in recipient cells.
H1299 “donor” cells were transfected with control constructs, NDFIP1 only, p53R273H only and NDFIP1+p53R273H and exosomes were collected and added to recipient cells at 24 and 48hrs post-transfection. Recipient cells were harvested 1hr after the last exosome addition for p53 staining or functional assays.

5.4 Results

5.4.1 Uptake of exosomes by recipient cells

We investigated the possibility that mt-p53 protein is transferred between cells through exosomes, by testing whether donor exosomes were taken-up by designated acceptor (recipient) cells (Section 5.3.2). By using a p53 null cell line (H1299) as recipient, we were able to visualize uptake of exosomal mt-p53 and its localization in the recipient cell by immunofluorescence (Fig 5.2A). Specifically, we could observe p53 staining predominantly in the cytoplasm and to a lesser degree in the nucleus of the recipient cells confirming successful uptake of exosomes (Fig 5.2B,C).

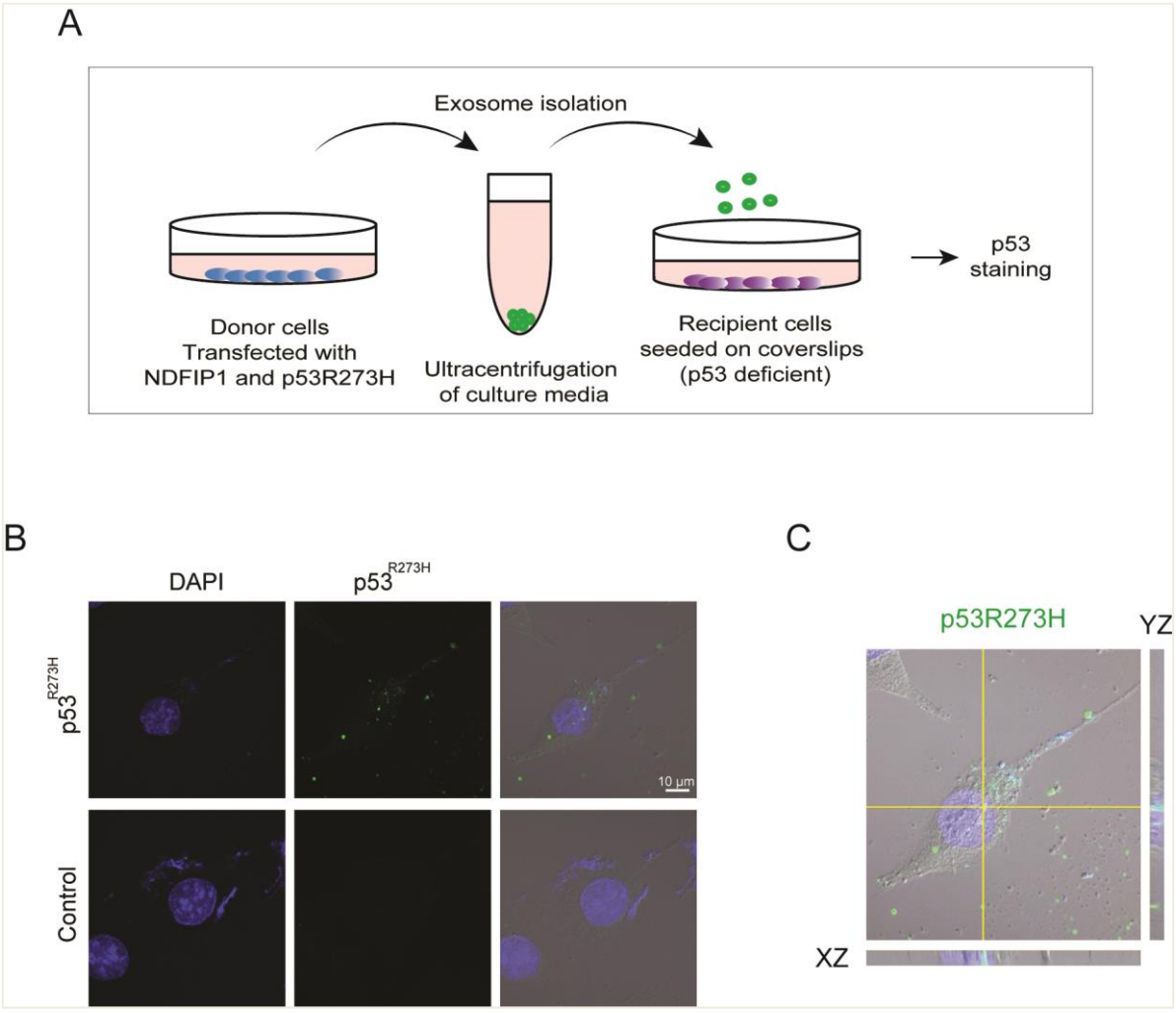


Figure 5.2: Mutant p53 in exosomes is taken up by H1299 'recipient' cells.
A. Experimental setup to detect uptake of vesicles: Exosomes were isolated from H1299 "donor" cells co-transfected with NDFIP1 and p53R273H or control (blank plasmid) and then incubated with "recipient" H1299 cells (seeded on coverslips) for 24hrs, followed by p53 staining. **B.** Confocal images of H1299 "recipient" cells incubated with exosomes and stained for p53 showing presence of exogenous mt-p53 (Green). **C.** Orthogonal section of a recipient cells showing localization of p53 in the nucleus.

For proof of concept, we repeated this experiment with the glioblastoma cell line, LN18par cell line as donor cells. We isolated exosomes from LN18 cells transfected with NDFIP1 and mt-p53 (R175H) and exposed H1299 cells to these exosomes for 24 hours. These recipient H1299 cells were also identified by confocal microscopy to have internalized p53 protein (Fig 5.3).

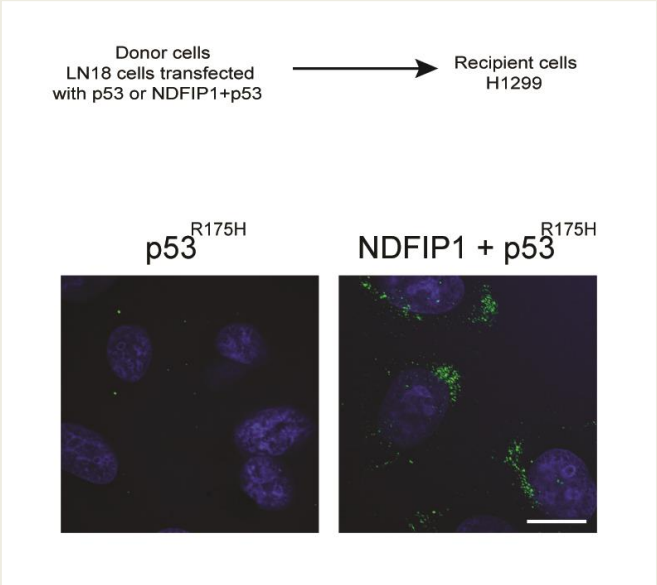


Figure 5.3: Mutant p53 in exosomes of LN18 'donor' cells is taken up by H1299 'recipient' cells.
Confocal images showing presence of p53 in H1299 recipient cells incubated with exosomes from donor LN18 cells transfected with p53R175H or NDFIP1 and p53R175H. Scale bar = 10µm.

5.4.2 Mutant p53 in exosomes confers neomorphic, 'gain of function' (GOF) properties to recipient $p53^{-/-}$ cells

Ascertaining functionality of internalized mt-p53 exosome cargo is fundamental to characterizing the relevance of this process in cancer. We speculated that the possible modes by which this exosomal mt-p53 could impose neomorphic transformation, include: altering transcriptional repertoire by binding to many transcription factors (Ets1, Ets2, NF-Y, Sp1, NF-kB, VDR, SREBP) and altering the transcription of their target genes and therefore modifying cell physiology [242].

Relevant to influencing recipient cell transcription, many mt-p53 target genes are components of the cell cycle and can drive cell growth and proliferation [37, 44]. Also, many mt-p53 regulated genes can directly or indirectly influence the

migratory/invasive capacity of cells and trigger an aggressive phenotype [41]. We observed partial nuclear localization of internalized mt-p53, which prompted us to monitor the levels of specific target genes known to be upregulated in the presence of mt-p53. In addition, relevant to altered cell physiology, we examined the influence of exosomes on the capacity of recipient cells to perform transwell invasion.

The uptake experiments were performed as described in section 5.3.3. Exosomes loaded with mt-p53 (isolated from cells transfected with NDFIP1+p53R273H) were added twice sequentially onto p53-null H1299 cells (24hrs and 48hrs after cell seeding) and these recipient cells were harvested one hour after the second exosome addition. The controls for this experiment consisted of recipient cells exposed to exosomes containing either no mt-p53, NDFIP1 only or residual mt-p53, isolated from H1299 donor cells transfected with empty vector (pcDNA), NDFIP1 or p53R273H only respectively (Fig 5.3A). Significant increase in mRNA expression of known mt-p53 target genes *CycA, CycB2, AURKB* and *MAPK1* was observed indicating an upregulation of genes driving G2/M progression (Fig 5.4C) [193, 243].

Mt-p53 expression in cells is known to impart higher invasive and migratory capabilities primarily due to the suppressive effects on p63. We examined whether exogenous mt-p53 transferred through exosomes can increase the invasive capacity of recipient cells through a matrigel invasion assay. Recipient cells incubated with exosomes (as explained above) were trypsinized and plated on matrigel coated transwells and allowed to migrate across a serum gradient for 24 hrs. The transwells were fixed and the nuclei of cells were stained by DAPI. The relative number of recipient cells that successfully crossed the matrigel was found to be higher in transwells seeded with recipient cells exposed to mt-p53 containing exosomes when compared to that of cells exposed to control exosomes (Fig 5.4D). Taken together, these results suggest that exogenous mt-p53 protein that is taken up by recipient cells is functional and increases gene expression levels favouring cell proliferation and also potentiates the invasive capacity of these recipient cells in a matrigel invasion assay.

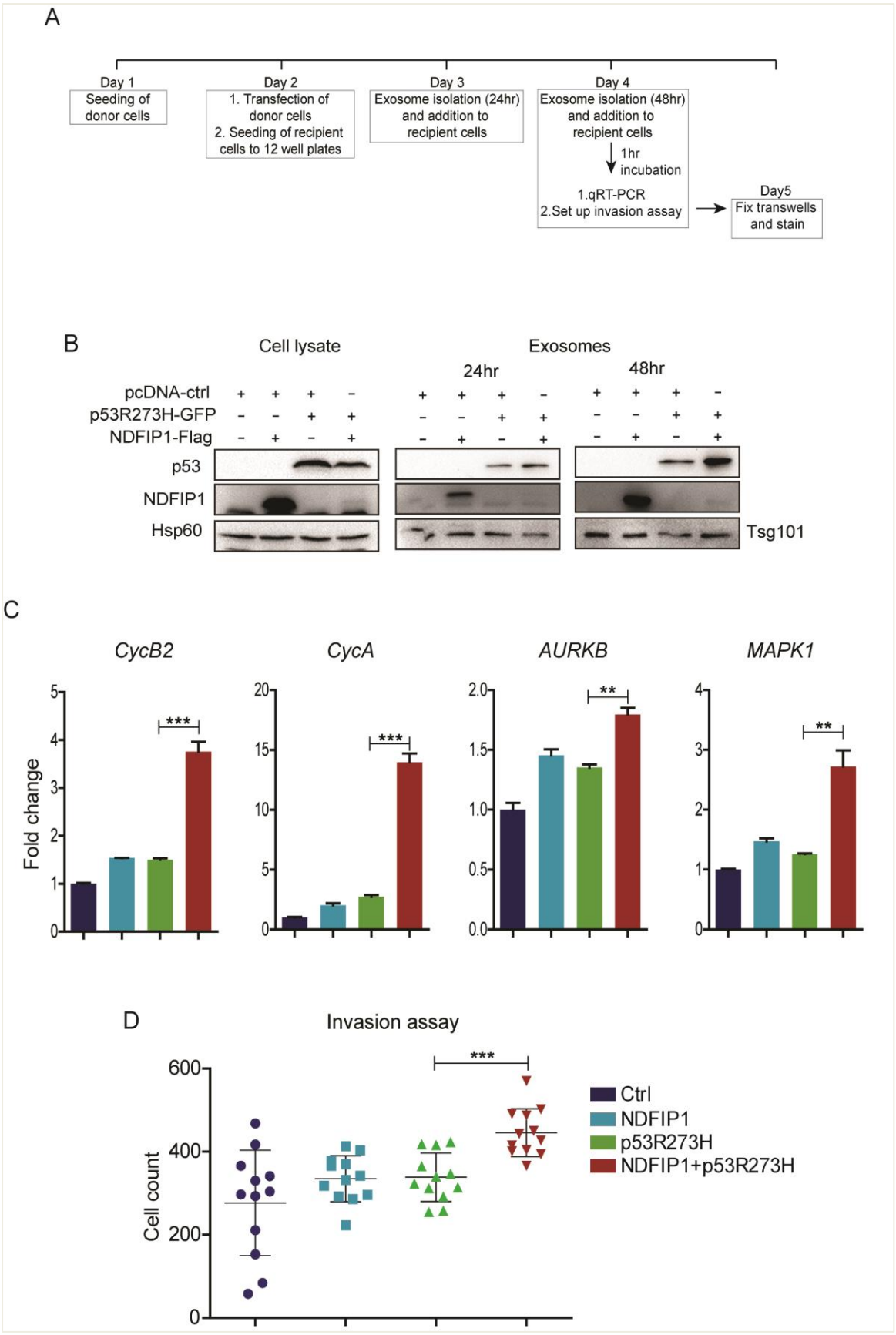


Figure 5.4: Mutant p53 in exosomes exhibits gain of function (GOF) properties in recipient p53$^{-/-}$ cells.

A. Experimental setup to examine functional effects of exosomal mt-p53: Exosomes were isolated from H1299 “donor” cells transfected with control constructs and NDFIP1+p53R273H at 24 and 48hrs post-transfection. Exosomes were added to recipient p53-null H1299 cells twice (24 and 48hrs after cell seeding) and harvested 1hr after the last exosome treatment. **B.** Western blot showing presence of mt-p53 in donor cell lysates and exosomes collected at 24 and 48hr. **C.** Quantitative real time PCR analysis of the expression of mt-p53 target genes, *CycB2*, *CycA*, *AURKB* and *MAPK1* in H1299 “recipient” cells. **D.** Graph showing the cell count (in 6 fields) in transwell filters at the end of the invasion assay.

5.4.3 NDFIP1-mtp53 in cancer

In healthy cells, NDFIP1 has been demonstrated to play a pivotal role in modulating cellular localization and ubiquitination of tumour suppressor PTEN, however its role in a cancer context has not previously been delineated. Through data mining of various freely available cancer databases, we evaluated NDFIP1 alterations with respect to cancer types and related parameters. Firstly, we analyzed TCGA data for *NDFIP1* expression and cancer survival and segregated the data based on p53 mutation (which includes “3'UTR”, “5'UTR”, “Frame_Shift_Del”, “Frame_Shift_Ins”, “Missense_Mutation”, “Nonsense_Mutation”, “Splice_Region” and “Splice_Site”). We observed reduction in overall survival of breast cancer samples with NDFIP1(High)-p53mt datasets over samples with mt-p53-NDFIP1 (Low) or wt-p53-NDFIP1 (High/Low) over a span of 7 years in all breast cancer types, particularly in luminal subtype of breast cancer (Fig 5.5A, B). We hypothesize that a combination of high NDFIP1 and mt-p53 can result in aggressive tumours due to the oncogenic effects of exosomal mt-p53 resulting in poor survival.

To gain more understanding of NDFIP1 in cancer, we investigated whether NDFIP1 is altered in cancer samples using cBIOPORTAL database. We queried NDFIP1 against a pan-cancer dataset and the resulting graph representing the percentage of NDFIP1 alteration frequency vs cancer type is shown in Fig 5.5C. NDFIP1 alteration frequency refers to the percentage of genetic alterations observed in NDFIP1 genomic locus derived from primary tumour sequencing data relating to mutation, deletion, fusion and amplification. In a dataset of renal clear cell carcinoma samples, 6% (31 out of 512 cases) of patient samples show amplification of NDFIP1 [244, 245]. We also queried NDFIP1 in Oncomine database and found a dataset of mesothelioma showing significantly higher expression of NDFIP1 when compared to the corresponding normal samples (of lung and pleura) [246, 247].

It is important to note that NDFIP1 expression in cells is not a clear indication of its ability to ubiquitinate proteins and recruitment to exosomes as NDFIP1 by itself lacks enzymatic activity and so the expression levels of the cognate NEDD4 E3 ligases are of importance.

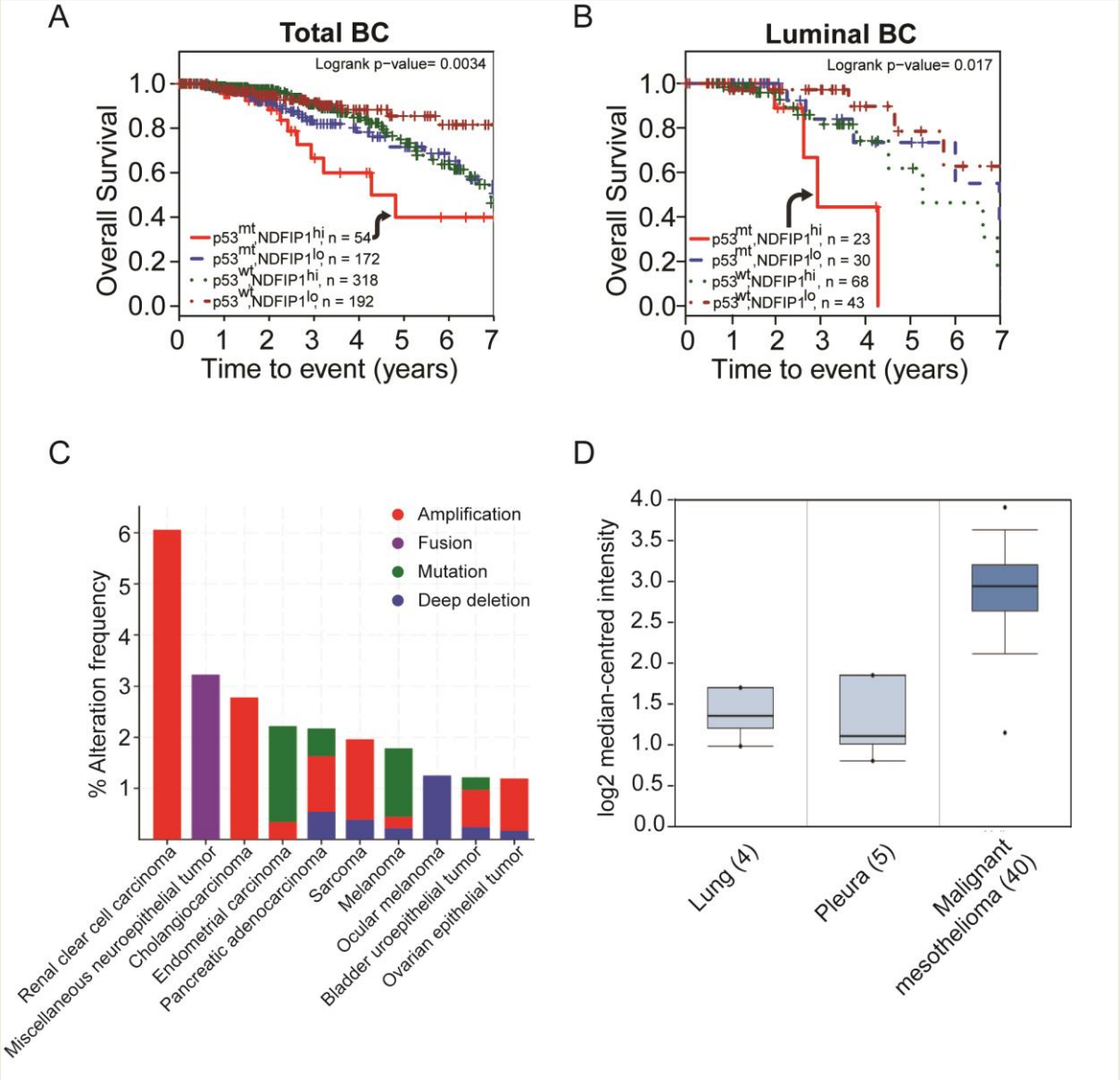


Figure 5.5: NDFIP1-mutant p53 axis in cancer.
Kaplan-Meier plot of survival rates for breast cancer patients segregated based on NDFIP1 expression (grouped based on median) and p53 mutation. The plot shows overall survival over 7 years for all breast cancer types **(A)** and luminal breast cancer **(B)**. Mutations in p53 together with high expression of NDFIP1 (red solid line) is associated with poor survival. **(C)** Graph showing the top 10 cancer types with NDFIP1 alterations and frequency of alterations observed for each tumour. **(D)** Box-plot showing NDFIP1 expression in normal samples (lung and pleura) vs malignant mesothelioma from Gordon mesothelioma dataset from Oncomine. The number in brackets represents the number of samples in Gordon

mesothelioma dataset. (P-value = 4.07E-10, Fold change=2.982 and reporter used 217800_s_at).

5.5 Discussion

Results of this chapter provide proof of concept for our hypothesis that oncogenic mt-p53 protein is transferred between cells through exosomes and confer neomorphic, gain of function activities in the exosome recipient cells. The key phenotypes examined were altered transcriptional capacity and modified cellular invasive capacity.

From our results, it is clear that a small amount of exosomal mt-p53 is transported to the nucleus of recipient cells. Nuclear localization of exosomal contents have been observed previously in a number of studies. In principle, these proteins are found to be functional in the nucleus in DNA recombination and transcriptional regulation as proven in the case of uptake of Cre or beta-catenin respectively [170, 248, 249]. Consistent to our observation of mt-p53 in the nucleus, we have observed higher levels of mt-p53 target genes playing a role in G2/M transition of cell cycle, two of which are regulated by the mt-p53-NF-Y complex. Increase in *CycB2, CycA* expression is expected due to mt-p53-NF-Y complex binding to CCAAT sites in the respective promoters [250, 251]. On the other hand, *AURKB* and *MAPK1* expression levels also increase in presence of mt-p53 supposedly through Sp1/Ets-1 and E2F1, respectively [243]. While CycB2 and CycA function through the corresponding CDKs in phosphorylating Rb, AURKB is important for mitotic events such as chromosome condensation and cytokinesis [252]. The activation of ERK pathway has been associated with many pathways relating to cell proliferation, survival and migration (reviewed in [253]). Follow-up experiments to check whether increase in G2/M cyclins or *MAPK1* would accelerate cell proliferation rates will be important to confirm the outcome. Also, considering that exosomes with wild type p53 have a growth-inhibitory effect on target cells, it is important to examine whether mt-p53 has a converse effect [234]. The cell lines mentioned in Table 5.1 will be of great use in future experiments. Future studies will also focus on quantitating the percentage of cells that take up mt-p53 and the proportion of nuclear localization.

In addition to transcriptional changes, cells treated with exosomal mt-p53 exhibit a highly invasive phenotype when compared to controls. A plausible explanation of this remarkable phenomenon relates to the reported capacity of mt-p53 to sequester its family member p63 (an ability that is not exhibited by wild type p53) [254]. Consistently, we speculate that mt-p53 bearing exosomes can impart invasive and metastatic capabilities through its engagement of p63, which in turn blocks p63 transcriptional and tumour suppressive activities. Importantly, p63 inactivation leads to upregulation of NF-κB2, which in turn results in upregulation of CXC chemokines important for cell migration [255, 256]. Many other mechanisms of mt-p53 mediated invasion have been identified – for example, mt-p53-SMAD complex driving TGF-β dependent cell invasion pathway, Rho-ROCK pathway, Integrin recycling reviewed in [41]. Additional experiments are needed to discern whether these pathways increase the invasive capacity of recipient cells (NDFIP1+mt-p53) compared to empty vector control in the context of H1299 cells.

Mt-p53 has functional roles in both nucleus and cytoplasm of cells and regulates different pathways in both compartments. Therefore, there is a need to look beyond the roles of mt-p53 in nucleus. Cytoplasmic mt-p53 is known to inhibit autophagy and can promote TNF-α induced NF-kB pathway [257, 258]. It would be of interest to examine the effects of mt-p53 on these pathways in the target recipient cells. In addition, the recipient cells could be subjected to alterations in metabolic pathways such as glucose transport and gain chemoresistance in response to mt-p53 [259]. In fact, studies on intra-tumoural heterogeneity have revealed that paracrine effects between subclones cooperating to sustain the tumour [38, 260]. In the case of colorectal cancer, conditioned media from resistant cells protect sensitive cells from inhibition of EGFR by cetuximab [261]. It is possible that mt-p53 transfer between tumour cells in a heterogenous population can result in a transfer of resistance phenotype against multiple drugs.

Our experiments describe the transfer of exosomes between tumour cells. Importantly however, there are also many reports on tumour derived exosomes affecting stromal cells, macrophages and cells at distant organs. Indeed, the progression of a tumour from a benign state to an invasive/metastatic state needs tumour cells communicating (in the form of secreted factors, extracellular vesicles,

chemokines) with neighbouring stromal cells and distant cells in pre-metastatic organs. One study described effects of certain p53 mutations pertaining to modifying cancer associated fibroblasts (CAFs) to support the growth and dissemination of tumour cells by modifying the secretome [125]. Another study by Cooks et al has shown that mt-p53 can reprogram macrophages to a more tumour supportive state by increasing the levels of exosomal miR-1246 [126].

The oncogenic potential of mt-p53 could extend to its own dissemination through exosomes and might play a previously undetermined role in modifying surrounding cells of the tumour microenvironment and cells of distant pre-metastatic sites by preparing a favourable environment for tumour cells to invade. Many questions also arise as to the receptors on the exosomes carrying mt-p53 and the identity of cells that take up the exosomes in a physiological setting. To substantiate the exosomal effects of mt-p53 in tumour and stromal cells, *in vivo* experiments must be performed to mimic the effect of tumour derived exosomes in influencing invasion and metastasis in physiological conditions. The possibility of exosomal mt-p53 promoting invasion to secondary organs by initiating the establishment of a pre-metastatic niche needs to be examined further. Intravenous injections of exosomes with varying mt-p53 levels followed by the injection of luciferase tagged cancer cells in NSG mice would facilitate tracking of cells and the establishment of metastasis in mice. Cell line models mentioned in Table 5.1 will be valuable in exploring NDFIP1-mt-p53 axis in propagating metastasis, establishment of secondary cancers and other mt-p53 GOF.

From a clinical point of view, mt-p53 is a well-established promoter of aggressive phenotypes in cancer and is a valuable predictor of patient prognosis, particularly in breast cancers [262]. From available literature, we have some insights into the possible link connecting NDFIP1 expression levels to tumour growth. High NDFIP1 levels positively correlate with poor survival in the case of head and neck carcinomas and better survival rate in renal cell carcinoma (https://www.proteinatlas.org/ENSG00000131507-NDFIP1/pathology) [263]. Interestingly, previous studies have revealed that NDFIP1 overexpression is not favourable for cell growth, possibly due to its effect on PTEN [152, 175]. Therefore, further stratification of the data on the basis of TP53 alterations is required to confirm these findings. Frequent loss of PTEN combined with TP53 mutations are observed

in some cancers and it would be interesting to study the effect of NDFIP1 in this context.

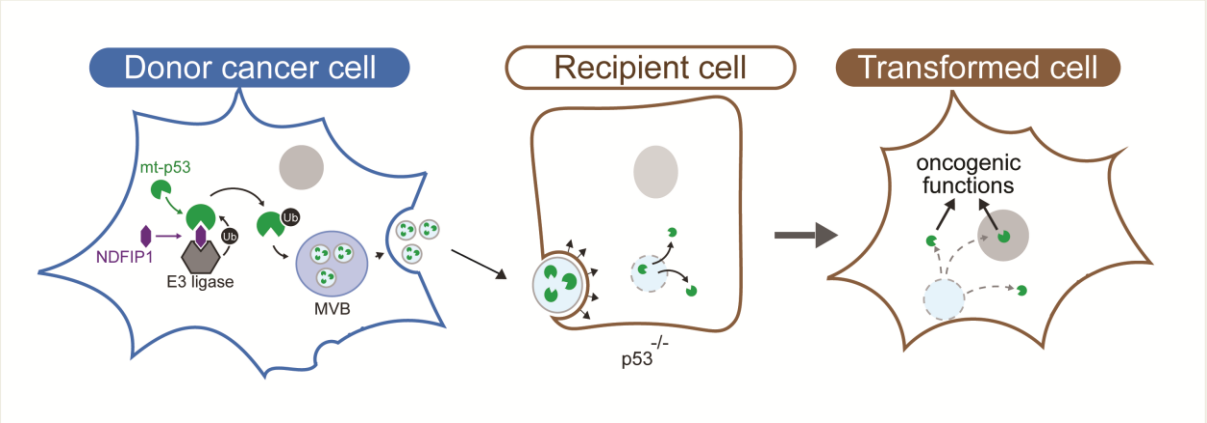


Figure 5.6: Model depicting transfer of oncogenic mt-p53 between cancer cells through exosomes.
NDFIP1 in cancer cells drives export of mt-p53 protein into exosomes, which are released into the tumour microenvironment. These exosomes are internalized by recipient cells where these exogenous mt-p53 proteins execute their oncogenic functions resulting in an aggressive recipient cell.

Based on our current results, we propose an NDFIP1 driven model for the transfer of mt-p53 between tumor cells and the subsequent transformation of the exosome recipient cells into a more pathogenic type (Fig 5.7). In the light of our discovery of mt-p53 in exosomes, questions arise as to whether exosomal mt-p53 transfer can aid the process in an endocrine and paracrine manner and both *in-vitro* and *in-vivo* experiments are needed to address these questions.

Chapter 6
Concluding remarks and future directions

Targeting mutant p53 protein is of great clinical importance due to its high mutation rates and associated accumulation and oncogenic properties. Studies targeting the mt-p53 expression and enabling its degradation have proven to be successful in improving survival in mice models. Specific regulatory mechanisms controlling mt-p53 stability exist but are currently unknown and unravelling these mechanisms can enable the development of improved therapies. In this study, we have identified a novel pathway of mt-p53 trafficking through an E3 ligase adaptor, NDFIP1.

This work is built upon a powerful high-throughput RNAi screen designed to identify mt-p53 regulators. The extensive data mining and bioinformatic analysis described in this thesis unveiled many interesting candidates never previously linked to mt-p53. Not unexpectedly, some regulators identified in the screen had previously been associated with p53. This is consistent with the key E3 ligase of p53, MDM2, which is also capable of targeting mt-p53 (although with different kinetics) [68]. Remarkably, few candidates from the screen were identified to decrease mt-p53 levels. One possible explanation for this could be that mt-p53 is extremely stable and protected by chaperones and multiple pathways need to be targeted to reduce its levels. Secondly, the negative regulators of mt-p53 could be expressed at low levels, that siRNA against these candidates have no effect on mt-p53. From the results of this screen and current known interactors, we have gained an understanding of the pathways that could affect mt-p53 protein and this information can be used to build a comprehensive map of mt-p53 regulation.

- Among the top pathways enriched based on the candidate list were RNA related pathways - Spliceosome, RNA transport and RNA degradation. It is possible that these pathways affect both wild type and mt-p53 and this can be explored by checking if these pathways influence mt-p53 directly or indirectly through regulators of mt-p53.

- One of the interesting pathways enriched from the RNAi screen candidates is Sphingolipid metabolism. Three key enzymes of this pathway may play a role in regulating mt-p53 levels possibly through indirect mechanisms. Although metabolites of this pathway are known to affect p53, these have not been explored in the context of the mutants [203].

- We validated 23 candidates based on mt-p53 immunoblotting in two cell lines and based upon its function as an E3 ligase adaptor in controlling substrate protein levels, we chose NDFIP1 as a potential candidate to pursue.

It is important to note that the RNAi screen has been extremely sensitive in identifying even small changes in mt-p53 levels. The NDFIP1 is a fabulous example of the unexpected, where initially we considered its potential as a tumour suppressor due to its capability of reducing mt-p53 levels. However, further studies revealed the presence of a trafficking pathway directed by NDFIP1 and the resulting dissemination of mt-p53 into the tumour microenvironment. At least, in cancer cells, NDFIP1 seems to be hijacked into the oncogenic juggernaut of mt-p53 cancer promoting machinery and its activities may have wider implications in tumour-stroma interactions.

In the second part of this work, we performed specific experiments to decipher the mechanism of NDFIP1 effects on mt-p53. We found that NDFIP1 interacts with mt-p53, promotes its ubiquitination and subsequently packages mt-p53 into exosomes. NDFIP1 was also found to have a similar effect on p53(wild type) possibly through the same mechanism. Many aspects of NDFIP1-mt-p53 pathway are still unknown.

- NDFIP1-p53 complex is found to be localized in specific compartments of the endosomes even though p53 is not generally found in endosomes. By studying the interaction between NDFIP1 and p53 mutants with their nuclear export signal (NES) disrupted, we can further elucidate how NDFIP1 and mt-p53 interact in the endosomal compartments and whether nucleocytoplasmic shuttling of mt-p53 is necessary for interaction with NDFIP1.

- More experiments such as ubiquitination assays need to be performed to identify the E3 ligase that ubiquitinates mt-p53 in the presence of NDFIP1 and whether it leads to mono or polyubiquitination. It is also important to check if ubiquitination is absolutely important for exosomal loading of mt-p53.

- Through deletion constructs, we need to examine the actual domains involved in NDFIP1-mt-p53 interaction. This will give more insights into whether NDFIP1 competes/complexes with other proteins (MDM2 or CHIP) for interaction with mt-p53.

- Other questions that arise with respect to NDFIP1-p53 interaction and subsequent release in exosomes relate to the upstream signals that can trigger this pathway. NDFIP1 protein levels are known to be upregulated in conditions of stress in neuronal cells – ischemia, DNA damage and high iron levels [151, 165, 172]. Cancer cells also have high levels of DNA damage and oxidative stress and it will be of interest to check if NDFIP1 is upregulated in such conditions. Recent studies have shown that chemotherapy can alter the contents of exosomes [264] and it would be worthwhile to check if the NDFIP1-mt-p53 pathway is affected by therapeutic agents.

- Since exosomes are found in most body fluids, it would be interesting to explore whether mt-p53 can be found in exosomes isolated from plasma of cancer patients, in which case mt-p53 and NDFIP1 in exosomes can be valuable as biomarkers for predicting patient prognosis, response to chemotherapy and predicting relapse [130]. Whether NDFIP1 is a tumour suppressor or a tumour promoting gene would largely depend upon the mutational status of p53 and the status of PTEN in the tumour. Improvement in technologies for purification and sensitive detection of exosomal proteins can give way to reliable use of mt-p53 and NDFIP1 as a biomarker.

To examine the functional outcome of mt-p53 in exosomes, we developed a set of cell line models and assays to identify mt-p53 function in recipient cells that take up exosomes. Unlike a previous study which identified that exosomal K-Ras to be non-functional in recipient cells [265], we have evidence that mt-p53 transferred between

cells retains functionality. We observed significant increase in mt-p53 target genes involved in G2/M progression in the recipient cells and an enhancement of invasive potential of these cells. These results have opened up new questions -

- Patocs et al., 2007 had identified increased p53 expression (suggestive of p53 mutation) in breast cancer associated stromal cells and attributed this to genetic alterations in these stromal cells [266]. However the existence of p53 mutations in stromal cells is disputed [267]. It would be worthwhile to revisit the data on mt-p53 expression in stromal cells taking into consideration exosomal mt-p53 transfer. Other studies have observed mt-p53 dependent tumour promoting roles of cancer associated fibroblasts (CAFs). Addadi et al., 2010 showed that p53 mutant CAFs promote tumour growth, and Arandkar et al., 2018 observed that p53 in CAFs exhibit a mt-p53-like conformation [268, 269]. Based on these studies, it would be imperative to explore this further as it would have significant implications to cancer therapy.

- Our work has singularly focused on tumour-tumour interactions and the implications of mt-p53 suggest a paracrine GOF mechanism. Further experiments must be performed to examine the fate of exosomal mt-p53 (originating from cancer cells) in recipient normal cells with wild type form of p53. Is mt-p53 stable in recipient cells and whether a dominant negative effect is observed in addition to GOF?

- Different p53 mutants have different levels of aggressiveness and this is assumed to be due to differential stability and interaction affinities with protein partners [37]. Does the type of mt-p53 affect what paracrine effects takes place in recipient cells? Furthermore, examining tissue distribution of exosomes and identifying recipient cells responsive to exosomal mt-p53 would be helpful in predicting future metastatic sites.

- Considering the broad effects of mt-p53 GOF (listed in section 1.3.2), it is possible that the epigenetic and metabolic changes in stromal cells of the tumour microenvironment could be due to exosomes with mt-p53.

- Further studies on the mechanisms of exosomal uptake can identify receptors or proteins that could blocked/inhibited as a way of therapeutically targeting mt-p53 dissemination through exosomes.

- Until now, suppressive effects of mutant p53 on immune cells have always been associated to be due to its non-autonomous effects on the TNF-α/NF-kB pathway or through miRNAs [270, 271]. Based on our discovery of inter-cellular mt-p53 transfer, we need to investigate whether exogenous mt-p53 is transferred into tumour associated immune cells and whether it is capable of compounding an immune-suppressive milieu through autonomous mechanisms. Considering the gaining importance of immunotherapy, the ramifications of mt-p53 dissemination on immune cells should be explored.

Recent studies have identified proteins with significant functions such as oncogenic tyrosine kinases (MET in melanoma), immunosuppressors (PD-L1 in melanoma), angiogenic factors (VEGF-A in glioblastoma) being transported through exosomes and promoting metastasis, immune evasion and angiogenesis [140, 272, 273]. This existing evidence of cancer promoting abilities of exosomes is compounded by our current findings on mt-p53 and this opens up a new area of research in the field of p53 with bigger implications in multiple cancers. Also, our study reveals a new pathway which is distinct from pathways of degradation and mt-p53 reactivation, which current therapies are focused on. Much is still unknown about this pathway and is also made complicated by the sheer number of p53 mutants and tumour types to study. There is an urgent need to study how current therapies could a play a role in modulating exosomes and in particular mt-p53. Investigating the mechanics of mt-p53 loading into the exosomes and uptake of exosomes can open new avenues of therapeutic targeting of mt-p53 expressing tumours.

Chapter 7

Appendix

I. Cloning of shRNA oligos into FH1T vector

1. Annealing of oligos

1µL of forward and reverse oligos (100µM) were diluted to 10µL with annealing buffer (750mM KCl, 200mM Tris-HCl). The oligos were subjected to 37^0C, 30min; 95^0C to 5^0C, 15min (decrement); 25^0C, forever.

2. Cloning into FH1T vector

FH1T-cherry vector (5µg) was sequentially digested with BsmBI (NEB) followed by digestion with XhoI (NEB). The digested vector was purified using Qiagen gel purification kit. The annealed oligos and the purified vector digest were ligated using T4 DNA ligase (NEB). Insertion of shRNA was confirmed by sequencing.

II. Cloning of hNDFIP1 into FUV-cherry vector

1. Amplication of NDFIP1

Reaction	
2x Phusion HF buffer	25µL
NDFIP1 Forward primer	0.5µM
NDFIP1 Reverse primer	0.5µM
Template DNA	50ng
DMSO	1.5uL
Make up to 50µL with Nuclease free water	

The template DNA used was NDFIP1-His construct already containing human NDFIP1.
PCR conditions: 98^0C 30s 1x; (98^0C, 5s; 60^0C, 30s; 72^0C, 32s) 32x; 72^0C, 10 min, 1x, 4^0C, forever.

2. Cloning into pTRE vector

pTRE vector (5µg) was digested sequentially with BamHI (Buffer E, Promega) and then with EcoRI (Buffer H, Promega). All digests were conducted at 37^0C for 3hrs. The PCR product was digested first with BglII and followed with EcoRI digestion. The digested plasmid and PCR product were run on an agarose gel followed by extraction by Qiagen Gel purification kit. The digested plasmid and PCR product were ligated using T4 DNA ligase (NEB) at 16^0C overnight.

3. Sub-cloning into FUV1-cherry

pTRE-NDFIP1 was digested with PacI (NEB) followed by gel purification of the TRE-NDFIP1 fragment (Tet response element and NDFIP1 coding region). FUV1-cherry

vector was also digested with PacI and the purified vector digest was ligated with TRE-NDFIP1 fragment using T4 DNA ligase. The orientation of the resulting clones was confirmed by sequencing with FUVseqR.

III. Site directed mutagenesis in VN-p53 vector:

Mutations: R175H CGC –> CAC, R273H CGT –> CAT
Total vector size = VN vector (5216bp) + p53 CDS (1182bp) = 6398bp

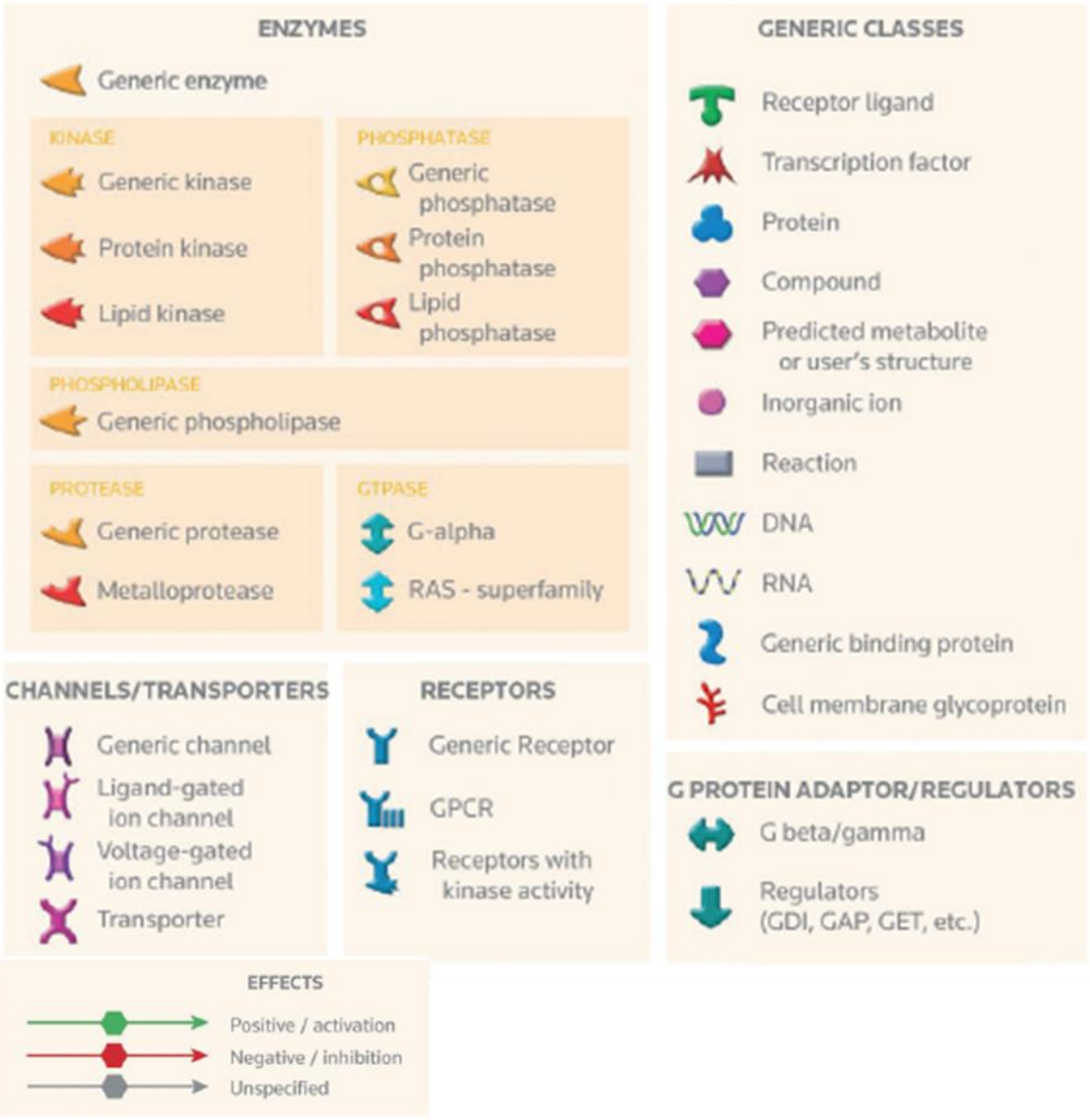
SDM reaction	µL	Final concentration
QF mix	12.5	1X
10 µM Fwd Primer	1.25	0.5uM
10 µM Rev Primer	1.25	0.5uM
Template DNA	1	1-25ng
NF Water	9	
Total volume	25	

Reaction conditions	^{0}C	
Initial denaturation	98	30s
25X	98	10s
	50-72*	30s
	72	3min 15 sec
Final extension	72	2min
	4	

*Annealing temp used – R273H - 64^{0}C, R175H - 66^{0}C.
The PCR product was ligated using the NEB protocol for KLD reaction (Kinase, Ligase and DpnI reaction) and competent cells were transformed with the reaction mixture and plasmid DNA was isolated from the resulting colonies and sequenced.

IV. METACORE legend

The following figure is from METACORE quick reference guide describing the network objects and their function.


ENZYMES
Generic enzyme
KINASE
Generic kinase
Protein kinase
Lipid kinase
PHOSPHATASE
Generic phosphatase
Protein phosphatase
Lipid phosphatase
PHOSPHOLIPASE
Generic phospholipase
PROTEASE
Generic protease
Metalloprotease
GTPASE
G-alpha
RAS - superfamily
GENERIC CLASSES
Receptor ligand
Transcription factor
Protein
Compound
Predicted metabolite or user's structure
Inorganic ion
Reaction
DNA
RNA
Generic binding protein
Cell membrane glycoprotein
CHANNELS/TRANSPORTERS
Generic channel
Ligand-gated ion channel
Voltage-gated ion channel
Transporter
RECEPTORS
Generic Receptor
GPCR
Receptors with kinase activity
G PROTEIN ADAPTOR/REGULATORS
G beta/gamma
Regulators (GDI, GAP, GET, etc.)
EFFECTS
Positive / activation
Negative / inhibition
Unspecified

References

1. Brücher, B.L.D.M., et al., *Imagine a world without cancer.* BMC cancer, 2014. **14**: p. 186-186.

2. Morash, M., et al., *The Role of Next-Generation Sequencing in Precision Medicine: A Review of Outcomes in Oncology.* Journal of personalized medicine, 2018. **8**(3): p. 30.

3. Dai, X., et al., *Breast cancer intrinsic subtype classification, clinical use and future trends.* American journal of cancer research, 2015. **5**(10): p. 2929-2943.

4. Russnes, H.G., et al., *Breast Cancer Molecular Stratification: From Intrinsic Subtypes to Integrative Clusters.* The American Journal of Pathology, 2017. **187**(10): p. 2152-2162.

5. Nakshatri, H., E. Srour, and S. Badve, *Breast Cancer Stem Cells and Intrinsic Subtypes: Controversies Rage On*. Vol. 4. 2009. 50-60.

6. Valastyan, S. and R.A. Weinberg, *Tumor metastasis: molecular insights and evolving paradigms.* Cell, 2011. **147**(2): p. 275-292.

7. Arteaga, C.L., et al., *Treatment of HER2-positive breast cancer: current status and future perspectives.* Nature Reviews Clinical Oncology, 2011. **9**: p. 16.

8. Park, J.H., J.H. Ahn, and S.B. Kim, *How shall we treat early triple-negative breast cancer (TNBC): from the current standard to upcoming immuno-molecular strategies.* ESMO Open, 2018. **3**(Suppl 1): p. e000357.

9. Rack, B., et al., *Circulating tumor cells predict survival in early average-to-high risk breast cancer patients.* J Natl Cancer Inst, 2014. **106**(5).

10. Lodish, H.F., *Molecular cell biology*. 4th ed. 2000, New York: W.H. Freeman. xxxvi, 1084, G-17, I-36 p.

11. Vogelstein, B., D. Lane, and A.J. Levine, *Surfing the p53 network.* Nature, 2000. **408**(6810): p. 307-10.

12. Vousden, K.H. and C. Prives, *Blinded by the Light: The Growing Complexity of p53.* Cell, 2009. **137**(3): p. 413-431.

13. Kim, M.P. and G. Lozano, *Mutant p53 partners in crime.* Cell Death And Differentiation, 2017. **25**: p. 161.

14. Beckerman, R. and C. Prives, *Transcriptional regulation by p53.* Cold Spring Harb Perspect Biol, 2010. **2**(8): p. a000935.

15. Zilfou, J.T. and S.W. Lowe, *Tumor suppressive functions of p53.* Cold Spring Harbor perspectives in biology, 2009. **1**(5): p. a001883-a001883.

16. Brooks, C.L. and W. Gu, *New insights into p53 activation.* Cell Research, 2010. **20**: p. 614.

17. Ranjan, A. and T. Iwakuma, *Emerging Non-Canonical Functions and Regulation of p53.* International journal of molecular sciences, 2018. **19**(4): p. 1015.

18. Liu, B., Y. Chen, and D.K. St Clair, *ROS and p53: a versatile partnership.* Free radical biology & medicine, 2008. **44**(8): p. 1529-1535.

19. Brady, C.A. and L.D. Attardi, *p53 at a glance.* Journal of Cell Science, 2010. **123**(15): p. 2527.

20. Wang, D.B., et al., *p53 and mitochondrial function in neurons.* Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 2014. **1842**(8): p. 1186-1197.

21. Haupt, Y., et al., *Mdm2 promotes the rapid degradation of p53.* Nature, 1997. **387**(6630): p. 296-9.

22. Kubbutat, M.H., S.N. Jones, and K.H. Vousden, *Regulation of p53 stability by Mdm2.* Nature, 1997. **387**(6630): p. 299-303.

23. Wu, X., et al., *The p53-mdm-2 autoregulatory feedback loop.* Genes Dev, 1993. **7**(7a): p. 1126-32.

24. Sionov, R.V. and Y. Haupt, *The cellular response to p53: the decision between life and death.* Oncogene, 1999. **18**(45): p. 6145-57.

25. Jones, S.N., et al., *Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53.* Nature, 1995. **378**(6553): p. 206-8.

26. Meek, D.W. and C.W. Anderson, *Posttranslational modification of p53: cooperative integrators of function.* Cold Spring Harbor perspectives in biology, 2009. **1**(6): p. a000950.

27. Scheffner, M., et al., *The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53.* Cell, 1990. **63**(6): p. 1129-36.

28. Truant, R., et al., *Direct interaction of the hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response element-directed transactivation.* J Virol, 1995. **69**(3): p. 1851-9.

29. Li, F.P., et al., *A cancer family syndrome in twenty-four kindreds.* Cancer Res, 1988. **48**(18): p. 5358-62.

30. Olivier, M., et al., *Li-Fraumeni and related syndromes: correlation between tumor type, family structure, and TP53 genotype.* Cancer Res, 2003. **63**(20): p. 6643-50.

31. Nichols, K.E., et al., *Germ-line p53 mutations predispose to a wide spectrum of early-onset cancers.* Cancer Epidemiol Biomarkers Prev, 2001. **10**(2): p. 83-7.

32. Chompret, A., et al., *P53 germline mutations in childhood cancers and cancer risk for carrier individuals.* British journal of cancer, 2000. **82**(12): p. 1932-1937.

33. Petitjean, A., et al., *Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database.* Hum Mutat, 2007. **28**(6): p. 622-9.

34. van Oijen, M.G.C.T. and P.J. Slootweg, *Gain-of-Function Mutations in the Tumor Suppressor Gene p53.* Clinical Cancer Research, 2000. **6**(6): p. 2138.

35. Sabapathy, K., *The contrived mutant p53 oncogene – beyond loss of functions.* Frontiers in Oncology, 2015. **5**.

36. Oren, M. and V. Rotter, *Mutant p53 gain-of-function in cancer.* Cold Spring Harb Perspect Biol, 2010. **2**(2): p. a001107.

37. Freed-Pastor, W.A. and C. Prives, *Mutant p53: one name, many proteins.* Genes & development, 2012. **26**(12): p. 1268-1286.

38. Brosh, R. and V. Rotter, *When mutants gain new powers: news from the mutant p53 field.* Nature Reviews Cancer, 2009. **9**: p. 701.

39. Lang, G.A., et al., *Gain of Function of a p53 Hot Spot Mutation in a Mouse Model of Li-Fraumeni Syndrome.* Cell, 2004. **119**(6): p. 861-872.

40. Lozano, G., *Mouse models of p53 functions.* Cold Spring Harbor perspectives in biology, 2010. **2**(4): p. a001115-a001115.

41. Muller, P.A.J., K.H. Vousden, and J.C. Norman, *p53 and its mutants in tumor cell migration and invasion.* The Journal of Cell Biology, 2011. **192**(2): p. 209.

42. Di Minin, G., et al., *Mutant p53 Reprograms TNF Signaling in Cancer Cells through Interaction with the Tumor Suppressor DAB2IP.* Molecular Cell, 2014. **56**(5): p. 617-629.

43. Haupt, S., et al., *Promyelocytic leukemia protein is required for gain of function by mutant p53.* Cancer Res, 2009. **69**(11): p. 4818-26.

44. Muller, P.A.J. and K.H. Vousden, *Mutant p53 in cancer: new functions and therapeutic opportunities.* Cancer cell, 2014. **25**(3): p. 304-317.

45. Kim, M.P., Y. Zhang, and G. Lozano, *Mutant p53: Multiple Mechanisms Define Biologic Activity in Cancer.* Frontiers in oncology, 2015. **5**: p. 249-249.

46. Vaughan, C., et al., *p53: its mutations and their impact on transcription.* Sub-cellular biochemistry, 2014. **85**: p. 71-90.

47. Mantovani, F., L. Collavin, and G. Del Sal, *Mutant p53 as a guardian of the cancer cell.* Cell Death & Differentiation, 2019. **26**(2): p. 199-212.

48. Cordani, M., et al., *Mutant p53 proteins alter cancer cell secretome and tumour microenvironment: Involvement in cancer invasion and metastasis.* Cancer Lett, 2016. **376**(2): p. 303-9.

49. Nigro, J.M., et al., *Mutations in the p53 gene occur in diverse human tumour types.* Nature, 1989. **342**(6250): p. 705-8.

50. Soussi, T., *p53 Antibodies in the sera of patients with various types of cancer: a review.* Cancer Res, 2000. **60**(7): p. 1777-88.

51. Olive, K.P., et al., *Mutant p53 Gain of Function in Two Mouse Models of Li-Fraumeni Syndrome.* Cell, 2004. **119**(6): p. 847-860.

52. Terzian, T., et al., *The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss.* Genes & development, 2008. **22**(10): p. 1337-1344.

53. Vijayakumaran, R., et al., *Regulation of Mutant p53 Protein Expression.* Front Oncol, 2015. **5**: p. 284.

54. Frum, R.A. and S.R. Grossman, *Mechanisms of mutant p53 stabilization in cancer.* Subcell Biochem, 2014. **85**: p. 187-97.

55. Lopez-Diaz, F.J., et al., *Coordinate transcriptional and translational repression of p53 by TGF-beta1 impairs the stress response.* Mol Cell, 2013. **50**(4): p. 552-64.

56. Yan, W., et al., *Histone deacetylase inhibitors suppress mutant p53 transcription via histone deacetylase 8.* Oncogene, 2013. **32**(5): p. 599-609.

57. Takagi, M., et al., *Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin.* Cell, 2005. **123**(1): p. 49-63.

58. Gurtner, A., et al., *Dysregulation of microRNA biogenesis in cancer: the impact of mutant p53 on Drosha complex activity.* J Exp Clin Cancer Res, 2016. **35**: p. 45.

59. Zhang, Y., et al., *Gain-of-function miRNA signature by mutant p53 associates with poor cancer outcome.* Oncotarget, 2016. **7**(10): p. 11056-66.

60. Garibaldi, F., et al., *Mutant p53 inhibits miRNA biogenesis by interfering with the microprocessor complex.* Oncogene, 2016. **35**(29): p. 3760-70.

61. Hershko, A. and A. Ciechanover, *The ubiquitin system.* Annu Rev Biochem, 1998. **67**: p. 425-79.

62. David, Y., et al., *E3 ligases determine ubiquitination site and conjugate type by enforcing specificity on E2 enzymes.* J Biol Chem, 2011. **286**(51): p. 44104-15.

63. Voges, D., P. Zwickl, and W. Baumeister, *The 26S proteasome: a molecular machine designed for controlled proteolysis.* Annu Rev Biochem, 1999. **68**: p. 1015-68.

64. Jain, A.K. and M.C. Barton, *Making sense of ubiquitin ligases that regulate p53.* Cancer biology & therapy, 2010. **10**(7): p. 665-672.

65. Midgley, C.A. and D.P. Lane, *p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding.* Oncogene, 1997. **15**(10): p. 1179-89.

66. Shimizu, H., et al., *The conformationally flexible S9-S10 linker region in the core domain of p53 contains a novel MDM2 binding site whose mutation increases ubiquitination of p53 in vivo.* J Biol Chem, 2002. **277**(32): p. 28446-58.

67. Wallace, M., et al., *Dual-site regulation of MDM2 E3-ubiquitin ligase activity.* Molecular cell, 2006. **23**(2): p. 251-263.

68. Lukashchuk, N. and K.H. Vousden, *Ubiquitination and degradation of mutant p53.* Mol Cell Biol, 2007. **27**(23): p. 8284-95.

69. Whitesell, L., et al., *The physical association of multiple molecular chaperone proteins with mutant p53 is altered by geldanamycin, an hsp90-binding agent.* Mol Cell Biol, 1998. **18**(3): p. 1517-24.

70. Blagosklonny, M.V., et al., *Mutant conformation of p53 translated in vitro or in vivo requires functional HSP90.* Proc Natl Acad Sci U S A, 1996. **93**(16): p. 8379-83.

71. Li, D., et al., *Functional inactivation of endogenous MDM2 and CHIP by HSP90 causes aberrant stabilization of mutant p53 in human cancer cells.* Molecular cancer research : MCR, 2011. **9**(5): p. 577-588.

72. Muller, P., et al., *Chaperone-dependent stabilization and degradation of p53 mutants.* Oncogene, 2008. **27**(24): p. 3371-83.

73. Kovacs, J.J., et al., *HDAC6 regulates Hsp90 acetylation and chaperone-dependent activation of glucocorticoid receptor.* Mol Cell, 2005. **18**(5): p. 601-7.

74. Li, D., N.D. Marchenko, and U.M. Moll, *SAHA shows preferential cytotoxicity in mutant p53 cancer cells by destabilizing mutant p53 through inhibition of the HDAC6-Hsp90 chaperone axis.* Cell Death Differ, 2011. **18**(12): p. 1904-13.

75. Zhao, D., et al., *Molecularly targeted therapies for p53-mutant cancers.* Cellular and molecular life sciences : CMLS, 2017. **74**(22): p. 4171-4187.

76. Parrales, A., et al., *DNAJA1 controls the fate of misfolded mutant p53 through the mevalonate pathway.* Nature cell biology, 2016. **18**(11): p. 1233-1243.

77. Calderwood, S.K., et al., *Heat shock proteins in cancer: chaperones of tumorigenesis.* Trends Biochem Sci, 2006. **31**(3): p. 164-72.

78. Li, D., et al., *A gain-of-function mutant p53-HSF1 feed forward circuit governs adaptation of cancer cells to proteotoxic stress.* Cell Death Dis, 2014. **5**: p. e1194.

79. Xiao, X., et al., *HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice.* Embo j, 1999. **18**(21): p. 5943-52.

80. Cancer Genome Atlas Research, N., et al., *The Cancer Genome Atlas Pan-Cancer analysis project.* Nat Genet, 2013. **45**(10): p. 1113-20.

81. Tracz-Gaszewska, Z., et al., *Molecular chaperones in the acquisition of cancer cell chemoresistance with mutated TP53 and MDM2 up-regulation.* Oncotarget, 2017. **8**(47): p. 82123-82143.

82. Wiech, M., et al., *Molecular Mechanism of Mutant p53 Stabilization: The Role of HSP70 and MDM2.* PLOS ONE, 2012. **7**(12): p. e51426.

83. Buckley, N.E., et al., *S100A2 is a BRCA1/p63 coregulated tumour suppressor gene with roles in the regulation of mutant p53 stability.* Cell death & disease, 2014. **5**(2): p. e1070-e1070.

84. Leng, R.P., et al., *Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation.* Cell, 2003. **112**(6): p. 779-91.

85. Sheng, Y., et al., *Molecular basis of Pirh2-mediated p53 ubiquitylation.* Nat Struct Mol Biol, 2008. **15**(12): p. 1334-42.

86. Yan, W., et al., *Arsenic trioxide reactivates proteasome-dependent degradation of mutant p53 protein in cancer cells in part via enhanced expression of Pirh2 E3 ligase.* PLoS One, 2014. **9**(8): p. e103497.

87. Liu, J., et al., *E3 ubiquitin ligase TRIM32 negatively regulates tumor suppressor p53 to promote tumorigenesis.* Cell Death Differ, 2014. **21**(11): p. 1792-804.

88. Hock, A.K. and K.H. Vousden, *The role of ubiquitin modification in the regulation of p53.* Biochim Biophys Acta, 2014. **1843**(1): p. 137-49.

89. Ben-Nissan, G. and M. Sharon, *Regulating the 20S proteasome ubiquitin-independent degradation pathway.* Biomolecules, 2014. **4**(3): p. 862-884.

90. Asher, G., et al., *P53 hot-spot mutants are resistant to ubiquitin-independent degradation by increased binding to NAD(P)H:quinone oxidoreductase 1.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(25): p. 15065-15070.

91. Oh, E.-T. and H.J. Park, *Implications of NQO1 in cancer therapy.* BMB reports, 2015. **48**(11): p. 609-617.

92. Glickman, M.H. and A. Ciechanover, *The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction.* Physiol Rev, 2002. **82**(2): p. 373-428.

93. Rodriguez, O.C., et al., *Dietary downregulation of mutant p53 levels via glucose restriction: mechanisms and implications for tumor therapy.* Cell Cycle, 2012. **11**(23): p. 4436-46.

94. Vakifahmetoglu-Norberg, H., et al., *Chaperone-mediated autophagy degrades mutant p53.* Genes Dev, 2013. **27**(15): p. 1718-30.

95. Choudhury, S., et al., *Dissecting the pathways that destabilize mutant p53: the proteasome or autophagy?* Cell Cycle, 2013. **12**(7): p. 1022-9.

96. Qin, B., et al., *DBC1 functions as a tumor suppressor by regulating p53 stability.* Cell Rep, 2015. **10**(8): p. 1324-34.

97. Yue, X., et al., *BAG2 promotes tumorigenesis through enhancing mutant p53 protein levels and function.* Elife, 2015. **4**.

98. Higashitsuji, H., et al., *The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquitylation and degradation of p53.* Cancer Cell, 2005. **8**(1): p. 75-87.

99. Zhou, X., et al., *Ribosomal proteins: functions beyond the ribosome.* J Mol Cell Biol, 2015. **7**(2): p. 92-104.

100. Xiong, X., et al., *Ribosomal protein S27-like and S27 interplay with p53-MDM2 axis as a target, a substrate and a regulator.* Oncogene, 2011. **30**(15): p. 1798-1811.

101. Zhang, Y., et al., *Negative regulation of HDM2 to attenuate p53 degradation by ribosomal protein L26.* Nucleic Acids Res, 2010. **38**(19): p. 6544-54.

102. Zhang, J., et al., *ABRO1 suppresses tumourigenesis and regulates the DNA damage response by stabilizing p53.* Nat Commun, 2014. **5**.

103. Yuan, J., et al., *USP10 regulates p53 localization and stability by deubiquitinating p53.* Cell, 2010. **140**(3): p. 384-96.

104. Melnikova, V.O., et al., *Mutant p53 is constitutively phosphorylated at Serine 15 in UV-induced mouse skin tumors: involvement of ERK1/2 MAP kinase.* Oncogene, 2003. **22**(38): p. 5958-66.

105. Yi, Y.W., et al., *Targeting mutant p53 by a SIRT1 activator YK-3-237 inhibits the proliferation of triple-negative breast cancer cells.* Oncotarget, 2013. **4**(7): p. 984-94.

106. Fei, Q., et al., *Histone methyltransferase SETDB1 regulates liver cancer cell growth through methylation of p53.* Nat Commun, 2015. **6**: p. 8651.

107. Pariat, M., et al., *Proteolysis by calpains: a possible contribution to degradation of p53.* Mol Cell Biol, 1997. **17**(5): p. 2806-15.

108. Zhou, X., Q. Hao, and H. Lu, *Mutant p53 in cancer therapy—the barrier or the path.* 2018.

109. Puca, R., et al., *Restoring p53 active conformation by zinc increases the response of mutant p53 tumor cells to anticancer drugs.* Cell Cycle, 2011. **10**(10): p. 1679-89.

110. Haupt, S. and Y. Haupt, *Manipulation of the tumor suppressor p53 for potentiating cancer therapy.* Semin Cancer Biol, 2004. **14**(4): p. 244-52.

111. Lambert, J.M., et al., *PRIMA-1 reactivates mutant p53 by covalent binding to the core domain.* Cancer Cell, 2009. **15**(5): p. 376-88.

112. Russo, D., et al., *PRIMA-1 cytotoxicity correlates with nucleolar localization and degradation of mutant p53 in breast cancer cells.* Biochem Biophys Res Commun, 2010. **402**(2): p. 345-50.

113. Liu, D.S., et al., *Inhibiting the system xC−/glutathione axis selectively targets cancers with mutant-p53 accumulation.* Nature Communications, 2017. **8**: p. 14844.

114. Liu, D.S., et al., *Inhibiting the system xC(-)/glutathione axis selectively targets cancers with mutant-p53 accumulation.* Nat Commun, 2017. **8**: p. 14844.

115. Alexandrova, E.M., et al., *Improving survival by exploiting tumour dependence on stabilized mutant p53 for treatment.* Nature, 2015. **523**(7560): p. 352-356.

116. Fu, S., et al., *Phase I study of pazopanib and vorinostat: a therapeutic approach for inhibiting mutant p53-mediated angiogenesis and facilitating mutant p53 degradation.* Ann Oncol, 2015. **26**(5): p. 1012-8.

117. Zhu, J., et al., *Gain-of-function p53 mutants co-opt chromatin pathways to drive cancer growth.* Nature, 2015. **525**(7568): p. 206-211.

118. Cordani, M., et al., *Mutant p53 proteins counteract autophagic mechanism sensitizing cancer cells to mTOR inhibition.* Molecular oncology, 2016. **10**(7): p. 1008-1029.

119. Maman, S. and I.P. Witz, *A history of exploring cancer in context.* Nature Reviews Cancer, 2018. **18**(6): p. 359-376.

120. De Palma, M., D. Biziato, and T.V. Petrova, *Microenvironmental regulation of tumour angiogenesis.* Nature Reviews Cancer, 2017. **17**: p. 457.

121. Balkwill, F.R., M. Capasso, and T. Hagemann, *The tumor microenvironment at a glance.* Journal of Cell Science, 2012. **125**(23): p. 5591.

122. Spranger, S. and T.F. Gajewski, *Impact of oncogenic pathways on evasion of antitumour immune responses.* Nature Reviews Cancer, 2018. **18**: p. 139.

123. Guo, G. and Y. Cui, *New perspective on targeting the tumor suppressor p53 pathway in the tumor microenvironment to enhance the efficacy of immunotherapy.* Journal for immunotherapy of cancer, 2015. **3**: p. 9-9.

124. Downing, S., et al., *Elevated Levels of Prostate-Specific Antigen (PSA) in Prostate Cancer Cells Expressing Mutant p53 is Associated with Tumor Metastasis.* Molecular Carcinogenesis, 2003. **38**(3): p. 130-140.

125. Novo, D., et al., *Mutant p53s generate pro-invasive niches by influencing exosome podocalyxin levels.* Nature Communications, 2018. **9**(1): p. 5069.

126. Cooks, T., et al., *Mutant p53 cancers reprogram macrophages to tumor supporting macrophages via exosomal miR-1246.* Nature Communications, 2018. **9**(1): p. 771.

127. Uehara, I. and N. Tanaka, *Role of p53 in the Regulation of the Inflammatory Tumor Microenvironment and Tumor Suppression.* Cancers, 2018. **10**(7): p. 219.

128. Bebelman, M.P., et al., *Biogenesis and function of extracellular vesicles in cancer.* Pharmacology & Therapeutics, 2018. **188**: p. 1-11.

129. van Niel, G., G. D'Angelo, and G. Raposo, *Shedding light on the cell biology of extracellular vesicles.* Nature Reviews Molecular Cell Biology, 2018. **19**: p. 213.

130. Colombo, M., G. Raposo, and C. Théry, *Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles.* Annual Review of Cell and Developmental Biology, 2014. **30**(1): p. 255-289.

131. Raposo, G. and W. Stoorvogel, *Extracellular vesicles: Exosomes, microvesicles, and friends.* The Journal of Cell Biology, 2013. **200**(4): p. 373.

132. Hessvik, N.P. and A. Llorente, *Current knowledge on exosome biogenesis and release.* Cellular and molecular life sciences : CMLS, 2018. **75**(2): p. 193-208.

133. Mathieu, M., et al., *Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication.* Nature Cell Biology, 2019. **21**(1): p. 9-17.

134. Davies, B.A., et al., *Membrane protein targeting to the MVB/lysosome.* Chemical reviews, 2009. **109**(4): p. 1575-1586.

135. Peinado, H., et al., *Pre-metastatic niches: organ-specific homes for metastases.* Nat Rev Cancer, 2017. **17**(5): p. 302-317.

136. Minciacchi, V.R., M.R. Freeman, and D. Di Vizio, *Extracellular Vesicles in Cancer: Exosomes, Microvesicles and the Emerging Role of Large Oncosomes.* Seminars in Cell & Developmental Biology, 2015. **40**: p. 41-51.

137. Becker, A., et al., *Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis.* Cancer Cell, 2016. **30**(6): p. 836-848.

138. Di Vizio, D., et al., *Oncosome Formation in Prostate Cancer: Association with a Region of Frequent Chromosomal Deletion in Metastatic Disease.* Cancer Research, 2009. **69**(13): p. 5601.

139. Zijlstra, C. and W. Stoorvogel, *Prostasomes as a source of diagnostic biomarkers for prostate cancer.* J Clin Invest, 2016. **126**(4): p. 1144-51.

140. Peinado, H., et al., *Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET.* Nat Med, 2012. **18**(6): p. 883-91.

141. Tickner, J.A., et al., *Functions and Therapeutic Roles of Exosomes in Cancer.* Frontiers in Oncology, 2014. **4**(127).

142. Scheffner, M. and S. Kumar, *Mammalian HECT ubiquitin-protein ligases: biological and pathophysiological aspects.* Biochim Biophys Acta, 2014. **1843**(1): p. 61-74.

143. Jolliffe, C., et al., *Identification of multiple proteins expressed in murine embryos as binding partners for the WW domains of the ubiquitin-protein ligase Nedd4*. Vol. 351 Pt 3. 2000. 557-65.

144. Cristillo, A.D., et al., *Cloning and characterization of N4WBP5A, an inducible, cyclosporine-sensitive, Nedd4-binding protein in human T lymphocytes.* J Biol Chem, 2003. **278**(36): p. 34587-97.

145. Yang, B. and S. Kumar, *Nedd4 and Nedd4-2: closely related ubiquitin-protein ligases with distinct physiological functions.* Cell death and differentiation, 2010. **17**(1): p. 68-77.

146. Foot, N. and S. Kumar, *NDFIP1 and NDFIP2*, in *Encyclopedia of Signaling Molecules*, S. Choi, Editor. 2018, Springer International Publishing: Cham. p. 3390-3395.

147. Harvey, K.F., et al., *N4WBP5, a potential target for ubiquitination by the Nedd4 family of proteins, is a novel Golgi-associated protein.* J Biol Chem, 2002. **277**(11): p. 9307-17.

148. Riling, C., et al., *Itch WW Domains Inhibit Its E3 Ubiquitin Ligase Activity by Blocking E2-E3 Ligase Trans-thiolation.* Journal of Biological Chemistry, 2015. **290**(39): p. 23875-23887.

149. Mund, T. and H.R. Pelham, *Control of the activity of WW-HECT domain E3 ubiquitin ligases by NDFIP proteins.* EMBO Rep, 2009. **10**(5): p. 501-7.

150. Li, Y., et al., *Rab5 and Ndfip1 are involved in Pten ubiquitination and nuclear trafficking.* Traffic, 2014. **15**(7): p. 749-61.

151. Lackovic, J., et al., *Differential regulation of Nedd4 ubiquitin ligases and their adaptor protein Ndfip1 in a rat model of ischemic stroke.* Exp Neurol, 2012. **235**(1): p. 326-35.

152. Howitt, J., et al., *Ndfip1 represses cell proliferation by controlling Pten localization and signaling specificity.* Journal of Molecular Cell Biology, 2015. **7**(2): p. 119-131.

153. Hammond, V.E., et al., *Ndfip1 is required for the development of pyramidal neuron dendrites and spines in the neocortex.* Cereb Cortex, 2014. **24**(12): p. 3289-300.

154. Oliver, P.M., et al., *Ndfip1 protein promotes the function of itch ubiquitin ligase to prevent T cell activation and T helper 2 cell-mediated inflammation.* Immunity, 2006. **25**(6): p. 929-940.

155. Kurzweil, V., A. Tarangelo, and P.M. Oliver, *Gastrointestinal microbiota do not significantly contribute to T cell activation or GI inflammation in Ndfip1-cKO mice.* PLoS One, 2012. **7**(4): p. e34478.

156. Ramon, H.E., et al., *The ubiquitin ligase adaptor Ndfip1 regulates T cell-mediated gastrointestinal inflammation and inflammatory bowel disease susceptibility.* Mucosal Immunol, 2011. **4**(3): p. 314-24.

157. Layman, A.A.K., et al., *Ndfip1 restricts mTORC1 signalling and glycolysis in regulatory T cells to prevent autoinflammatory disease.* Nat Commun, 2017. **8**: p. 15677.

158. Mund, T. and H.R. Pelham, *Regulation of PTEN/Akt and MAP kinase signaling pathways by the ubiquitin ligase activators Ndfip1 and Ndfip2.* Proc Natl Acad Sci U S A, 2010. **107**(25): p. 11429-34.

159. Beck, A., et al., *Nedd4 family interacting protein 1 (Ndfip1) promotes death of pancreatic beta cells.* Biochem Biophys Res Commun, 2015. **465**(4): p. 851-6.

160. Wang, Y., X. Tong, and X. Ye, *Ndfip1 negatively regulates RIG-I-dependent immune signaling by enhancing E3 ligase Smurf1-mediated MAVS degradation.* J Immunol, 2012. **189**(11): p. 5304-13.

161. Howitt, J., et al., *Divalent metal transporter 1 (DMT1) regulation by Ndfip1 prevents metal toxicity in human neurons.* Proc Natl Acad Sci U S A, 2009. **106**(36): p. 15489-94.

162. Kang, Y., et al., *Regulation of the human ether-a-go-go-related gene (hERG) potassium channel by Nedd4 family interacting proteins (Ndfips).* Biochem J, 2015. **472**(1): p. 71-82.

163. Murray, S.S., et al., *Ubiquitin Regulation of Trk Receptor Trafficking and Degradation.* Mol Neurobiol, 2019. **56**(3): p. 1628-1636.

164. Trimpert, C., et al., *NDFIP allows NEDD4/NEDD4L-induced AQP2 ubiquitination and degradation.* PLOS ONE, 2017. **12**(9): p. e0183774.

165. Low, L.H., et al., *Nedd4 family interacting protein 1 (Ndfip1) is required for ubiquitination and nuclear trafficking of BRCA1-associated ATM activator 1 (BRAT1) during the DNA damage response.* J Biol Chem, 2015. **290**(11): p. 7141-50.

166. Dalton, H.E., et al., *Drosophila Ndfip is a novel regulator of Notch signaling.* Cell death and differentiation, 2011. **18**(7): p. 1150-1160.

167. Yao, W., et al., *WW domain-mediated regulation and activation of E3 ubiquitin ligase Suppressor of Deltex.* Journal of Biological Chemistry, 2018. **293**(43): p. 16697-16708.

168. Putz, U., et al., *Nedd4 Family-interacting Protein 1 (Ndfip1) Is Required for the Exosomal Secretion of Nedd4 Family Proteins.* Journal of Biological Chemistry, 2008. **283**(47): p. 32621-32627.

169. Putz, U., et al., *The Tumor Suppressor PTEN Is Exported in Exosomes and Has Phosphatase Activity in Recipient Cells.* Science Signaling, 2012. **5**(243): p. ra70-ra70.

170. Sterzenbach, U., et al., *Engineered Exosomes as Vehicles for Biologically Active Proteins.* Mol Ther, 2017. **25**(6): p. 1269-1278.

171. Tian, J., et al., *Lower Expression of Ndfip1 Is Associated With Alzheimer Disease Pathogenesis Through Decreasing DMT1 Degradation and Increasing Iron Influx.* Frontiers in Aging Neuroscience, 2018. **10**(165).

172. Howitt, J., et al., *Increased Ndfip1 in the substantia nigra of Parkinsonian brains is associated with elevated iron levels.* PLoS One, 2014. **9**(1): p. e87119.

173. Altin, J.A., et al., *Ndfip1 mediates peripheral tolerance to self and exogenous antigen by inducing cell cycle exit in responding CD4+ T cells.* Proc Natl Acad Sci U S A, 2014. **111**(6): p. 2067-74.

174. Besse, A., et al., *MiR-338-5p sensitizes glioblastoma cells to radiation through regulation of genes involved in DNA damage response.* Tumor Biology, 2016. **37**(6): p. 7719-7727.

175. Peng, J., H. Liu, and C. Liu, *MiR-155 Promotes Uveal Melanoma Cell Proliferation and Invasion by Regulating NDFIP1 Expression.* Technology in cancer research & treatment, 2017. **16**(6): p. 1160-1167.

176. Collaud, S., et al., *Lung neuroendocrine tumors: correlation of ubiquitinylation and sumoylation with nucleo-cytosolic partitioning of PTEN.* BMC Cancer, 2015. **15**(1): p. 74.

177. Tung, Y.-T., et al., *Mir-17~92 Governs Motor Neuron Subtype Survival by Mediating Nuclear PTEN.* Cell Reports, 2015. **11**(8): p. 1305-1318.

178. Debernardi, S., et al., *MicroRNA miR-181a correlates with morphological sub-class of acute myeloid leukaemia and the expression of its target genes in global genome-wide analysis.* Leukemia, 2007. **21**: p. 912.

179. Bernassola, F., et al., *The HECT family of E3 ubiquitin ligases: multiple players in cancer development.* Cancer Cell, 2008. **14**(1): p. 10-21.

180. Laine, A. and Z. Ronai, *Regulation of p53 localization and transcription by the HECT domain E3 ligase WWP1.* Oncogene, 2006. **26**: p. 1477.

181. Liu, K., et al., *Protective effects of Ndfip1 on MPP(+)-induced apoptosis in MES23.5 cells and its underlying mechanisms.* Exp Neurol, 2015. **273**: p. 215-24.

182. Rivlin, N., et al., *Rescue of embryonic stem cells from cellular transformation by proteomic stabilization of mutant p53 and conversion into WT conformation.* Proc Natl Acad Sci U S A, 2014. **111**(19): p. 7006-11.

183. Nie, J., et al., *Smad ubiquitylation regulatory factor 1/2 (Smurf1/2) promotes p53 degradation by stabilizing the E3 ligase MDM2.* J Biol Chem, 2010. **285**(30): p. 22818-30.

184. Huang, D.W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nature Protocols, 2008. **4**: p. 44.

185. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists.* Nucleic Acids Res, 2009. **37**(1): p. 1-13.

186. Mi, H., et al., *PANTHER version 11: expanded annotation data from Gene Ontology and Reactome pathways, and data analysis tool enhancements.* Nucleic Acids Research, 2017. **45**(D1): p. D183-D189.

187. Oliveros, J.C., *Venny. An interactive tool for comparing lists with Venn's diagrams.* http://bioinfogp.cnb.csic.es/tools/venny/index.html. 2007-2015.

188. Hulsen, T., J. de Vlieg, and W. Alkema, *BioVenn - a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams.* BMC Genomics, 2008. **9**: p. 488.

189. Herold, M.J., et al., *Inducible and reversible gene silencing by stable integration of an shRNA-encoding lentivirus in transgenic rats.* Proc Natl Acad Sci U S A, 2008. **105**(47): p. 18507-12.

190. Spandidos, A., et al., *PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification.* Nucleic Acids Res, 2010. **38**(Database issue): p. D792-9.

191. Spandidos, A., et al., *A comprehensive collection of experimentally validated primers for Polymerase Chain Reaction quantitation of murine transcript abundance.* BMC Genomics, 2008. **9**: p. 633.

192. Wang, X. and B. Seed, *A PCR primer bank for quantitative gene expression analysis.* Nucleic Acids Res, 2003. **31**(24): p. e154.

193. Di Agostino, S., et al., *Gain of function of mutant p53: the mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation.* Cancer Cell, 2006. **10**(3): p. 191-202.

194. Ye, J., et al., *Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction.* BMC Bioinformatics, 2012. **13**: p. 134.

195. Livak, K.J. and T.D. Schmittgen, *Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method.* Methods, 2001. **25**(4): p. 402-408.

196. Schindelin, J., et al., *Fiji: an open-source platform for biological-image analysis.* Nat Methods, 2012. **9**(7): p. 676-82.

197. Suh, Y.-A., et al., *Multiple stress signals activate mutant p53 in vivo.* Cancer research, 2011. **71**(23): p. 7168-7175.

198. Szklarczyk, D., et al., *The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible.* Nucleic Acids Res, 2017. **45**(D1): p. D362-d368.

199. Hepburn, L.A., et al., *Targeting the spliceosome for cutaneous squamous cell carcinoma therapy: a role for c-MYC and wild-type p53 in determining the degree of tumour selectivity.* Oncotarget, 2018. **9**(33): p. 23029-23046.

200. Siebring-van Olst, E., et al., *A genome-wide siRNA screen for regulators of tumor suppressor p53 activity in human non-small cell lung cancer cells identifies components of the RNA splicing machinery as targets for anticancer treatment.* Mol Oncol, 2017. **11**(5): p. 534-551.

201. Wang, X. and R. Simon, *Identification of potential synthetic lethal genes to p53 using a computational biology approach.* BMC medical genomics, 2013. **6**: p. 30-30.

202. Heffernan-Stroud, L.A. and L.M. Obeid, *p53 and regulation of bioactive sphingolipids.* Adv Enzyme Regul, 2011. **51**(1): p. 219-28.

203. Fekry, B., et al., *C16-ceramide is a natural regulatory ligand of p53 in cellular stress response.* Nature Communications, 2018. **9**(1): p. 4149.

204. Jorgensen, P.M., et al., *Characterisation of the human APC1, the largest subunit of the anaphase-promoting complex.* Gene, 2001. **262**(1-2): p. 51-9.

205. Sivakumar, S. and G.J. Gorbsky, *Spatiotemporal regulation of the anaphase-promoting complex in mitosis.* Nature reviews. Molecular cell biology, 2015. **16**(2): p. 82-94.

206. Iannetti, A., et al., *Regulation of p53 and Rb Links the Alternative NF-κB Pathway to EZH2 Expression and Cell Senescence.* PLOS Genetics, 2014. **10**(9): p. e1004642.

207. He, Y., et al., *The anaphase-promoting complex/cyclosome is an E3 ubiquitin ligase for Mdm2.* Cell Cycle, 2014. **13**(13): p. 2101-9.

208. Hixon, M.L., et al., *Gain of function properties of mutant p53 proteins at the mitotic spindle cell cycle checkpoint.* Histol Histopathol, 2000. **15**(2): p. 551-6.

209. Lima, E.M., et al., *Methylation status of ANAPC1, CDKN2A and TP53 promoter genes in individuals with gastric cancer.* Braz J Med Biol Res, 2008. **41**(6): p. 539-43.

210. He, M.-L., et al., *Multiple gene dysfunctions lead to high cancer-susceptibility: evidences from a whole-exome sequencing study.* American journal of cancer research, 2011. **1**(4): p. 562-573.

211. Ying, J., et al., *DLEC1 is a functional 3p22.3 tumour suppressor silenced by promoter CpG methylation in colon and gastric cancers.* British journal of cancer, 2009. **100**(4): p. 663-669.

212. Li, L., et al. *Epigenomic characterization of a p53-regulated 3p22.2 tumor suppressor that inhibits STAT3 phosphorylation via protein docking and is frequently methylated in esophageal and other carcinomas*. Theranostics, 2018. **8**, 61-77 DOI: 10.7150/thno.20893.

213. Ahn, Y.-H., et al., *Map2k4 functions as a tumor suppressor in lung adenocarcinoma and inhibits tumor cell invasion by decreasing peroxisome proliferator-activated receptor γ2 expression.* Molecular and cellular biology, 2011. **31**(21): p. 4270-4285.

214. Garritano, S., et al. *More targets, more pathways and more clues for mutant p53*. Oncogenesis, 2013. **2**, e54 DOI: 10.1038/oncsis.2013.15.

215. Lee, S., et al., *Disrupting Na+,HCO3–-cotransporter NBCn1 (Slc4a7) delays murine breast cancer development*. Vol. 35. 2015.

216. Shearwin-Whyatt, L.M., et al., *N4WBP5A (Ndfip2), a Nedd4-interacting protein, localizes to multivesicular bodies and the Golgi, and has a potential role in protein trafficking.* Journal of Cell Science, 2004. **117**(16): p. 3679.

217. Putz, U., et al., *PTEN secretion in exosomes.* Methods, 2015. **77-78**: p. 157-63.

218. Jeffries, K.A. and N.I. Krupenko, *Chapter Seven - Ceramide Signaling and p53 Pathways*, in *Advances in Cancer Research*, C.E. Chalfant and P.B. Fisher, Editors. 2018, Academic Press. p. 191-215.

219. Gulati, S., et al., *Sterols and sphingolipids: dynamic duo or partners in crime?* Progress in lipid research, 2010. **49**(4): p. 353-365.

220. Parrales, A., E. Thoenen, and T. Iwakuma, *The interplay between mutant p53 and the mevalonate pathway.* Cell Death & Differentiation, 2018. **25**(3): p. 460-470.

221. Kleiger, G. and T. Mayor, *Perilous journey: a tour of the ubiquitin-proteasome system.* Trends Cell Biol, 2014. **24**(6): p. 352-9.

222. Pant, V. and G. Lozano, *Limiting the power of p53 through the ubiquitin proteasome pathway.* Genes Dev, 2014. **28**(16): p. 1739-51.

223. O'Keefe, K., H. Li, and Y. Zhang, *Nucleocytoplasmic shuttling of p53 is essential for MDM2-mediated cytoplasmic degradation but not ubiquitination.* Mol Cell Biol, 2003. **23**(18): p. 6396-405.

224. Kerppola, T.K., *Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells.* Nature Protocols, 2006. **1**: p. 1278.

225. Wandinger-Ness, A. and M. Zerial, *Rab proteins and the compartmentalization of the endosomal system.* Cold Spring Harb Perspect Biol, 2014. **6**(11): p. a022616.

226. Sluimer, J. and B. Distel, *Regulating the human HECT E3 ligases.* Cellular and molecular life sciences : CMLS, 2018. **75**(17): p. 3121-3141.

227. Feng, Y., B. Press, and A. Wandinger-Ness, *Rab 7: an important regulator of late endocytic membrane traffic.* J Cell Biol, 1995. **131**(6 Pt 1): p. 1435-52.

228. Kucera, A., O. Bakke, and C. Progida, *The multiple roles of Rab9 in the endolysosomal system.* Communicative & integrative biology, 2016. **9**(4): p. e1204498-e1204498.

229. Moreno-Gonzalo, O., C. Villarroya-Beltri, and F. Sánchez-Madrid, *Post-translational modifications of exosomal proteins.* Front Immunol, 2014. **5**: p. 383.

230. Savina, A., et al., *Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner.* Traffic, 2005. **6**(2): p. 131-43.

231. Yu, X., T. Riley, and A.J. Levine, *The regulation of the endosomal compartment by p53 the tumor suppressor gene.* The FEBS Journal, 2009. **276**(8): p. 2201-2212.

232. Shapira, I., et al., *P53 mutations in triple negative breast cancer upregulate endosomal recycling of epidermal growth factor receptor (EGFR) increasing its oncogenic potency.* Critical Reviews in Oncology/Hematology, 2013. **88**(2): p. 284-292.

233. Lee, S.H., et al., *p53, secreted by K-Ras-Snail pathway, is endocytosed by K-Ras-mutated cells; implication of target-specific drug delivery and early diagnostic marker.* Oncogene, 2009. **28**(19): p. 2005-14.

234. Burdakov, V.S., et al., *Exosomes Transfer p53 between Cells and Can Suppress Growth and Proliferation of p53-Negative Cells.* Cell and Tissue Biology, 2018. **12**(1): p. 20-26.

235. Chaudhary, N. and S. Maddika, *WWP2-WWP1 ubiquitin ligase complex coordinated by PPM1G maintains the balance between cellular p73 and DeltaNp73 levels.* Mol Cell Biol, 2014. **34**(19): p. 3754-64.

236. Attali, I., et al., *Ubiquitylation-dependent oligomerization regulates activity of Nedd4 ligases.* The EMBO journal, 2017. **36**(4): p. 425-440.

237. Zocco, D., et al., *Extracellular Vesicles as Shuttles of Tumor Biomarkers and Anti-Tumor Drugs.* Frontiers in Oncology, 2014. **4**(267).

238. Iraci, N., et al., *Focus on Extracellular Vesicles: Physiological Role and Signalling Properties of Extracellular Membrane Vesicles.* International journal of molecular sciences, 2016. **17**(2): p. 171-171.

239. Van Meir, E.G., et al., *Analysis of the p53 gene and its expression in human glioblastoma cells.* Cancer Res, 1994. **54**(3): p. 649-52.

240. Flaman, J.M., et al., *A simple p53 functional assay for screening cell lines, blood, and tumors.* Proc Natl Acad Sci U S A, 1995. **92**(9): p. 3963-7.

241. Masuda, H., et al., *Rearrangement of the p53 gene in human osteogenic sarcomas.* Proc Natl Acad Sci U S A, 1987. **84**(21): p. 7716-9.

242. Stiewe, T. and T.E. Haran, *How mutations shape p53 interactions with the genome to promote tumorigenesis and drug resistance.* Drug Resistance Updates, 2018. **38**: p. 27-43.

243. Vaughan, C.A., et al., *Gain-of-function p53 activates multiple signaling pathways to induce oncogenicity in lung cancer cells.* Molecular oncology, 2017. **11**(6): p. 696-711.

244. Cerami, E., et al., *The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data.* Cancer Discov, 2012. **2**(5): p. 401-4.

245. Gao, J., et al., *Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal.* Sci Signal, 2013. **6**(269): p. pl1.

246. Rhodes, D.R., et al., *ONCOMINE: a cancer microarray database and integrated data-mining platform.* Neoplasia, 2004. **6**(1): p. 1-6.

247. Gordon, G.J., et al., *Identification of novel candidate oncogenes and tumor suppressors in malignant pleural mesothelioma using large-scale transcriptional profiling.* Am J Pathol, 2005. **166**(6): p. 1827-40.

248. Dovrat, S., et al., *14-3-3 and beta-catenin are secreted on extracellular vesicles to activate the oncogenic Wnt pathway.* Mol Oncol, 2014. **8**(5): p. 894-911.

249. Zomer, A., et al., *Studying extracellular vesicle transfer by a Cre-loxP method.* Nature Protocols, 2015. **11**: p. 87.

250. Zwicker, J., et al., *Cell cycle regulation of the cyclin A, cdc25C and cdc2 genes is based on a common mechanism of transcriptional repression.* The EMBO journal, 1995. **14**(18): p. 4514-4522.

251. Salsi, V., et al., *Interactions between p300 and multiple NF-Y trimers govern cyclin B2 promoter function.* J Biol Chem, 2003. **278**(9): p. 6642-50.

252. Goldenson, B. and J.D. Crispino, *The aurora kinases in cell cycle and leukemia.* Oncogene, 2015. **34**(5): p. 537-545.

253. Kohno, M. and J. Pouyssegur, *Targeting the ERK signaling pathway in cancer therapy.* Annals of Medicine, 2006. **38**(3): p. 200-211.

254. Strano, S., et al., *Physical interaction with human tumor-derived p53 mutants inhibits p63 activities.* J Biol Chem, 2002. **277**(21): p. 18817-26.

255. Yeudall, W.A., et al., *Gain-of-function mutant p53 upregulates CXC chemokines and enhances cell migration.* Carcinogenesis, 2012. **33**(2): p. 442-51.

256. Neilsen, P.M., et al., *Mutant p53 uses p63 as a molecular chaperone to alter gene expression and induce a pro-invasive secretome.* Oncotarget, 2011. **2**(12): p. 1203-1217.

257. Bellazzo, A., G. Di Minin, and L. Collavin, *Cytoplasmic gain-of-function mutant p53 contributes to inflammation-associated cancer.* Molecular & Cellular Oncology, 2015. **2**(4): p. e1002719.

258. Morselli, E., et al., *Mutant p53 protein localized in the cytoplasm inhibits autophagy.* Cell Cycle, 2008. **7**(19): p. 3056-61.

259. Liu, J., et al., *Tumor suppressor p53 and its mutants in cancer metabolism.* Cancer letters, 2015. **356**(2 Pt A): p. 197-203.

260. McGranahan, N. and C. Swanton, *Clonal Heterogeneity and Tumor Evolution: Past, Present, and the Future.* Cell, 2017. **168**(4): p. 613-628.

261. Hobor, S., et al., *TGFα and Amphiregulin Paracrine Network Promotes Resistance to EGFR Blockade in Colorectal Cancer Cells.* Clinical Cancer Research, 2014. **20**(24): p. 6429.

262. Robles, A.I., J. Jen, and C.C. Harris, *Clinical Outcomes of TP53 Mutations in Cancers.* Cold Spring Harb Perspect Med, 2016. **6**(9).

263. Uhlen, M., et al., *A pathology atlas of the human cancer transcriptome.* Science, 2017. **357**(6352).

264. Keklikoglou, I., et al., *Chemotherapy elicits pro-metastatic extracellular vesicles in breast cancer models.* Nature Cell Biology, 2019. **21**(2): p. 190-202.

265. Luhtala, N. and T. Hunter, *Failure to detect functional transfer of active K-Ras protein from extracellular vesicles into recipient cells in culture.* PLOS ONE, 2018. **13**(9): p. e0203290.

266. Patocs, A., et al., *Breast-Cancer Stromal Cells with TP53 Mutations and Nodal Metastases.* New England Journal of Medicine, 2007. **357**(25): p. 2543-2551.

267. Qiu, W., et al., *No evidence of clonal somatic genetic alterations in cancer-associated fibroblasts from human breast and ovarian carcinomas.* Nature Genetics, 2008. **40**: p. 650.

268. Addadi, Y., et al., *p53 status in stromal fibroblasts modulates tumor growth in an SDF1-dependent manner.* Cancer research, 2010. **70**(23): p. 9650-9658.

269. Arandkar, S., et al., *Altered p53 functionality in cancer-associated fibroblasts contributes to their cancer-supporting features.* Proceedings of the National Academy of Sciences, 2018. **115**(25): p. 6410.

270. Cui, Y. and G. Guo, *Immunomodulatory Function of the Tumor Suppressor p53 in Host Immune Response and the Tumor Microenvironment.* International journal of molecular sciences, 2016. **17**(11): p. 1942.

271. Quigley, D., et al., *Lymphocyte Invasion in IC10/Basal-Like Breast Tumors Is Associated with Wild-Type <em>TP53</em&gt.* Molecular Cancer Research, 2015. **13**(3): p. 493.

272. Zhao, C., et al., *Hypoxic glioblastoma release exosomal VEGF-A induce the permeability of blood-brain barrier.* Biochem Biophys Res Commun, 2018. **502**(3): p. 324-331.

273. Chen, G., et al., *Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response.* Nature, 2018. **560**(7718): p. 382-386.