The clinical significance of cyclin E1 deregulation in high grade serous ovarian cancer and basal-like breast cancer

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Abstract

High grade serous ovarian cancer (HGSOC) and basal like breast cancer (BLBC) are genomically unstable and aggressive cancers that frequently co-occur and share common molecular features. Of these molecular characteristics are P53 inactivation occurring in almost all cases of HGSOC and BLBC, *BRCA1/2* inactivation reported in more than 50% of both cancers and *CCNE1* amplification reported in up to 30% and 8% of HGSOC and BLBC respectively.

Both HGSOC and BLBC are currently grouped in many clinical trials to test new drugs or drug combination, for instance, PARP inhibitors in the context of *BRCA1*/*BRCA2* mutation. We asked whether deregulated cyclin E1 (*CCNE1* amplification and/or its encoding protein, cyclin E1, overexpression) is an additional biomarker that can potentially be used to group patients with both diseases for therapeutic purposes. We studied two well characterised cohorts of 262 HGSOC and 222 familial breast cancer (BLBC enriched) samples of formalin fixed paraffin embedded sections. HGSOC and the BLBC enriched cohort were from patients enrolled in the Australian ovarian (AOCS) and the Kathleen Cuningham Foundation Consortium for research into Familial Breast cancer (KConFab) respectively. Using automated tissue based assay and an in situ hybridization probe that spans 19q12 locus harbouring *CCNE1*, we assessed the level of *CCNE1* amplification. We also assessed the expression of cyclin E1 and a cyclin E1 degradation associated protein FBXW7 and a cyclin E1 deubiquitinase, USP28, by immunohistochemistry, as possible drivers of high cyclin E1 expression in amplified and non-amplified cyclin $E1^{hi}$ subsets. We also assessed the expression of URI1 in our HGSOC cohort. URI1 is a protein encoded by the *URI1* gene which co-localise with *CCNE1* on 19q12 locus.

In HGSOC, we identified seemingly two separate subsets of cyclin $E1^{hi}$ tumors that have different pathological and biological characteristics as well as different clinical outcomes. These are the amplified/cyclin $E1^{hi}$ group that had amplification and high expression of cyclin E1, low expression of FBXW7, higher genomic instability, intact *BRCA1/2* and worse outcome. The other is the non-amplified/cyclin $E1^{hi}$ tumors that typically had high expression of cyclin E1 in the absence of amplification, high USP28 expression, lower genomic instability, more prevalent *BRCA1/2* loss and more favorable outcome compared to the amplified group.

Next we assessed cyclin E1 deregulation in the overlapping groups *BRCA1* mutant breast cancer and BLBC. Both subtypes had significantly higher expression of cyclin E1 and amplification compared to other breast cancer types. However, the intensity of cyclin E1 expression and level of 19q12 amplification were lower in BLBC compared to those observed in HGSOC. Moreover, in *BRCA1* mutant breast cancer and BLBC patients, only high expression of cyclin E1 was associated with lower overall survival while amplification did not seem to impact outcome. These observations were further supported by our meta-analysis that included our cohorts as well as other published datasets. In the meta-analysis, both *CCNE1* amplification and cyclin E1 expression were found to be adverse prognostic factors in HGSOC while only high expression was associated with worse survival in BLBC patients.

In fact, both amplified and non-amplified cyclin $E1^{hi}$ BLBC subsets shared almost all cyclin E1 deregulation associated features as well as many features with the non-amplified cyclin $E1^{hi}$ HGSOC subset. Of these are the prevalence of high expression of the cyclin E1 deubiquitinase, USP28, *BRCA* inactivation, the lower genomic instability and cyclin $E1^{hi}$ linked adverse outcome.

In order to provide better therapeutic options for cyclin $E1^{hi}$ *BRCA1* mutant breast cancer/BLBC patients, we sought to further assess mechanisms behind the co-occurrence of cyclin E1 overexpression and *BRCA1* inactivation.

Using the KConFab cohort we have found that *BRCA1* loss correlated with decreased phosphorylation of cyclin E1, on Threonine 62, assessed by immunohistochemistry. We also showed by in vitro analysis that *BRCA1* loss in cell lines led to cell cycle specific stabilisation of cyclin E1 by reducing cyclin E1 T62 phosphorylation. Conversely, *BRCA1* overexpression increased T62 phosphorylation. Overexpression of cyclin E1 with an inactivated T62 site, to mimic loss of phosphorylation, increased cyclin E1 stability and resistance to Paclitaxel. These findings suggest that *BRCA1* regulates cyclin E1 stability in breast cancer cells via regulating T62 phosphorylation.

We next assessed a combination therapy that target cyclin E1 and *BRCA1* inactivation using CDK2 and PARP inhibitors. CDK inhibitors are suggested to induce DNA damage and therefore we hypothesised that CDK2 inhibition would enhance sensitivity of *BRCA1* deficient cells to PARP inhibition. Our finding is that CDK2 inhibition induced DNA damage and synergised with the PARP inhibitor Rucaparib in *BRCA1*

mutated cell lines. Combination treatment of xenograft are in progress but the preliminary data is supportive of our hypothesis.

Our results propose a new therapeutic strategy for *BRCA1*-mutant breast cancer/BLBC by combining CDK2 and PARP inhibitors to enhance synthetic lethality. As this group shares similarities with non-amplified cyclin $E1^{hi}$ HGSOC subset, we suggest that this combination is likely to be effective in the comparable HGSOC subset.

Declaration

This is to certify that

i) the thesis comprises only my original work towards the degree of PhD except where indicated in the preface,

ii) due acknowledgement has been made in the text to all other material used

iii) the thesis is fewer than 100,000 words in length, exclusive of tables, maps and bibliographies.

Diar G. Aziz Aziz

Preface

All of the work in this thesis is my own, with the following exceptions:

- Chapter 3: these were done by Dr. Liz Caldon and her lab
    - The graphical abstract
    - the cancer genome atlas data USP28-cyclin E1 expression/amplification correlations in ovarian cancer Figure 3, D&E
    - the cancer genome atlas data survival analysis in ovarian cancer Figure 4, G&H
    - the genomic instability analysis Figure 5
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Chapter 4:

- Figure 1-C, BRCA1 methylation cyclin E1 expression analysis
- Figure 2-A, schematic of cyclin E1 degradation
- Figure 3,4,5
- S4,S5,S6

- Chapter 5:
    - Figure 1A&B
    - Figure 2A
    - Figure 3D
    - Figure 4
    - S3
    - S4

All additional significant intellectual and/or technical contributions are duly noted by authorships in publication in Chapters 3, 4 and 5 of this thesis.

Publications

The following publications fully or partially arising from the work in this thesis have been prepared during this study:

Aziz, D.,Etemadmoghadam, D., Caldon, C. E. Au-Yeung, G. Deng, N. Hutchinson, R. Bowtell, D. Waring, P. **(2018)**. "19q12 amplified and non-amplified subsets of high grade serous ovarian cancer with overexpression of cyclin E1 differ in their molecular drivers and clinical outcomes." **Gynecologic Oncology 151**(2): 327-336 **(Published)**.

Au-Yeung, G., Lang, F., Azar, W. J., Mitchell, C., Jarman, K. E., Lackovic, K., **Aziz, D.**, Cullinane, C., Pearson, R. B., Mileshkin, L., Rischin, D., Karst, A. M., Drapkin, R., Etemadmoghadam, D., Bowtell, D. **(2017)**. "Selective Targeting of Cyclin E1-Amplified High-Grade Serous Ovarian Cancer by Cyclin-Dependent Kinase 2 and AKT Inhibition." **Clinical Cancer Research.** 23(7):1862-1874 **(Published).**

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Aziz, D., Etemadmoghadam, D., Au-Yeung, G., Muranyi, A., Gresshoff, I., Christie, M., Hutchinson, R., Ferraro, D., Stanislaw, S., Henricksen, L., Tubbs, A., Shanmugam, K., Bowtell, D., Waring, P." Co-expression of *CCNE1* and *URI1*, with intact *BRCA1/2*

function is important for 19q12 - amplified high-grade serous ovarian cancer" **Tucson Symposium (2016)**, (Abstract) **(Poster presentation).**

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Aziz D, Lee,C., Chin,V., Fernandez,K., kConFab Investigators, AOCS, Etemadmoghadam, D., Bowtell,D. Waring,P. Caldon, C. "High cyclin E1 protein is a shared biomarker for specific subsets of basal-like breast cancer and high grade serous ovarian cancer"**(under review).**

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List of Abbreviations

AB	antibody
Amp	amplified
AOCS	Australian Ovarian Cancer Study
ATCC	American Type Culture Collection
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia mutated and Rad3 related
BCR	BTB-Cul3-Rbx1
BLBC	basal like breast cancer
BRCA	breast cancer gene
CNA	copy number alteration
CC	cell conditioning
CC1	cell conditioning 1
CC2	cell conditioning 2
Cdc25A	cell division cycle 25C phosphatase A
CDK	cyclin dependent kinase
CGH	comparative genomic hybridization
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
CIN	chromosomal instability
CLS	centrosomal localization signal
CP110	coiled-coil protein of 110
CPDs	Cdc4-phosphodegrons
Cul3	Cullin 3
DDR	DNA damage response
DFS	disease free survival
DIG	digoxin
DMEM	Dulbecco's odified Eagle Medium
DNP	dinitrophenol
E2F	E2 factor
EOC	epithelial ovarian cancer

ER	estrogen receptor
ER+	estrogen receptor positive
FBXW7	F-box protein 7
FCS	fetal calf serum
FDA	Food and Drug Administration
FFL	feedforward loop
FFPE	formalin-fixed, paraffin-embedded
FFPET	formalin fixed paraffin embedded tissues
FIGO	International Federation of Gynecology and Obstetrics
FISH	fluorescence in situ hybridiasation
FL	full length
G1	GAP 1
G2	GAP 2
GD	genome doubled
GSK3	Glycogen synthase kinase 3
HER2	human epidermal growth factor receptor type 2
HG	high grade
HGSOC	high grade serous ovarian cancer
HR	homologous recombination
IDC	invasive ductal carcinoma
IHC	immunohistochemistry
ISH	*in situ* hybridization
KConFab	Kathleen Cuningham Foundation Consortium for Research into Familial Breast cancer
LG	low grade
LMW	low molecular weight
MAD	median absolute deviation
MAPK	mitogen-activated protein kinase
MCM	minichromosome maintenance
Met	metastatic
Min	minutes
M phase	mitosis phase

MVA	multivariate analysis
NBLBC	non-basal like breast cancer
NEG	negative
NGD	non-genome doubled
NHMRC	National Statement on Ethical Conduct in Human Research
NOS	not otherwise specified
NPM	nucleophosmin
NR	not reported
OC	ovarian cancer
OS	overall survival
OSE	ovarian surface epithelium
P	Phosphorylation
PAD	publically available data
PARP	poly (ADP ribose) polymerase
PBS	phosphate-buffered saline
PFS	progression free survival
PI3K	phosphoinositide-3-kinase
PKCα	protein kinase Cα activity
POS	positive
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
RB	retinoblastoma
RBBP8	RB binding protein 8, endonuclease
RFS	relapse free survival
RMA	robust multi-array averages
ROC	receiver operator curve
RPM	round per minute
RPPA	reverse phase protein lysate
Rsf-1	remodelling and spacing factor 1
S372	serine 372
S384	serine 384
SB	southern blot

SCF	Skp1-Cdc53/Cul1- F-box protein
SNP	single nucleotide pleomorphic
S phase	Synthesis phase
STIC	serous tubal intraepithelial carcinomas
T380	threonine 380
T62	threonine 62
T-ALL	T-cell acute lymphoblastic
TCA	trichloroacetic acid
TCGA	The Cancer Genome Atlas
TMA	tissue microarrays
TNBC	triple negative breast cancer
TOF	time of fixation
TTF	time to fixation
UPP	ubiquitin proteasome pathway
USP28	ubiquitin-specific-processing protease 28
UVA	univariate analysis
WB	western blot

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Chapter 1: Introduction and Literature review

1.1 Introduction

A major breakthrough in cancer treatment has been the introduction of targeted therapies that specifically target particular molecular defects and thus can block cancer cell growth, proliferation or survival. The last decade has witnessed the discovery of Poly (ADP ribose) polymerase (PARP) inhibitors that target *BRCA1*- and *BRCA2*-related hereditary ovarian and breast cancers (Dziadkowiec, K. N. *et al.* 2016). This success has encouraged the search for other molecular targets to advance the development of targeted therapy in both ovarian and breast cancer patients (Hanahan, D. and Weinberg, R. A. 2011).

One of the potential molecular targets that has recently attracted increasing interest is the amplification of the *CCNE1* gene. Amplification of the locus harbouring *CCNE1*, 19q12, has been described as one of the most frequent genetic changes in human cancers, including ovarian cancer (Beroukhim, R. *et al.* 2010, Macheret, M. and Halazonetis, T. D. 2018). *CCNE1* amplification is frequently associated with chemo-resistance and unfavourable clinical outcome (Nakayama, K. *et al.* 2007, Nakayama, N. *et al.* 2010, Cancer Genome Atlas Research Network 2011, Karst, A. M. *et al.* 2014). The *CCNE1* gene encodes cyclin E1, a cell cycle regulatory protein that promotes G_1/S phase transition during the cell cycle. Cyclin E1 binds to, and activates cyclin dependent kinase 2 (CDK2). The expression of cyclin E1 is periodic and is tightly regulated throughout the different phases of the cell cycle via balanced transcription and degradation (Siu, K. T. *et al.* 2012).

CCNE1 is an oncogene that is frequently expressed in various precancerous lesions and is known to have a tumorigenic effect on untransformed cells, including proposed precursor cells of ovarian cancer (Shaye, A. *et al.* 2009, Karst, A. M. *et al.* 2014, Kuhn, E. *et al.* 2016).

Ovarian cancer, predominantly high grade serous ovarian cancer (HGSOC), is the leading cause of cancer related death of gynecological origin and the fifth cause of overall cancer related death in women (Cancer Genome Atlas Research Network 2011). *CCNE1* amplification is reported to occur in 15-30% of HGSOC cases (Nakayama, K. *et al.* 2007, Nakayama, N. *et al.* 2010, Cancer Genome Atlas Research Network 2011, Karst, A. M. *et al.* 2014). *CCNE1*-amplified ovarian cancer is increasingly being recognized as a distinct subset that is associated with high cyclin E1 expression, intact

BRCA1 and *BRCA2*, and unfavorable outcome (Nakayama, N. *et al.* 2010, Cancer Genome Atlas Research Network 2011, Etemadmoghadam, D. *et al.* 2013). In those cases, amplification-linked cyclin E1 overexpression has been shown to drive cell cycle progression and result in resistance to platinum-based therapeutic agents (Etemadmoghadam, D. *et al.* 2010). Therefore, patients within this group have an urgent unmet clinical need for the development of specific targeted therapy.

Notably, suppression of *CCNE1* has been shown to result in G_1 arrest in *CCNE1* amplified ovarian cancer cell lines (Etemadmoghadam, D. *et al.* 2010, Nakayama, N. *et al.* 2010, Yang, L. *et al.* 2015). Accordingly, targeting cyclin E1 activity, mainly by CDK2 inhibitors, has attracted interest over the last few years and cyclin E1 is currently emerging as an important therapeutic target in *CCNE1* amplified HGSOC and other cancers cases (Natrajan, R. *et al.* 2012, Kanska, J. *et al.* 2016, Au-Yeung, G. *et al.* 2018). In fact, the selective reliance of tumor cells on individual cyclins and CDKs has recently been translated into novel targeted cancer therapies (Geng, Y. *et al.* 2018). For instance, new generation specific CDK4 and CDK6 inhibitors (Palbociclib, Ribociclib, and Abemaciclib) have received breakthrough therapy designation by the Food and Drug Administration (FDA) for use in hormone receptor positive metastatic breast cancer (Asghar, U. *et al.* 2015, Sherr, C. J. *et al.* 2016, Mills, C. C. *et al.* 2017, Otto, T. and Sicinski, P. 2017, Geng, Y. *et al.* 2018).

The core concept of targeting CDK2 activity with CDK2 inhibitors is driven by the observation that *CCNE1* amplified cancer cells are addicted to cyclin E1 expression for their survival (Etemadmoghadam, D. *et al.* 2010, Nakayama, N. *et al.* 2010, Torti, D. and Trusolino, L. 2011). This is in agreement with the frequently reported association between *CCNE1* amplification and high expression of cyclin E1 (Nakayama, N. *et al.* 2010, Etemadmoghadam, D. *et al.* 2013, Karst, A. M. *et al.* 2014, Noske, A. *et al.* 2015, Nakayama, K. *et al.* 2016, Noske, A. *et al.* 2017). However, high expression of cyclin E1 has been reported in the absence of *CCNE1* amplification in various cancer types (Siu, K. T. *et al.* 2012). It is not understood whether the impact of high cyclin E1 in the absence of amplification is similar to that associated with amplification in terms of tumor behavior and patients' outcome. Moreover, it is not established whether suppression of cyclin E1 activity in *CCNE1* non-amplified tumors will have similar consequences to those with amplification. In ovarian cancer, high cyclin E1 expression has been shown to occur in up to 50% of HGSOC cases, of which half only is linked to

CCNE1 amplification (Nakayama, N. *et al.* 2010, Karst, A. M. *et al.* 2014). This discrepancy implies that in HGSOC, as in other tumor types, high cyclin E1 expression can occur in the absence of its corresponding gene amplification due to other mechanisms. Suggested other mechanisms for cyclin E1 expression mainly incorporate disruption of cyclin E1 periodic degradation (Nakayama, N. *et al.* 2010, Noske, A. *et al.* 2015, Ayhan, A. *et al.* 2017, Noske, A. *et al.* 2017). A similar example to the ovarian scenario is the reportedly common high cyclin E1 expression (~45%) in the *BRCA*-related basal-like breast cancer (BLBC) (Foulkes, W. D. *et al.* 2004), despite the less frequent incidence of *CCNE1* gene amplification (5-8%) (Cancer Genome Atlas Research Network 2012, Natrajan, R. *et al.* 2012). According to analysis of the cancer genome atlas (TCGA) breast cancer dataset, BLBC is one of the most closely related cancer subtypes to HGSOC and shares numerous genetic changes including higher tendency for widespread chromosomal copy number alterations, *TP53* mutation, *BRCA1/2* pathway disruption and *CCNE1* amplification (Cancer Genome Atlas Research Network 2012). However, in ovarian cancer, *CCNE1* amplification is almost always mutually exclusive of *BRCA* inactivation, possibly due to synthetic lethality between both aberrations (Etemadmoghadam, D. *et al.* 2013). Conversely, about half of *BRCA1/2*- related BLBC exhibit high expression of cyclin E1 (Foulkes, W. D. *et al.* , Waddell, N. *et al.* 2010). Previous research has linked high expression of cyclin E1 in the majority of the cases of BLBC, and of breast cancer overall, to epigenetic aberrations and disrupted degradation (Akhoondi, S. *et al.* 2007, Akhoondi, S. *et al.* 2010, Moelans, C. B. *et al.* 2014, Schulein-Volk, C. *et al.* 2014, Tan, Y. *et al.* 2014) with reportedly less frequent *CCNE1* amplification in breast cancer (Cancer Genome Atlas Research Network 2012, Natrajan, R. *et al.* 2012). Learning from the BLBC example, we sought to seek explanations for high cyclin E1 expression in a proportion of HGSOC that lacked *CCNE1* amplification. We aimed to characterise those cases in terms of possible drivers, *BRCA1/2* relation and prognostic impact. Similarly, and prompted by the ovarian scenario of proposed synthetic lethality between *BRCA1/2* mutation and *CCNE1* amplification in HGSOC cases, we searched for similar *BRCA* - *CCNE1* relationship in breast cancer cases, particularly in the allegedly HGSOC comparable group, BLBC.

This literature review explains cyclin E1 regulation and function during the cell cycle in normal and neoplastic cells. Additionally, it highlights the prevalence of cyclin E1 abundance and dysregulation, the underlining mechanisms and the subsequent clinical impact in the two supposedly related types of cancer: ovarian (HGSOC) and breast (BLBC). For both ovarian and breast cancer, the literature has provided inconsistent results in terms of prevalence of high cyclin E1 and/or *CCNE1* amplification, correlation with histopathological features and impact on patient outcomes. This inconsistency could possibly be due to using various techniques, assay cut offs and cohorts. This review outlines the major studies on cyclin E1 expression and amplification in both ovarian and breast cancer and their impact. It also illustrates the correlation of cyclin E1 with *BRCA* inactivation in both tumor types. The review finally summarises the proposed therapeutic tools in targeting cyclin E1 deregulation, particularly the promising strategy of CDK2 inhibitors in combination with other treatments. The aims of this thesis are described at the end of this literature review.

1.2 Cell cycle and cyclin E1 regulation and deregulation

The cell cycle that shapes cell division in eukaryotic cells involves four phases, the preparation phase G1, the DNA replication phase S, a gap phase in which the cell prepares for mitosis, G2, and lastly the M phase (Mitosis) which involves segregation of the chromosomes and cytokinesis. Cytokinesis is cytoplasmic division and formation of two cells which have a diploid complement of chromosomes (Siu, K. T. *et al.* 2012). G0 is a latent or resting phase to which the cell may enter before entering into G1 (Siu, K. T. *et al.* 2012). G0 is either regarded as a continuation of G1 phase as the cell is not dividing and is not preparing to, or as a distinct inactive phase outside the cell cycle (Siu, K. T. *et al.* 2012).

The cell cycle is tightly regulated by restriction points that are fundamental for maintaining genetic stability through restricting inaccurate chromosome duplication and separation (Swanton, C. 2004). Therefore, failure of checkpoints to block the cell cycle after improper stimuli, could eventually result in cancer through the transmission of genetic defects acquired by parental cells to their daughter cells (Hanahan, D. and Weinberg, R. A. 2000). Consequently, cell cycle deregulation is a hallmark of cancer (Hanahan, D. and Weinberg, R. A. 2000). Two main classes of proteins that regulate cell cycle progression are cyclins and CDKs.

The cyclin family is a group of proteins that were originally described in 1983 as

proteins that cyclically accumulate and get destroyed during the cell cycle of sea urchin eggs (Evans, T. *et al.* 1983). In mammals, several different cyclin types have been described based on the extent of similarity in the protein sequence and their various association throughout different phases of the cell cycle (Morgan, D. O. 1995). The two main groups are the G1/S phase and the G2/M phase cyclins (Morgan, D. O. 1995). Cyclins oscillate during the different phases of the cell cycle (Morgan, D. O. 1995). While cyclins lack the enzymatic activity themselves, they target CDKs to particular subcellular locations through their substrates' specific binding sites (Morgan, D. O. 1995). The events of the cell cycle are tightly regulated by specificity of association of each cyclin to their related CDKs as well as the additional phosphorylation mediated regulation of CDKs by CDK inhibitor proteins (Morgan, D. O. 1995). A crucial element in maintaining periodic cyclin oscillations during different phases of the cell cycle is the balance between transcription and ubiquitination - mediated proteasome degradation (Noble, M. E. M. *et al.* 1997). However, the levels of CDKs are comparatively consistent during the various phases of the cell cycle (Noble, M. E. M. *et al.* 1997).

Each cyclin has particular functions that can overlap with other members of the cyclin family (Horton, L. E. and Templeton, D. J. 1997). Cyclin proteins in general are formed of three components: the cyclin Box and the N and C terminals (Horton, L. E. and Templeton, D. J. 1997). The cyclin Box is a 100 amino acid sequence, that tends to be similar in different cyclins, which contain the CDK bindings sites with specificity of substrate recognition provided by the cyclin Box (Horton, L. E. and Templeton, D. J. 1997) and C terminus (Kobayashi, H. *et al.* 1992). Each cyclin-CDK dimer targets specific substrates (Hochegger, H. *et al.* 2008). The substrate specificity is mainly defined by the different sequences of each cyclin, the periodicity of their expression during various phases of the cell cycle as well as by their specific cellular localization (Hochegger, H. *et al.* 2008).

The cyclin family include primary cyclins; A, B, D and E and secondary cyclins; C and H (Husdal, A. *et al.* 2006). Deregulation of the primary cyclins has been reported in various malignancies including breast and ovarian cancers (Nielsen, N. H. *et al.* 1996, Marone, M. *et al.* 1998, Poikonen, P. *et al.* 2005). Deregulation of those cyclins have been linked to oncogenesis and increased proliferation of malignant cells, however, reports have not been fully consistent. There are only a limited number of reports

concerning the secondary cyclins C and H in clinical materials. This review will cover the primary cyclins, mainly cyclin E1 as the main focus of this thesis.

The primary cyclin family includes G2/M mitotic cyclins A and B and the G1/S cyclins D and cyclin E (Fung, T. K. and Poon, R. Y. 2005). Cyclin A is both G1/S phase cyclin as well as G2/M cyclin. In mammalian cells, cyclins A (A1 and A2) and B (B1, B2 and B3) are considered mitotic cyclins because their synthesis and destruction occur around time of mitosis (Fung, T. K. and Poon, R. Y. 2005). Cyclin A starts accumulating in late G1 phase throughout S phase and G2 phase with cyclin B synthesis and destruction occur slightly after cyclin A (Fung, T. K. and Poon, R. Y. 2005).

Cyclin A binds and activates CDK1 and CDK2 promoting S phase entry through phosphorylating substrates of the DNA replication initiation complex, thus facilitating the initiation of DNA replication (Chibazakura, T. *et al.* 2011). Cyclin A1 is also present in meiosis, in very early embryos and has an essential function in spermatogenesis (Liu, D. *et al.* 1998). Cyclin A2 is expressed in all proliferating somatic cells and disruption of its gene leads to early embryonic lethality (Murphy, M. *et al.* 1997). Notably, cyclin A overexpression has been linked to amplification of both cyclin A and cyclin E genes (Husdal, A. *et al.* 2006) and is reported to drive poor outcome in breast cancer (Michalides, R. *et al.* 2002).

Cyclin B binds and activates only CDK1 (Jackman M, F. M., Pines J. 1995). The majority of dividing cells co - express both cyclin B1 and B2 , however these two proteins are not co - localised within the cell (Jackman M, F. M., Pines J. 1995) and only the lack of cyclin B1 has been shown to be lethal (Fung, T. K. and Poon, R. Y. 2005). Cyclin B3 expression is limited to the developing germ cells and in the adult testis (Fung, T. K. and Poon, R. Y. 2005). Cyclin B-CDK1 dimer formation initiates mitosis in animal cells. During interphase, cyclin B usually shuttles between the nucleus and the cytoplasm (Pines, O. G. a. J. 2010). Following its binding to CDK1 and formation of cyclin B-CDK complex, there is a 40 - fold increase in its import to the nucleus throughout the prophase up to the stage of nuclear lamina disassembly and nuclear envelop breakdown (Pines, O. G. a. J. 2010). Cyclin B is overexpressed in various malignancies including breast cancer. A meta-analysis assessment of clinical, pathological and prognostic significance of cyclin B in breast cancer has revealed that cyclin B overexpression is associated with higher rate of lymphatic invasion and is as an independent adverse prognostic factor that associates with shorter overall survival

(OS) and disease free survival (DFS) (Xi Sun, G. Z., Liang Shi, Ying Wang, Beicheng Sun, Qiang Ding 2017).

The second group of the primary cyclins is the G1/S cyclins (Swanton, C. 2004). The G1/S cyclins are the main group involved in early cell cycle regulation and includes the cyclin D group (D1, D2 and D3) and the cyclin E group (E1 and E2) (Swanton, C. 2004). Both cyclin D and E groups direct passaging through the cell cycle restriction point from G1 into S phase (Swanton, C. 2004).

Cyclin D is a major participant in signaling the progression of cells through the early G1 phase (Swanton, C. 2004). Cyclin D binds to, and activates CDK4 and CDK6 to form complexes that phosphorylate and partially inactivate the repressive retinoblastoma (RB) activity on E2F transcription factor (Swanton, C. 2004). In addition, cyclin D1 by itself plays a role in activating estrogen receptor (ER) transcriptional activity and is therefore a frequent finding in ER positive cancers including breast cancer (Neuman, E. *et al.* 1997). The increased ER activity is achieved by cyclin D1 association with ER receptor and by cyclin D1 interaction with a histone acetyltransferase independent of CDK4 action (Neuman, E. *et al.* 1997). Cyclin D overexpression is a common finding in most cancer types and members of cyclin D family are prognostic markers in pancreatic, endometrial, and head and neck cancer (Kornmann, M. *et al.* 1998, Namazie, A. *et al.* 2002, Khabaz, M. N. *et al.* 2017).

1.3 Cyclin E family

Cyclin E1 is the prototype of cyclin E that was first identified in 1991 (Koff, A. *et al.* 1991). Cyclin E1 has been found to play a crucial role in both cell cycle progression and oncogenesis (Möröy, T. and Geisen, C. 2004, Hwang, H. C. and Clurman, B. E. 2005). Cyclin E2 is the second member of cyclin E2 family which was first described in 1998 and is mostly viewed to have a redundant function with cyclin E1 (Möröy, T. and Geisen, C. 2004, Hwang, H. C. and Clurman, B. E. 2005).

Cyclin E1 is encoded by *CCNE1* gene located at 19q12 locus while cyclin E2 is encoded by *CCNE2* located at 8.q22.1 While the genes encoding cyclin E1 and cyclin E2 are different, the protein sequences and functional motives are highly similar (Pearson, W. 1990). This similarity and the nuclear co-localization of both proteins support the notion of functional redundancy between cyclin E1 and cyclin E2 (Caldon, C. E. and Musgrove, E. 2010).

Importantly, amplification of *CCNE1* and *CCNE2* genes and overexpression of their corresponding proteins cyclin E1 and cyclin E2 respectively have been reported in various human cancers (Schraml, P. *et al.* 2003, Shaye, A. *et al.* 2009, Gao, S. *et al.* 2013, Patch, A. M. *et al.* 2015, Huang, L. *et al.* 2016, Nakayama, K. *et al.* 2016, Geng, Y. *et al.* 2018). Notably, a study of somatic copy-number alteration across human cancers has revealed that 19q12, the locus carrying *CCNE1*, is one of the most commonly amplified foci in various human cancers (Beroukhim, R. *et al.* 2010).

1.3.1 Cyclin E1 function and substrates

Cyclin E regulates multiple cellular processes at various developmental stages. During embryogenesis, cyclin E1 is found to be essential for supporting endoreduplication and generation of polyploid cells such as megakaryocytes, that generate platelets and trophoblast giant cells, essential for placental formation (Geng, Y. *et al.* 2003, Parisi, T. *et al.* 2003). As a result, cyclin E1 ablation result in embryonic lethality due to its effects on the placenta (Geng, Y. *et al.* 2003, Parisi, T. *et al.* 2003). Additionally, extensive research has proven that cyclin E1 plays an important role in various cellular processes in a kinase dependent and independent manner.

The kinase dependent function of cyclin E1 is basically mediated via cyclin E1 –CDK2 complex and mostly involves phosphorylation of various substrates including RB, Smad3, centriolar coiled-coil protein of 110 kDa (CP110), c-Myc, p27 and p21, BRCA1 and nucleophosmin (NPM) and possibly others (Swanton, C. 2004, Schildkraut, J. M. *et al.* 2008, Siu, K. T. *et al.* 2012, Wiedemeyer, W. R. *et al.* 2014).

First, cyclin E1-CDK2 mediated RB phosphorylation, in cooperation with cyclin D-CDK4/6, is a key regulatory step in the cell cycle that positively regulates cell proliferation through stimulating the cell cycle progression from G1 phase to S phase (Swanton, C. 2004, Siu, K. T. *et al.* 2012, Wiedemeyer, W. R. *et al.* 2014). Hyperphosphorylation of RB contributes to the inactivation of RB repressive activity on E2F transcription factor (Swanton, C. 2004, Siu, K. T. *et al.* 2012, Nik, N. N. *et al.* 2014). Release of the E2F transcription factor leads to the expression of proteins that are involved in the S- phase entry and DNA synthesis and progression, including cyclin E1 itself, and these steps are collectively titled the RB pathway (Figure 1-1) (Swanton, C. 2004, Siu, K. T. *et al.* 2012, Nik, N. N. *et al.* 2014, Rundle, S. *et al.* 2017).

Secondly, and in a similar effect, cyclin E1-CDK2 promotes cell cycle progression by mediating phosphorylation of another protein called Smad3 (Matsuura, I. *et al.* 2004,

Cooley, A. *et al.* 2010). Smad3 is a key mediator of transforming growth factor-β signaling pathway, which can inhibit cell cycle progression (Massagué, J. *et al.* 2000, Derynck, R. *et al.* 2001). Therefore, cyclin E1- CDK2 mediated phosphorylation of Smad3 promotes cell cycle progression (Matsuura, I. *et al.* 2004, Cooley, A. *et al.* 2010).

Third, c-Myc is another important target for cyclin E1-CDK2 mediated phosphorylation that has significantly changed our understanding of the role of cyclin E1 in oncogenesis (Hydbring, P. *et al.* 2010). This is because cyclin E1-CDK2 mediated phosphorylation of c-Myc counteracts oncogene-induced senescence through controlling a transcriptional program (Hydbring, P. *et al.* 2010). Curiously, the target phosphate for CDK2 mediated phosphorylation of c-Myc, serine-62, is the same that regulates Myc stability via FBXW7 ubiquitin ligase pathway (Sears, R. *et al.* 2000, Welcker, M. *et al.* 2004, Yada, M. *et al.* 2004).

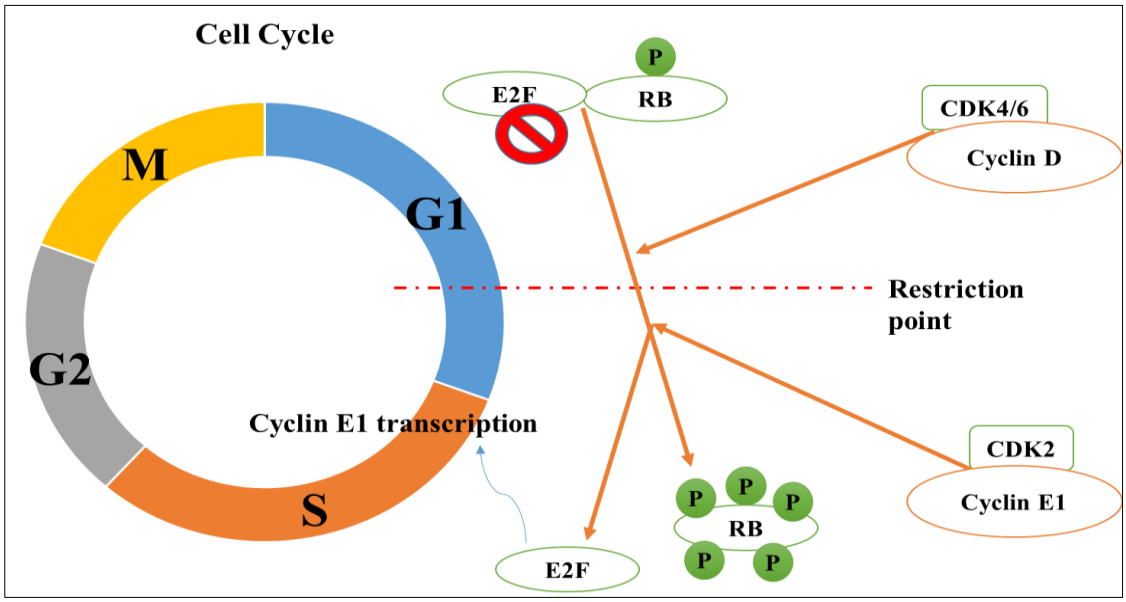

Figure 1-1 RB pathway and G1-S transition: RB phosphorylation must be initiated by cyclin D-CDK4/CDK6 and maintained by cyclin E1-CDK2, RB phosphorylation releases its inhibitory action on E2F and E2F initiates the transcription of cell cycle related genes including cyclin E1

Therefore, the c-Myc phosphodegron is seemingly essential for both transcriptional activity and protein stability (Hemann, M. T. *et al.* 2005). Consequently, for tumors that rely on CDK2 for evasion of oncogene-induced senescence in particular, CDK

inhibition make a possible option for an effective anticancer strategy (Ruffner, H. *et al.* 1999).

Fourthly, cyclin E1-CDK2 catalyzes protein degradation controlled by the SCF^{Skp2} ubiquitin ligase by phosphorylating p27 and p21 in G_1 and S phases, respectively (Sheaff, R. J. *et al.* 1997, Carrano, A. C. *et al.* 1999, Tsvetkov, L. M. *et al.* 1999, Malek, N. P. *et al.* 2001, Bornstein, G. *et al.* 2003). Additionally, cyclin E1-CDK2 complex also phosphorylates proteins involved in DNA synthesis and DNA repair including BRCA1 and plays a role in apoptosis regulation in -response to DNA damage (Ruffner, H. *et al.* 1999).

Lastly, cyclin E1/CDK2 phosphorylates NPM, which following its phosphorylation dissociates from an unduplicated centrosome and initiates the centrosome duplication (Okuda, M. *et al.* 2000, Tokuyama, Y. *et al.* 2001, Swanton, C. 2004, Schildkraut, J. M. *et al.* 2008, Nakayama, N. *et al.* 2010, Nik, N. N. *et al.* 2014). Since cyclin E1 interacts with the centrosome, overexpression of cyclin E1 results in polyploidy that might eventually contribute to genomic instability (Schildkraut, J. M. *et al.* 2008, Etemadmoghadam, D. *et al.* 2013). Another cyclin E1-CDK2 substrate is CP110, a protein that is essential for duplication of the centrioles and centrosome separation (Chen, Z. *et al.* 2002).

Further to its central role in centrosome duplication through NPM phosphorylation, cyclin E1 is believed to have a domain, of 20 amino acids, called the centrosomal localization signal (CLS). CLS plays a crucial role in targeting centrosome and promoting S phase entry and DNA synthesis in a CDK2 independent way (Matsumoto, Y. and Maller, J. L. 2004).

The kinase dependent functions of cyclin E1 in cell cycle progression are complemented by kinase independent roles in the initiation of DNA replication. Essentially, the kinase independent function of cyclin E1 is to facilitate the loading of a DNA helicase named minichromosome maintenance (MCM) protein complex (Geng, Y. *et al.* 2007). MCM is required in DNA pre-replication complex during G0/S phase progression (Carpentieri, F. *et al.* 2002). Cyclin E1 mediated MCM incorporation into DNA pre-replication complex is thought to be a kinase independent function of cyclin E1 (Geng, Y. *et al.* 2003, Roberts, J. M. and Sherr, C. J. 2003, Geng, Y. *et al.* 2007). This is in agreement with the reported failure of cyclin E1 null cells to incorporate MCM and initiate DNA synthesis (Geng, Y. *et al.* 2003, Geng, Y. *et al.* 2007) .

Conversely, cells that lacked CDK, whether by knockdown or CDK2 inhibitors, were able to initiate DNA synthesis (Roberts, J. M. and Sherr, C. J. 2003). In addition to the previously described kinase independent function of cyclin E1 (Geng, Y. *et al.* 2007), Geng *et al* has recently shown hepatic cancer cells that expressed "kinase dead" cyclin E–CDK2 complexes proliferated normally (Geng, Y. *et al.* 2018). These recent observations imply that E cyclins are crucial for liver cancer cell proliferation and progression in a kinase independent form (Geng, Y. *et al.* 2018).

Overall, the roles of cyclin E1, kinase dependent and independent, highlight its importance as a potential promising therapeutic target for cancer treatment (Siu, K. T. *et al.* 2012).

1.3.2 Cyclin E1 Periodicity

The level of cyclin E1 mRNA expression, abundance of its protein and the catalytic activity of cyclin E1-CDK2 are periodic throughout the cell cycle (Dulic, V. *et al.* 1992, Koff, A. *et al.* 1992). This periodicity is achieved partly via *CCNE1* gene transcription, that is by itself cell cycle dependent (Ohtani, K. *et al.* 1995, Geng, Y. *et al.* 1996) and by post transcriptional regulation via the tightly regulated ubiquitin mediated degradation (Clurman, B. E. *et al.* 1996, Won, K. A. and Reed, S. I. 1996).

Oscillation of cyclin E1 starts upon mitogenic signaling to signal cell proliferation leading to rapid synthesis in G1 phase (Lew, D. J. *et al.* 1991, Dulic, V. *et al.* 1992, Koff, A. *et al.* 1992). Cyclin E1 protein accumulation in late G1 phase is responsible for S phase entry timing (Lew, D. J. *et al.* 1991, Dulic, V. *et al.* 1992, Koff, A. *et al.* 1992). When cyclin E1 is downregulated in late S phase and G2 further mitogen signaling is required for upregulation of cyclin E1 as part of the initiation of a new cell cycle (Lew, D. J. *et al.* 1991, Dulic, V. *et al.* 1992, Koff, A. *et al.* 1992). The periodicity of cyclin E1 expression underlies its significance in normal cell cycle progression in the control of cell cycle restriction point (Lew, D. J. *et al.* 1991, Dulic, V. *et al.* 1992, Koff, A. *et al.* 1992).

1.3.3 Cyclin E1 synthesis

In humans, the cyclin E1 protein is 395 amino acids and approximately 50 kDa molecular weight (Koff, A. *et al.* 1991, Lew, D. J. *et al.* 1991). The promotor of cyclin E1 gene harbors an E2F binding site (Weinberg, R. A. 1995). E2F is a transcription factor that is regulated by cell cycle regulatory proteins including cyclin E1 (Weinberg, R. A. 1995). Firstly, cyclin D-CDK4/6 together with cyclin E1/CDK2 mediated

phosphorylation of RB occur during the G1 phase (Weinberg, R. A. 1995). This releases repression of the E2F transcription factor (Weinberg, R. A. 1995). Once E2F is released and its transcriptional program is activated, it in turn enhances the expression of the responsive genes, including cyclin E1 itself (Weinberg, R. A. 1995). These findings imply that the net effect of cyclin D on cyclin E1 is to bolster its gene expression (Lukas, J. *et al.* 1995, Leng, X. *et al.* 1997, Geng, Y. *et al.* 1999). Overall, cyclin D-CDK4/6 expression and activation is mitogen dependent and consequently, cyclin E1 expression is indirectly mitogen dependent too (Sherr, C. J. 1995).

C-myc, a proto-oncogene that is involved in activating cyclin E1, is another cell proliferation-associated target of mitogen mediated induction (Dubik, D. *et al.* 1987, Murphy, L. J. *et al.* 1987). C-myc has been proven necessary and sufficient for G_1/S-phase progression (Henriksson, M. and Lüscher, B. 1996) as c-myc activation results in activation of cyclin D and cyclin E1 associated kinases (Steiner, P. *et al.* 1995, Rudolph, B. *et al.* 1996, Pérez-Roger, I. *et al.* 1997). Activation of cyclin E1-CDK2 by c-myc occur through various mechanisms including modification of cyclin E1-CDK2 complex to a form that is responsive to cdc25 phosphatase activation (Steiner, P. *et al.* 1995) or through increasing the level of cyclin E1 protein (Jansen-Dürr, P. *et al.* 1993, Pérez-Roger, I. *et al.* 1997) or inhibiting cyclin dependent kinase inhibitor p27 from association with cyclin E1-CDK2 complex (Vlach, J. *et al.* 1996, Pérez-Roger, I. *et al.* 1997).

Importantly, mitogenic signals have a rate limiting impact on cyclin E1 synthesis during G1 phase (Santoni-Rugiu, E. *et al.* 2000, Geng, Y. *et al.* 2003). However, when cyclin E1 is overexpressed, the progression of cell cycle becomes liberated of the usually rate limiting effect of mitogenic signals (Santoni-Rugiu, E. *et al.* 2000). Evading mitogenic signals is strongly perceived as an important mechanism in cyclin E1 oncogenesis (Hwang, H. C. and Clurman, B. E. 2005).

1.3.4 Dysregulated synthesis of cyclin E1

Dysregulated synthesis of cyclin E1 encompasses various mechanisms, of which the most common is amplification of its gene locus on chromosome 19q12 (Akama, Y. *et al.* 1995, Kitahara, K. *et al.* 1995, Marone, M. *et al.* 1998, Donnelian, R. and Chetty, R. 1999, Etemadmoghadam, D. *et al.* 2009, Beroukhim, R. *et al.* 2010). 19q12 has been reported as one of the most frequently amplified foci in various cancer types (Beroukhim, R. *et al.* 2010). Various publications have equally reported *CCNE1*

amongst the 5 most frequently amplified genes in this locus, at least in breast and ovarian cancer, the other four genes are *POP4*, *PLEKHF1*, *C19orf12*, and *C19orf2* (*URI1*) (Etemadmoghadam, D. *et al.* 2009, Beroukhim, R. *et al.* 2010, Etemadmoghadam, D. *et al.* 2010). The *CCNE1* gene, however, is strongly perceived as the key oncogenic target in the 19q12 locus based on a sum of observations that include the fact that deregulated cyclin E1 is known to deregulate the cell cycle, promote genomic instability and play a significant role in carcinogenesis (Etemadmoghadam, D. *et al.* 2010). However, this observation per se is not sufficient to exclude other genes as possible drivers of 19q12 amplification (Etemadmoghadam, D. *et al.* 2010). For instance, the gene neighboring *CCNE1*, *C19orf2* has been reported to encode URI1, member of the prefoldin family of molecular chaperones (Parusel, C. T. *et al.* 2006). URI1 is proposed to be involved in chromatin remodeling and endogenous genotoxic DNA damage repair and prevention, and hence protects against genomic instability (Parusel, C. T. *et al.* 2006). Additionally, URI1 has been shown to play a role regulating the mTOR/S6K1 survival pathway (Djouder, N. *et al.* 2007). Even though these interesting biological associations nurture the possibility that *C19orf2* is a driver of 19q12 locus, the literature findings are inconsistent. In agreement with the notion that *CCNE1* is the main, if not the only, oncogenic driver within the 19q12 locus2, Nakayama et al has reported an increase in apoptosis and decrease in the growth of *CCNE1* amplified ovarian cancer cells when subjected to *CCNE1* siRNA (Nakayama, N. *et al.* 2010). They further reported no impact on cell lines that had overexpression of cyclin E1 that did not associate with *CCNE1* amplification (Nakayama, N. *et al.* 2010). This was later validated by Etemadmoghadam and colleagues who have described "addiction" (Weinstein, I. B. 2002, Weinstein, I. B. and Joe, A. 2008) of *CCNE1* amplified ovarian cancer cells to cyclin E1 expression (Etemadmoghadam, D. *et al.* 2010). They also reported specific sensitivity of those cell lines to *CCNE1* attenuation; in contrast, they report no evidence that *URI1* is the driver (Etemadmoghadam, D. *et al.* 2010). This is in conflict with Theurillat et al who have reported *URI1* as an "addicting" oncogene that is selectively required for *URI1* amplified ovarian cancer cells survival (Theurillat, J. P. *et al.* 2011)

A second mechanism for dysregulated synthesis and expression of cyclin E1 is the so called type 4 feedforward loop (FFL) (Cohen, E. E. *et al.* 2009). FFL, in general, comprises of boosting rapid activation via stimulator and decelerating inhibition upon

stimulator removal (Cohen, E. E. *et al.* 2009). A FFL for cyclin E1 has been identified in squamous cell carcinoma cells of the head and neck (Cohen, E. E. *et al.* 2009) and is initiated via protein kinase Cα activity (PKCα). In this example of FFL, PKCα is the stimulator that enhances cyclin E1 synthesis by activation of the mitogen-activated protein kinase (MAPK) signaling pathway and suppresses an inhibitor of cyclin E1 synthesis,miR-15a, to bolster cyclin E1 expression and DNA synthesis (Cohen, E. E. *et al.* 2009, Siu, K. T. *et al.* 2012). In addition, Ras activation through MAPK signaling further enhances cyclin E1 expression through inhibiting its FBXW7 mediated degradation (Minella, A. C. *et al.* 2005).

1.3.5 Cyclin E1 degradation

The post transcriptional component of cyclin E1 periodicity is tightly regulated by ubiquitin mediated degradation (Clurman, B. E. *et al.* 1996, Won, K. A. and Reed, S. I. 1996). Normally the half-life of cyclin E1 is just below 30 minutes, however this can be extended up to more than two hours through proteasomal inhibition (Clurman, B. E. *et al.* 1996, Won, K. A. and Reed, S. I. 1996). The fast and periodic degradation of cyclin E1 protein is responsible for maintaining equivalent level of the proteins to their corresponding mRNA (Clurman, B. E. *et al.* 1996, Won, K. A. and Reed, S. I. 1996).

At least two distinct ubiquitin-proteasome pathways are involved in cyclin E1 degradation, the Cul3 pathway that targets monomeric non-phosphorylated cyclin E1 (Singer, J. D. *et al.* 1999, McEvoy, J. D. *et al.* 2007, Siu, K. T. *et al.* 2012) and the SCF^{Fbxw7} pathway that targets CDK2 bound phosphorylated cyclin E1 (Koepp, D. M. *et al.* 2001, Strohmaier, H. *et al.* 2001).

1.3.5.1 The Cul-3 ubiquitin-proteasome pathway

The Cul3 pathway targets cyclin E1, at least in early embryogenesis (Hwang, H. C. and Clurman, B. E. 2005). The Cul3 pathway is responsible for monomeric cyclin E1 ubiquitination and leads to its degradation via the formation of a BTB-Cul3-Rbx1 (BCR) ubiquitin ligase complex (Furukawa, M. *et al.* 2003, Geyer, R. *et al.* 2003, Pintard, L. *et al.* 2004, Wimuttisuk, W. *et al.* 2014).

Notably, monomeric cyclin E1 is highly labile and tends to accumulate quickly following proteasome inhibition (Hwang, H. C. and Clurman, B. E. 2005). Loss of Cul3 has been found to result in excess cyclin E1 expression, but unexpectedly in reduced cell viability as well (Singer, J. D. *et al.* 1999, McEvoy, J. D. *et al.* 2007). The reduced cell viability associated with Cul3 loss is anticipated to be due to cells entering

an apoptotic pathway, possibly as a result of loss of the oscillating balance of cell cycle regulators (McEvoy, J. D. *et al.* 2007). In agreement with this statement is the finding that deregulated cell cycle components, or an increase in G1 cyclins, act as apoptotic signals (Slingerland, J. and Pagano, M. 1998). In addition, the Cul3 associated increase in apoptotic cells could well be attributed to the effect of the increased cyclin E1 as the latter has been proposed to promote cell apoptosis besides its well described role in cell proliferation (Mazumder, S. *et al.* 2004).

The regulation of cyclin E1 by the Cul3 pathway is highly affected by cell type and differentiation (Kossatz, U. *et al.* 2010). For instance, differentiating hepatic progenitor cells that lack Cul3 accumulate significant DNA damage together with high cyclin E1 level that result in cellular senescence (Kossatz, U. *et al.* 2010). In contrast, undifferentiated liver progenitor cells rely on SCF^{Fbxw7} for regulating cyclin E1 expression (Kossatz, U. *et al.* 2010).

1.3.5.2 The SCF^{Fbxw7} ubiquitin-proteasome pathway

The ubiquitin proteasome pathway (UPP) is responsible for substrate, including cyclin E1, ubiquitination through the formation of ubiquitin-protein conjugates (Nash, P. *et al.* 2001, Tu, Y. *et al.* 2012). UPP incorporates three constituents that are responsible for a series of ubiquitin transfer reactions: a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a specificity factor (E3) (Hershko, A. *et al.* 1983, Hershko, A. and Ciechanover, A. 1998). As their names imply, the ubiquitin ligases E3 are enzymes essential for substrate specificity and are responsible for the last step for ubiquitin transfer (Nash, P. *et al.* 2001). E3 enzyme binding sites are called degrons and are responsible for the recognition of substrates (Hershko, A. and Ciechanover, A. 1998). E3 recognition of a substrate, cyclin E1 in this case, depends on its post-translational modification in the form of phosphorylation that in turn direct substrates to Skp1-Cdc53/Cul1- F-box protein (SCF) complexes (Tyers, M. and Jorgensen, P. 2000).

SCF complexes target a wide range of substrates through adapter subunits that are specific to particular substrates called F-box proteins (Bai, C. *et al.* 1996). F-box proteins are 40-amino-acid motif which act as a Skp1 binding site (Tyers, M. and Jorgensen, P. 2000). Skp1, sequentially, links F-box proteins to a core ubiquitination complex including scaffold protein Cdc53/Cul1 (Tyers, M. and Jorgensen, P. 2000). F-box proteins capture phosphorylated substrates via carboxy-terminal protein-protein

interaction regions, including WD40 repeat domains (Skowyra, D. *et al.* 1997). An example of this group is the F-box protein 7 (FBXW7), also referred to as Cdc4 and Sel-10, that targets phosphorylated substrates including the ubiquitinated CDK2 bound cyclin E1.

1.3.6 FBXW7

FBXW7 is a member of the F-box protein family and is a substrate recognition component of the SCF E3 ubiquitin ligase (Cao, J. *et al.* 2016). FBXW7 was first described in 1973 by Hartwell as part of his research of genetic control of the cell cycle in yeast (Hartwell, L. H. *et al.* 1973, Cao, J. *et al.* 2016). *FBXW7* is located on 4q32, a chromosomal region that is frequently deleted in cancers (Welcker, M. and Clurman, B. E. 2008, Cao, J. *et al.* 2016). The *FBXW7* gene contains 4 untranslated and 13 coding exons that span approximately 210 kb of the human genome (Cao, J. *et al.* 2016). The FBXW7 protein contains WD40 domains, which form a canonical structure that can bind various substrates in a phosphorylation dependent manner (Clurman, B. E. *et al.* 1996, Won, K. A. and Reed, S. I. 1996, Koepp, D. M. *et al.* 2001, Strohmaier, H. *et al.* 2001, Welcker, M. *et al.* 2003, Ye, X. *et al.* 2004, Cao, J. *et al.* 2016). The FBXW7 protein also contains an F-box domain, a 5 residue tail, and a-helical linker domain (Cao, J. *et al.* 2016).

1.3.6.1 FBXW7 transcription and isoforms

Three transcripts are encoded by the *FBXW7* gene (α, −β and −γ) (Spruck, C. H. *et al.* 2002, Cao, J. *et al.* 2016). These were found to be the products of alternative splicing, a regulated process during which a single gene encodes for multiple proteins (Lee, Y. and Rio, D. C. 2015). The three isoforms contain a D domain, an F box domain, and a WD40-repeat domain (Cao, J. *et al.* 2016). Each mRNA consists of one exon that is specific to the particular isoform, associated with 10 C-terminal shared exons, encodes for F-box and the substrate recognition motifs, and a unique N terminal exon (Cao, J. *et al.* 2016, Kitade, S. *et al.* 2016). Collectively, this creates three protein isoforms that differ only at their N termini (Cao, J. *et al.* 2016, Kitade, S. *et al.* 2016). This genomic organization is highly conserved in mammals (Welcker, M. and Clurman, B. E. 2008, Kossatz, U. *et al.* 2010). Each isoform occupies a distinct subcellular location and is variably expressed in mammals' tissues (Strohmaier, H. *et al.* 2001, Matsumoto, A. *et al.* 2006). The α isoform is found in the nucleoplasm, and is ubiquitously expressed at a higher level than β and γ isoforms (Strohmaier, H. *et al.* 2001, Matsumoto, A. *et al.*

2006) and is responsible for most of the function of FBXW7 (Cao, J. *et al.* 2016). FBXW7 β isoform is localized to the cytoplasmic membrane with its expression detected at high levels in non-proliferating cells such as the brain and the skeletal muscles and is therefore believed to play a role in cell cycle arrest and to be important for cell senescence (Cao, J. *et al.* 2016). FBXW7 γ in turn, is found in the nucleolus and although its expression is limited to cardiac and skeletal muscles , FBXW7 γ has been found to be essential for degradation of proteins that are important for cell division, mainly cyclin E1 (Matsumoto, A. *et al.* 2006, van Drogen, F. *et al.* 2006, Welcker, M. and Clurman, B. E. 2008, Kossatz, U. *et al.* 2010). Each of the three isoforms is thought to have its own promoter and to be therefore, under isoform specific transcriptional control (Matsumoto, A. *et al.* 2006). *FBXW7* β is reported to be a transcriptional target of p53 (Matsumoto, A. *et al.* 2006, Sionov, R. V. *et al.* 2013), however, the expression of both *FBXW7* α and *FBXW7 γ* genes has been shown to be p53 independent and the mechanisms that regulate their expression are still uncharacterized (Cao, J. *et al.* 2016).

1.3.6.2 FBXW7 mediated degradation of cyclin E1

The SCF^{Fbxw7} ubiquitin-proteasome system controls the abundance of many short-lived regulatory proteins , including CDK2 bound cyclin E1(Clurman, B. E. *et al.* 1996, Won, K. A. and Reed, S. I. 1996, Koepp, D. M. *et al.* 2001, Strohmaier, H. *et al.* 2001, Welcker, M. *et al.* 2003, Ye, X. *et al.* 2004, Cao, J. *et al.* 2016), in addition to oncogenes and tumor suppressor genes involved in the cell cycle (Kitagawa K, K. M. 2016) as well as the misfolded protein response (Cao, J. *et al.* 2016). Cyclin E1 is recognized by the nuclear isoforms of the E3 ubiquitin ligase family, namely, FBXW7 α and γ (Koepp, D. M. *et al.* 2001, van Drogen, F. *et al.* 2006). FBXW7 targets cyclin E1 that is phosphorylated and CDK2-bound. Cyclin E1/CDK2 accumulates in mid - S phase and cyclin E1 is usually targeted for either degradation (Koepp, D. M. *et al.* 2001, Strohmaier, H. *et al.* 2001) or sequestration in the nucleolus (Bhaskaran, N. *et al.* 2013). The role of FBXW7 in the degradation of the oncogenic protein, cyclin E1, has raised the suspicion that *FBXW7* is a tumor suppressor gene (Akhoondi, S. *et al.* 2007, Welcker, M. and Clurman, B. E. 2008). As tumorigenesis can be initiated by loss of one FBXW7 alleles, FBXW7 is regarded a haplo-insufficient tumor suppressor (Akhoondi, S. *et al.* 2007, Welcker, M. and Clurman, B. E. 2008). Additional FBXW7 substrates include those involved in cell division, growth, survival and differentiation,

for instance c-Myc, Notch, c-Jun, PGC-1α and Mcl-1 (Gupta-Rossi, N. *et al.* 2001, Koepp, D. M. *et al.* 2001, Strohmaier, H. *et al.* 2001, Nateri, A. S., Riera-Sans, L., Da Costa, C., Behrens, A. 2004, Welcker, M. *et al.* 2004, Yada, M. *et al.* 2004, Olson , B. L. *et al.* 2008, Inuzuka, H. *et al.* 2011, Inuzuka, H. *et al.* 2011, Wertz, I. E. *et al.* 2011). Phosphorylation of SCF^{FBXW7} substrates occurs within motifs named Cdc4-phosphodegrons (CPDs) that trigger substrate's degradation process (Orlicky, S. *et al.* 2003). CPDs contain both a central phosphorylated residue and a negatively charged residue in the +4 position, and both residues contact FBXW7 WD40 repeats (Nash, P. *et al.* 2001, Welcker, M. *et al.* 2003, Ye, X. *et al.* 2004, Hao, B. *et al.* 2007).

Cyclin E1 contains two CPDs that have at least four phosphorylation sites which participate in optimizing FBXW7 mediated degradation of cyclin E1 (Welcker, M. *et al.* 2003). These are the C-terminal degron phosphorylation sites centered on threonine 380 (T380) in addition to a serine 372 (S372) and serine 384 (S384) as well as a single N-terminal phosphorylation site on threonine 62 (T62) (Clurman, B. E. *et al.* 1996, Won, K. A. and Reed, S. I. 1996, Koepp, D. M. *et al.* 2001, Nash, P. *et al.* 2001, Strohmaier, H. *et al.* 2001, Welcker, M. *et al.* 2003, Ye, X. *et al.* 2004).

The C terminal degron has been studied extensively and is usually considered the high affinity degron as well as the dominant signal for cyclin E1 degradation (Welcker, M. and Clurman, B. E. 2008). This is because it contains the T380 residue that contacts FBXW7 more extensively at +4 phosphate (Hao, B. *et al.* 2007). The kinase primarily responsible for phosphorylating T380 is GSK3 (Welcker, M. *et al.* 2003). For that reason, activation of GSK3 has been reported to increase cyclin E1 degradation while GSK3 inhibition inhibits cyclin E1 turn over (Welcker, M. *et al.* 2003, Ye, X. *et al.* 2004, Welcker, M. and Clurman, B. E. 2008). Equally, the kinase responsible for S384 phosphorylation is exclusively CDK2, therefore CDK2 is responsible for regulating cyclin E1 stability via its own activity (Welcker, M. *et al.* 2003, Ye, X. *et al.* 2004, Welcker, M. and Clurman, B. E. 2008). Notably, ablation of T380-equivalent in a mouse model resulted in genetic instability and cyclin E1 associated tumorigenesis that were exacerbated by mutations of the p53-p21 pathway (Loeb, K. R. *et al.* 2005).

Overall, the T62 degron is the least characterized of the phosphorylation sites and is frequently perceived as the low affinity degron (Loeb, K. R. *et al.* 2005, Siu, K. T. *et al.* 2012). However, the few studies that have focused on T62 were able to highlight its biological significance (Welcker, M. *et al.* 2003, Loeb, K. R. *et al.* 2005). Firstly,

Welcker and colleagues proved that mutation of the T62 residue is as effective at blocking cyclin E1 turnover as mutation of the residues in the larger C terminal phosphodegron (Welcker, M. *et al.* 2003). This is probably because T62 is the only phosphorylation site of the N terminal degron whereas the large degron has multiple major phosphorylation sites (Welcker, M. *et al.* 2003). Loeb and colleagues reported that in vivo disruption of C degron, via ablation of T380-equivalent in mice, was not sufficient to stabilize cyclin E1 (Strohmaier, H. *et al.* 2001, Loeb, K. R. *et al.* 2005, Minella, A. C. *et al.* 2008). These findings variably suggest a role of the N terminal residue, T62, in maintaining FBXW7 mediated cyclin E1 degradation that is, without doubt, more than previously anticipated (Strohmaier, H. *et al.* 2001, Loeb, K. R. *et al.* 2005, Minella, A. C. *et al.* 2008). However, the kinase responsible for phosphorylating T62 site is still unknown, as are the circumstances in which T62 is phosphorylated.

In summary, disruption of both C and N degrons have been reported to result in disturbed phosphorylation of cyclin E1 and therefore its FBXW7 mediated degradation (Minella, A. C. *et al.* 2008, Siu, K. T. *et al.* 2012). Consequently, disturbed cyclin E1 periodicity results, accompanied by continuous accumulation of cyclin E1 as well as continual entrance of cells into the cell cycle from quiescence (Minella, A. C. *et al.* 2008, Siu, K. T. *et al.* 2012). Moreover, loss of the two phosphodegrons has been reported to cause proliferative anomalies in both hematopoietic and epithelial cells in addition to fibroblast immortalisation (Minella, A. C. *et al.* 2008). However, disruption of either C or N phosphodegron alone did not seem to have the same impact as simultaneous disruption of both degrons (Loeb, K. R. *et al.* 2005, Minella, A. C. *et al.* 2008, Siu, K. T. *et al.* 2012).

1.3.7 Disrupted degradation of cyclin E1 by FBXW7 and others

Cyclin E1 degradation can be suppressed or disturbed via various mechanisms that interfere with its ubiquitination or phosphorylation (Hermida, M. A. *et al.* 2017). These mechanisms include inactivation of GSK3, the kinase responsible for phosphorylating C and N termini of cyclin E1 (Hermida, M. A. *et al.* 2017). GSK3 is inactivated by the phosphoinositide-3-kinase (PI3K)/Akt pathway through its Akt mediated phosphorylation (Hermida, M. A. *et al.* 2017). Inactivation of GSK3 activity consequently leads to suppression of FBXW7 mediated degradation of cyclin E1 and results in cyclin E1 abundance (Welcker, M. and Clurman, B. E. 2008). Additionally, Akt activation downregulates the cyclin E1 inhibitor, P27, by inducing its proteolysis

resulting in an increase in cyclin E1-CDK2 activity and cell cycle progression (Liang, J. *et al.* 2002). Conversely, Akt inactivation results in increased GSK3 activity (Liang, J. *et al.* 2002). Curiously, a significant co-occurrence of *CCNE1* and *AKT2*-amplification has been reported, which may partially be attributed to co-localisation of both genes on chromosome 19q (Au-Yeung, G. *et al.* 2018).

Another cause of disrupted cyclin E1 degradation is loss of FBXW7 function that leads to deregulation and accumulation of cyclin E1 relative to the cell cycle, i.e. persistently high cyclin E1 (Koepp, D. M. *et al.* 2001, Strohmaier, H. *et al.* 2001) . *FBXW7* mutations occur in about 6% of overall cancer types (Akhoondi, S. *et al.* 2007, Welcker, M. and Clurman, B. E. 2008) but with significant variation in tumour types (Welcker, M. and Clurman, B. E. 2008). Variation of *FBXW7* mutations involves both frequency and type of mutation, however, the exact explanation for such diversity has not been elucidated (Welcker, M. and Clurman, B. E. 2008). Nevertheless, this diversity might imply different functions and roles of FBXW7 substrates in various tissues (Welcker, M. and Clurman, B. E. 2008). *FBXW7* mutations are more common in cholangicarcinoma, T‑cell acute lymphoblastic leukaemia (T-ALL) and gastrointestinal, prostate and endometrial cancers (Akhoondi, S. *et al.* 2007). Conversely, *FBXW7* mutations are rarely found in other cancers including ovarian (Kwak, E. L. *et al.* 2005, Akhoondi, S. *et al.* 2007, Cancer Genome Atlas Research Network 2012) and breast cancer (Akhoondi, S. *et al.* 2007, Ibusuki, M. *et al.* 2011).

Suppression of FBXW7 function has frequently been reported in the absence of *FBXW7* mutation (Welcker, M. and Clurman, B. E. 2008, Popov, N. *et al.* 2014, Schulein-Volk, C. *et al.* 2014). Although, *FBXW7* mutations are infrequent in both ovarian cancer and breast cancer, loss of FBXW7 mRNA and protein expression has been reported in about 51% (Akhoondi, S. *et al.* 2010) and 21% of cases (Ibusuki, M. *et al.* 2011) respectively, and is correlated to cyclin E1 overexpression (Akhoondi, S. *et al.* 2007, Ibusuki, M. *et al.* 2011). FBWX7 can be inactivated by other mechanisms that result in oncogenic suppression of the SCF^{Fbw7} ubiquitin ligase, for instance by epigenetic inactivation via hyper-methylation (Figueroa, M. E. *et al.* 2009). Akhoondi and colleagues reported that *FBXW7* inactivation via promotor specific hypermethylation of its β isoform was associated with loss or significant reduction of FBXW7 expression *in vitro*, but also in up to 43% of breast cancer samples (Akhoondi, S. *et al.* 2010). However, it is reported

that α and γ isoforms of FBXW7, rather than β are involved in cyclin E1 degradation (Bhaskaran, N. *et al.* 2013).

Another inactivating mechanism of FBXW7 function is via microRNAs, including miR-22 (Xu, Y., Sengupta, T., Kukreja, L., Minella, A. C. 2010) and miR-27a that directly regulate *FBXW7* (Lerner, M. *et al.* 2011, Spruck, C. 2011). These microRNAs are dysregulated in T-ALL (Akhoondi, S. *et al.* 2007, Mavrakis, K. J. *et al.*).

Countering FBXW7 function are several negative regulators including ubiquitin-specific-processing protease 28 (USP28), that deubiquitinates FBXW7 substrates including and stabilization (Schulein-Volk, C. *et al.* 2014, Taranets, L. *et al.* 2015). Moreover, the dual regulation of FBXW7 function by USP28 is proven to be essential for maintaining cellular homeostasis since FBXW7 substrates are maintained at low levels via the FBXW7 – USP28 complex (Taranets, L. *et al.* 2015). In summary, inactivated USP28 triggers autocatalytic ubiquitination and degradation of FBXW7, and results in accumulation of FBXW7 substrates that can promotes oncogenic transformation (Taranets, L. *et al.* 2015). However, accumulation of FBXW7 substrates and enhancement of cell transformation have also been attributed to USP28 overexpression due to inhibition of substrate ubiquitination, though in association with FBXW7 accumulation as well due to blocks of its autocatalysis (Schulein-Volk, C. *et al.* 2014, Taranets, L. *et al.* 2015). Fittingly, abundance of USP28 has been found to correlate with overexpression of both FBXW7 as well as its substrates (Bhaskaran, N. *et al.* 2013)

FBXW7 can also be inactivated by PP2A-B55 β which dephosphorylates cyclin E1 (Tan, Y. *et al.* 2014), so that it is no longer targeted for degradation by the proteasome pathway.

Therefore, loss of FBXW7, loss or gain of USP28 and /or gain of PP2A-B55B expression following G_1/S transition are expected to result in accumulation of cyclin E1 during S phase. However, the two distinct studies that focused on disrupted FBXW7-mediated cyclin E1 degradation have shown disrupted cyclin E1 activity and cyclin E1 abundance to be more prominent in G_2/M- than in S phase (Grim, J. E., Gustafson, M.P., Hirata, R.K., Hagar, A.C., Swanger,J., Welcker, M., et al. 2008, Minella, A. C. *et al.* 2008).

Additionally, FBXW7 inactivation can be attributed to transcriptional suppression that has frequently been linked to p53 mutation (Kitade, S. *et al.* 2016). Basically, p53 loss

is associated with significantly reduced FBXW7 expression in the majority, if not all, cancers with *p53* mutations including serous ovarian cancers (Yokobori, T. *et al.* 2009, Kitade, S. *et al.* 2016). Moreover, nine, four and five recognized p53 DNA-responsive elements have been identified in promoter regions of α, β and γ isoforms of *FBXW7* respectively (Mao, J.-H. *et al.* 2004).

DNA hypermethylation of the promoter region of tumor suppressor genes, for instance *FBXW7*, is one of the epigenetic characteristics of cancer (Kitade, S. *et al.* 2016). Consistent with these observations, Kitade and colleagues have recently described an association between p53 status and hypermethylation of FBXW7 5′-upstream promoter region (Kitade, S. *et al.* 2016). In agreement with these findings are many *in vitro* and *in vivo* studies that have reported regulation of FBXW7 by p53 (Mao, J.-H. *et al.* 2004, Matsumoto, A. *et al.* 2006, Minella, A. C. *et al.* 2007, Onoyama, I. *et al.* 2007).

According to Mao et al, $p53^{+/-}$ mice harbored *FBXW7* mutation in about 10% and those $FBXW7^{+/-}$ mice exhibited increased susceptibility to radiation-associated tumorigenesis such as lymphoma (Mao, J.-H. *et al.* 2004). Yet, the majority of these tumors retained and expressed the wild-type allele of *FBXW7*, reinforcing the notion that *FBXW7* is a haplo-insufficient tumor suppressor gene (Mao, J.-H. *et al.* 2004). Moreover, in p53 deficient mice, FBXW7 loss have been reported to extend the spectrum of tumorigenesis in mice to involve those of epithelial tissues such as the lung, liver and ovary (Mao, J.-H. *et al.* 2004). However, Mao et al described such FBXW7 loss linked tumorigenesis to associate with higher expression of FBXW7 substrates such as Aurora-A , Notch and c- Jun but not cyclin E1 (Mao, J.-H. *et al.* 2004). The lack of cyclin E1 abundance following p53- mediated FBXW7 loss linked tumorigenesis is perplexing. One possible explanation is that p53- mediated regulation of FBXW7 is isoform specific involves mainly the β isoform (Matsumoto, A. *et al.* 2006) that does not control cyclin E1 degradation (Bhaskaran, N. *et al.* 2013). Nevertheless, Kitade and colleagues have also reported p53 mediated epigenetic inactivation and transcriptional suppression in α isoform of FBXW7 (Kitade, S. *et al.* 2016).

Regardless of the FBXW7 inactivation mechanism , loss of FBXW7 has frequently been associated with carcinogenesis, a characteristic that has been typically linked to deregulation of its oncogenic substrates including cyclin E1, and others (Mao, J.-H. *et al.* 2004, Akhoondi, S. *et al.* 2007, Kitade, S. *et al.* 2016).

In summary, over the last two decades, we have developed a better understanding of the complexity of the cellular processes that are responsible for regulating cyclin E1 synthesis, stability and activity (Siu, K. T. *et al.* 2012). Equally, an evolved understanding of the role of cyclin E1 in various cellular processes has emerged together with an appreciation of the span of the cellular phenotypes that stem from deregulated cyclin E1 (Siu, K. T. *et al.* 2012). Overall, these findings have formed a basis for continuing research to identify the critical role of cyclin E1 in promoting cellular transformation and cancer evolution along with potential tumor-specific mechanisms that results in cyclin E1 pathologic accumulation and activation (Siu, K. T. *et al.* 2012).

1.3.8 The oncogenic impact of deregulated cyclin E1

The oncogenic impact of deregulated cyclin E1 is hard to appreciate given the wide range of its substrates and variable cellular processes that it is involved in (Siu, K. T. *et al.* 2012). The major events and evidence that have been linked to cyclin E1 tumorigenesis include the disruption of the G1/S restriction point, the impact on cell cycle check points and DNA damage response and the subsequent genomic instability. The key event in deregulated cyclin E1 appears to be the release of its repressive activity on E2F and cell cycle progression i.e. release the G1/S transition restriction point regardless of cyclin D-CDK4/6 activity (Keenan, S. M. *et al.* 2004, Rundle, S. *et al.* 2017). Consequently, forced uncontrolled S phase entry leads to replication stress and eventually genomic instability.

1.3.8.1 Deregulated cyclin E1 effect on cell cycle checkpoints, DNA Damage Response (DDR) and replication stress.

DDR is the term applied to adaptive mechanisms adopted by cells in order to combat the threats of DNA damage (Jackson, S. P. and Bartek, J. 2009). DDR is achieved via first detecting the DNA damage followed by signaling their presence and promoting the affected cells to undergo growth arrest at cell cycle checkpoints to allow for DNA damage to be repaired (Blagosklonny, M. V. and Pardee, A. B. 2013). Since, damaged DNA is able to progress through both S and M phases (Blagosklonny, M. V. and Pardee, A. B. 2013), growth arrest normally occurs prior to S phase, i.e. at G1 phase to prevent damaged DNA from abnormal replication and also before M phase, i.e. G2 phase to prevent cells with DNA damage from entering mitosis (Chene, G. *et al.* 2014). Halting at either checkpoint provides opportunities for DNA repair and the prevention of

replicating damaged cells (Chene, G. *et al.* 2014)(Figure1-3). Restriction points that normally present in normal cells are commonly lost in cancer cells (Pardee, A. B. 1974). In normal cells, the G1/S phase check point is mainly controlled by p53. P53 regulates cyclin dependent kinase inhibitor $p21^{cip1}$, the main mediator of cell cycle arrest (Rundle, S. *et al.* 2017). $p21^{cip1}$ inhibits the kinase activity of CDK2 and down regulates cyclin E1-CDK2 substrates including RB (Rundle, S. *et al.* 2017). Cyclin E1 –CDK2 normally facilitates entry into S and hence controls the G1/S phase transition. Following DNA damage, Cyclin E - CDK2 normally plays a key role in inhibiting cell cycle progression (Lin, A. B. *et al.* 2017). Notably, p53 normally protect cells at the G1/S phase transition from the deleterious consequences of deregulated cyclin E1 activity

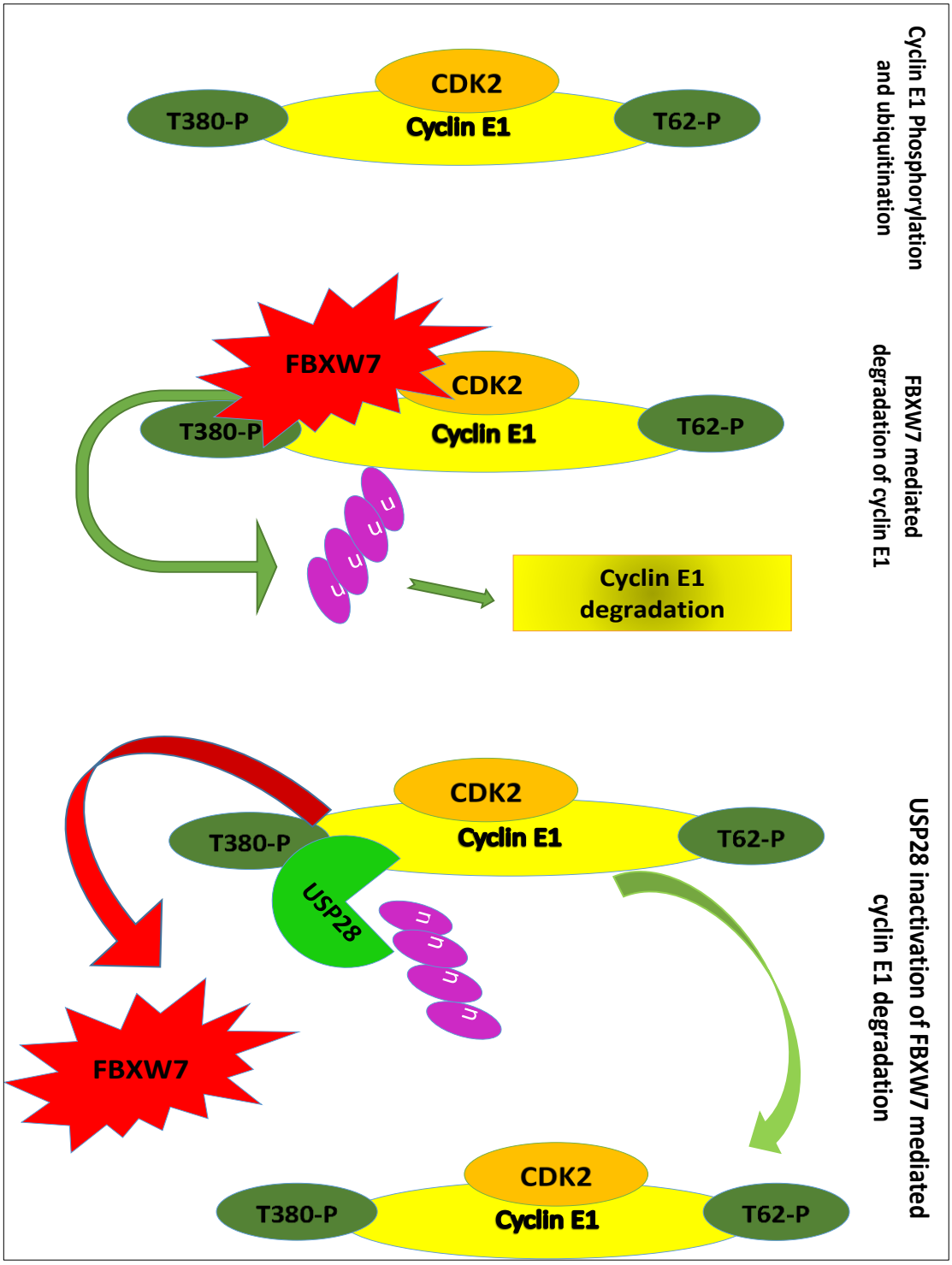

Figure 1-2: a schematic representation of FBXW7 mediated degradation of cyclin E1: cyclin E1 degradation is regulated by its phosphorylation and subsequent ubiquitination via FBXW7. This is usually balanced by USP27 that deubiquitinase cyclin E1 and help fine tune cyclin E1 expression during the cell cycle.

(Minella, A. C. *et al.*). Consequently, inactivation of p53 is a crucial enabler for cancer cells to tolerate excess cyclin E1 (Minella, A. C. *et al.*). Excess cyclin E1 leads to premature DNA replication origin firing, in addition to release of RB mediated restriction on cell cycle, and eventually replication stress (Zeman, M., K. and Cimprich, K., A. 2014, Rundle, S. *et al.* 2017). Replication stress is a terms applied to the slowing or stalling of replication fork progression (Zeman & Cimprich, 2014) that can be caused by various factors, many of which are attributed to oncogene activation (Schoonen, P. M. *et al.* 2019). Cyclin E1 - related replication stress is reported to result in cells entering mitosis, prior to the completion of S phase, with short un-replicated genomic segments (Bester, A. C. *et al.* 2011, Teixeira, Leonardo K. *et al.* 2015). These incompletely replicated regions are typically in regions of the genome that are replicated late resulting in a paucity of replication origins. (Teixeira, Leonardo K. *et al.* 2015). This leads to anaphase anomalies and eventually deletions of specific genomic regions (Teixeira, Leonardo K. *et al.* 2015).

As cyclin E1 related replication stress typically evades G1/S checkpoint cells are reliant on an intact G2/M checkpoint for their survival (Figure 1-3). G2/M transition is mediated by Cyclin B - CDK1 (Lin, A. B. *et al.* 2017). Repair of DNA damage detected at the G2 checkpoint is governed by two well-described and connected pathways. The ultimate outcome of both pathways is the inhibitory phosphorylation of CDK1 and delayed M entrance (Bartek, J. and Lukas, J.).

The first G2/M checkpoint DNA repair pathway is mediated by ataxia telangectasia mutated (ATM) that becomes activated in response to double-strand DNA damage, particularly those induced by ionizing radiation (Matsuoka, S. *et al.* 2000). Activation of ATM leads to activation of checkpoint kinase 2 (Chk2) which by itself inactivates cell division cycle 25C phosphatase A (Cdc25A) by proteolysis (Lin, A. B. *et al.* 2017). Chk2 also inactivates Cdc25C by phosphorylating it at Ser216 (Matsuoka, S. *et al.* 2000). This results in re-localization of Cdc25c from the nucleus to the cytoplasm and suppression of its phosphatase activity. Inactivated Cdc25A and Cdc25C release the repression on the inhibitory phosphorylation CDK2 and CDK1 and prevent cells from entering S and M phases respectively (Do, K. *et al.* 2013).

The second G2/M checkpoint DNA repair pathway is mediated by the ataxia telangiectasia mutated and Rad3 related (ATR) protein kinase that becomes activated following single strand DNA breaks (Johnson, N. *et al.* , Jazayeri, A. *et al.* 2005) as

well as by ATM when single strand DNA is generated by 5′ to 3′ resection of double strand DNA break (Jazayeri, A. *et al.* 2005). The main target for ATR mediated phosphorylation is checkpoint kinase 1 (Chk1), both ATR and Chk1 kinases are usually referred to as ATR-Chk1 pathway (Domínguez-Kelly, R. *et al.* 2011). Notably, Chk1 can be activated by ATM as well (Domínguez-Kelly, R. *et al.* 2011). Furthermore, Chk1 activation can be part of normal cell cycle progression but also as a response to DNA damage and stalled replication forks during replication stress (Domínguez-Kelly, R. *et al.* 2011). Active Chk1 enhances Wee1 kinase activity and the net outcome of these events is cell cycle arrest due to Wee1 mediated phosphorylation and inactivation of CDK1 permitting time for DNA repair prior to mitosis (Domínguez-Kelly, R. *et al.* 2011, Geenen, J. J. J. and Schellens, J. H. M. 2017, Lin, A. B. *et al.* 2017). Moreover, Wee1 also phosphorylates and inactivates cyclin E1 binding CDK2 (Domínguez-Kelly, R. *et al.* 2011). Therefore Wee1 plays a role in regulating DNA replication during S phase as well and maintain stalled replication forks (Domínguez-Kelly, R. *et al.* 2011) (Figure1-3).

Another critical component of the ATM - Chk2 and ATR - Chk1 -Wee1 DNA repair pathways is BRCA1 (Foray, N. *et al.* 2003). Following DNA damage, BRCA1 becomes hyperphosphorylated by ATM and ATR and facilitates their kinase ability to phosphorylate their downstream substrates including Chk2 and Chk1, respectively (Foray, N. *et al.* 2003). The presence of intact BRCA1 significantly increases resistance to DNA damaging agents, especially those causing double strand DNA break, for instance etoposide or bleomycin (Quinn, J. E. *et al.* 2003). On the other hand, intact BRCA1 increases sensitivity to antimicrotubule agents, for example, paclitaxel (Quinn, J. E. *et al.* 2003). However, BRCA1 mediates cell cycle arrest at G2/M in response to both groups of agents through either the induction or the inhibition of apoptosis respectively (Quinn, J. E. *et al.* 2003). Therefore, intact BRCA1 is essential for cell cycle arrest at G2, especially in cells with deregulated G1/S phase downstream of cyclin E1. This provides a rationale for why intact BRCA1 is crucial in the survival of *CCNE1* amplified cells (Etemadmoghadam, D. *et al.* 2013).

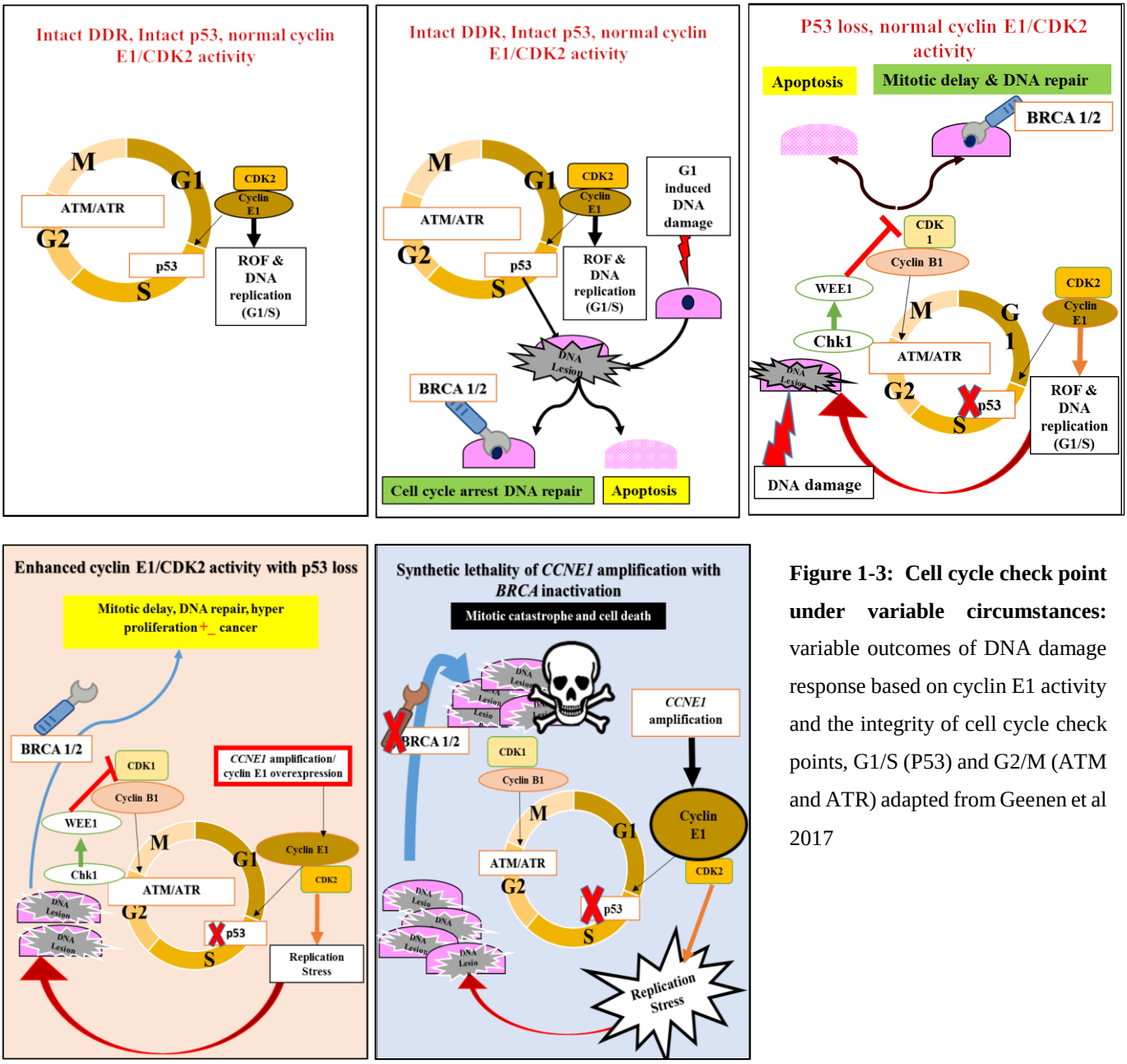

Figure 1-3: Cell cycle check point under variable circumstances: variable outcomes of DNA damage response based on cyclin E1 activity and the integrity of cell cycle check points, G1/S (P53) and G2/M (ATM and ATR) adapted from Geenen et al 2017

1.3.8.2 Deregulated cyclin E1 and genomic instability

Based on the initial studies on the function of cyclin E1, the carcinogenic impact of cyclin E1 was generally attributed to unrestricted cell proliferation or the reduced threshold for cells to respond to mitogen stimulation (Hwang, H. C. and Clurman, B. E. 2005). However, there is increasing evidence that this hypothesis in isolation is not accurate and the role of cyclin E1 in tumorigenesis has been strongly questioned following the emergence of reports confirming that CDK2 null and cyclin E1 and E2 knocked out mice (with rescued placenta) are born viable and healthy (Berthet, C. *et al.* 2003, Geng, Y. *et al.* 2003, Lents, N. H. and Baldassare, J. J. 2004).

More recently, the tumorigenic effect of cyclin E1 has been linked to genomic instability due to deregulated expression of cyclin E1, whether by disrupted degradation (Rajagopalan, H. *et al.* 2004) or *CCNE1* amplification (Hwang, H. C. and Clurman, B. E. 2005). Cyclin E1 associated genomic instability was first described in rodent and human cancer cell lines that showed aneuploidy due to ectopic cyclin E1 expression (Spruck, C. H. *et al.* 1999).

Generally, there is strong evidence that aberrant cyclin E1 leads to genomic instability, a hallmark of cancer (Hanahan, D. and Weinberg, R. A. 2011). However, the mechanisms by which cyclin E1 deregulation results in genomic instability is not fully elucidated. One possible explanation is cyclin E1 induced centrosome amplification causes genomic instability in cancer through aneuploidy (Kawamura, K. *et al.* 2004). Curiously, loss of p53 has also been shown to correlate with centrosome amplification and aneuploidy in mouse cells (Kawamura, K. *et al.* 2004). However, for human cancer cells, p53 inactivation per se does not impact the numeral integrity of centrosome or result in aneuploidy unless associated with disruption of cyclin E1 (Kawamura, K. *et al.* 2004). This is because cyclin E1 is an important additional regulatory mechanism that human cancer cells seem to rely on in order to maintain centrosome and karyotype numeral integrity (Kawamura, K. *et al.* 2004). Notably, the role of cyclin E1-CDK2 in centrosome replication is well established in Xenopus extracts and, according to many studies, in the mammalian centrosome cycle as well (Okuda, M. *et al.* 2000, Matsumoto, Y. and Maller, J. L. 2004, Hwang, H. C. and Clurman, B. E. 2005). However, the finding that cyclin E1-CDK2 null animals did not suffer centrosome and/or spindle anomalies has questioned the significance of cyclin E1 in normal

centrosome function (Okuda, M. *et al.* 2000). *In vitro* studies have shown that overexpression of cyclin E1, especially when coupled with p53 loss, results in centrosome amplification and aneuploidy linked to spindle defects (Kawamura, K. *et al.* 2004). However, cyclin E1-linked genetic instability have also been reported in the absence of spindle defects (Spruck, C. H. *et al.* 1999). Accordingly, the role of centrosomal anomalies in cyclin E1 associated cancer remains debatable (Okuda, M. *et al.* 2000).

Other suggested explanations of cyclin E1 induced genomic instability include replication stress associated with activated cyclin E1 (detailed above) (Bartkova, J. *et al.* 2006, Neelsen, K. J. *et al.* 2013). Alternatively, cyclin E1 linked genomic instability has been attributed to the mitotic aberrations associated with the ability of excess cyclin E1 to inhibit the activity of the ubiquitin ligase APC^{Cdh1} activity that persists as cells progress up to and through the early stages of mitosis, resulting in the abnormal accumulation of APC^{Cdh1}substrates including cyclin B, cdc20 and Seculin as cells enter mitosis (Keck, J. M. *et al.* 2007).

1.3.8.3 Deregulated cyclin E1 triggers malignant changes in precursor and putative precursor cells in *vitro* and in *vivo*

Many reports have confirmed the ability of aberrant cyclin E1 to malignantly transform cells in *vitro* and in *vivo* in different types of tissues. For instance, co-transfection of cyclin E1 with activated H-ras resulted in transformation of primary rat embryo fibroblasts (Haas, K. *et al.* 1997). The transformed cells exhibited the classical criteria for neoplastic cells: were highly tumorigenic in syngeneic rats, formed colonies in soft agar and were able to evade apoptosis in the setting of serum starvation and DNA damage (Haas, K. *et al.* 1997).

In breast cancer, an important role for cyclin E1 in mammary tumorigenesis was revealed in transgenic mice (Bortner, D. M. and Rosenberg, M. P. 1997). Human cyclin E1 transfection that was specifically directed to the mammary glands of pregnant and lactating mice induced tumorigenesis (Bortner, D. M. and Rosenberg, M. P. 1997). The lactating mammary glands of transgenic mice expressing cyclin E1 exhibited areas of hyperplasia that is usually not present in lactating glands of control mice (Bortner, D. M. and Rosenberg, M. P. 1997). Additionally, greater than 10% of female cyclin E1 transgenic mice developed mammary carcinomas (Bortner, D. M. and Rosenberg, M. P. 1997).

Importantly, cyclin E1 is reported to transform the putative cell of origin of serous ovarian cancer, the fallopian tube and secretory epithelial cell, in cooperation with other genetic aberrations (Karst, A. M. *et al.* 2014).

1.3.8.4 Deregulated cyclin E1 in precancerous lesions

There is growing evidence that cyclin E1 is a driver gene, at least in certain types of cancers. The concept has been enforced by the frequent observation of cyclin E1 expression in precancerous lesions and early stages of carcinogenesis in various human cancers. However, the mere expression of cyclin E1 does not necessarily reflect cyclin E1 activity, and therefore caution needs to be taken in interpreting cyclin E1 expression in such lesions.

In many occasions, the expression of cyclin E1 tends to build up with tumor evolution, or at least exist in premalignant lesion but not in adjacent normal tissue (Donnelian, R. and Chetty, R. 1999). Examples include, dysplastic lesions in the esophagus (Zhou, Z. *et al.* 2014), gastric cancer (Wang, M. *et al.* 2010), colorectal adenomas (Yasui, W. *et al.* 1996), neoplastic cells of the bronchial epithelium (Langenfeld, J. *et al.* 1996), premalignant conditions of the skin, though the expression is lower in squamous cell carcinomas and basal cell carcinomas (Toshinori, B. *et al.* 1997), cervical cancer carcinogenesis (Chen, I. T. *et al.* 1996, Cho, N. H. *et al.* 1997, Jones, D. L. *et al.* 1997, Ruesch, M. N. and Laimins, L. A. 1997, Quade, B. *et al.* 1998), endometrial cancer, though expressed in normal proliferative cells as well (Li, S. F. *et al.* 1996, Shiozawa, T. *et al.* 1996, Ahn, H. *et al.* 1998). In addition, cyclin E1 is reported in early lesions of both ovarian and breast tissue (Shaye, A. *et al.* 2009, Karst, A. M. *et al.* 2014). In breast cancer, cyclin E1 expression was reported in both ductal carcinoma in situ and invasive carcinoma and the expression of cyclin E1 were found to be concordant in the in situ and invasive components within the same tumor (Shaye, A. *et al.* 2009). The role of deregulated cyclin E1 in ovarian carcinogenesis is detailed below.

1.4 Cyclin E1 deregulation in ovarian cancer

1.4.1 Epidemiology of ovarian cancer

Ovarian cancer is the leading cause of cancer-associated death among neoplasms of gynecological origin (Delk, N. A. *et al.* 2009, Kurman, R. J. 2010, Rosai, J. 2011, McAlpine, J. N. *et al.* 2012, Maniar, P. k. *et al.* 2013, Nik, N. N. *et al.* 2014, Wiedemeyer, W. R. *et al.* 2014) with approximately 152,000 deaths are reported each year worldwide (Ferlay, J. *et al.* 2015). In Australia, ovarian cancer is estimated to be

the eighth most commonly diagnosed cancer in females in 2017 (Australian Institute of Health and Welfare 2017).

Overall, it is estimated that about 239,000 new cases of ovarian cancer are diagnosed annually worldwide (Australian Institute of Health and Welfare 2017). The disease usually affects post-menopausal women and the mean age at the time of diagnosis is 64 years while rarely presenting in women younger than 40 (Australian Institute of Health and Welfare 2017). The risk of developing ovarian cancer increases with increasing age and in Australian women the risk is estimated to be 1 in 82.2 by the age of 85 (Australian Institute of Health and Welfare 2017). Other risk factors include family history of breast or ovarian cancer, with 5-10% of ovarian cancers is attributed to heritable risk (Salehi, F. *et al.* 2008). Reproductive history is another factor that might influence women's risk of having ovarian cancer where infertility contributes to higher risk of ovarian cancer, while pregnancy, tubal ligation, and hysterectomy decrease risk (Salehi, F. *et al.* 2008). In addition, hormone replacement therapy increases the risk for ovarian cancer whereas oral contraceptive use has been proven to be protective against ovarian cancer (Salehi, F. *et al.* 2008). The huge impact of both reproductive factors and hormones on ovarian cancer risk strongly suggests a role of endocrine disrupters in ovarian cancer development (Salehi, F. *et al.* 2008). Other factors that could influence ovarian cancer development risk are smoking, obesity and diet (Salehi, F. *et al.* 2008).

The poor understanding of ovarian cancer tumorigenesis has significantly limited the ability to detect and treat ovarian cancer at earlier stages (Lengyel, E. 2010). Furthermore, ovarian cancer is usually asymptomatic in early stages, and hence the majority of ovarian cancer cases are diagnosed at late stage when the cancer has already spread to peritoneum or omentum (Lengyel, E. 2010). The relative 5-year survival rates for stage I ovarian cancer is up to 93%, while those of stage IV is about 19% (The American Cancer Society medical and editorial content team 2018).

1.4.2 The classification of ovarian cancer

Until the last few years, ovarian cancer, despite the known variation in its different subtypes, has been treated as single disease (Kurman, R. J. , Kurman, R. J. 2013, Nik, N. N. *et al.* 2014). Unsurprisingly, this has resulted in higher rate of treatment failure in ovarian cancer (Kurman, R. J. , Kurman, R. J. 2013, Nik, N. N. *et al.* 2014). However, the last few decades has witnessed significant advances in our understanding of ovarian cancer tumorigenesis and classification (Kurman, R. J. and Shih, I.-M. 2016). Ovarian

cancer is a complex group of diseases originating from various backgrounds by various mechanisms (Bowtell, D. D. 2010, Rosai, J. 2011, McAlpine, J. N. *et al.* 2012, Kurman, R. J. 2013). Ovarian tumors are primarily classified morphologically according to the cytological features of their cells (Goldblum, J. R. *et al.* 2018). The morphologic classification has been further supported by the latest molecular studies that have demonstrated, in most instances, the accuracy of such classification in reflecting both the cellular origin of these tumors as well as their underlying molecular aberrations (Herrington, C. S. 1999, Goldblum, J. R. *et al.* 2018). The 2014 WHO classification has incorporated a few minor changes to this classification, however the basic principle is that the four main types of tissue in the ovary, epithelium, germ cells, sex cords and stroma can cause a variety of neoplasms consistently, has remained the same (Kurman, R. J. *et al.* 2014). Accordingly the main ovarian tumor type is the epithelial tumors which are about 75% of all ovarian cancer cases (Kurman, R. J. *et al.* 2014). Second, the germ cell tumors account for 20% of all ovarian tumors (Kurman, R. J. *et al.* 2014).Third is the sex cord stromal tumors which form 5% of ovarian tumors where cells tend to differentiate either towards sex cord or specialized ovarian stroma (Young, R. H. and Scully, R. E. 1988, Goldblum, J. R. *et al.* 2018). The last group is the germ cell - sex cord- stromal tumors that constitutes, as the name implies, the three elements and mainly affects individuals with sexual abnormalities such as gonadal dysgenesis and constitute 1-2% of cases (Eifel, P. *et al.* 1982, Young, R. H. and Scully, R. E. 1988, Pfleiderer, A. 1993, Sasano, H. 1994, Tavassoli, F. A. 1994, Zheng, W. *et al.* 1997, Herrington, C. S. 1999, Roth, L. M. *et al.* 2000, Jondle, D. M. *et al.* 2002, Ulbright, T. M. 2005, Roth, L. M. and Talerman, A. I. 2006, Li, Y. *et al.* 2007, Kurman, R. J. *et al.* 2014, Goldblum, J. R. *et al.* 2018). Regardless of the different classification systems that are used to categorise ovarian cancer, they all reflect that ovarian cancer is a complex group of diseases originating from various backgrounds by various mechanisms (Bowtell, D. D. 2010, Rosai, J. 2011, McAlpine, J. N. *et al.* 2012, Kurman, R. J. 2013, Kurman, R. J. *et al.* 2014).

A recently proposed classification of epithelial ovarian cancer incorporates both the molecular and histopathological features for each type of ovarian cancer (Shih, I. M. and Kurman, R. J. 2004, Kurman, R. J. and Shih, I.-M. 2016). This is called the dualistic model of classification that, in summary, divides the group into those of type I of low malignant potential for which the prototype is low grade serous ovarian cancer. The

second group is type II of high malignant potential where HGSOC, the focus of this thesis, is prototypical.

HGSOC constitutes three quarters of all epithelial ovarian cancers and accounts for 90% of deaths due to ovarian carcinoma (McAlpine, J. N. *et al.* 2012, Maniar, P. k. *et al.* 2013). It is the leading cause of cancer-associated death among neoplasm of gynaecological origin (Delk, N. A. *et al.* 2009, Kurman, R. J. 2010, Rosai, J. 2011, McAlpine, J. N. *et al.* 2012, Maniar, P. k. *et al.* 2013, Nik, N. N. *et al.* 2014, Wiedemeyer, W. R. *et al.* 2014). In most of the cases, the prognosis is poor due to late diagnosis and rapid progression of the disease (McAlpine, J. N. *et al.* 2012, Maniar, P. k. *et al.* 2013). Despite the high response rate to first line treatment and improved treatment techniques in terms of surgical cyto-reduction and/or platinum based chemotherapy, 20%-25% of cases relapse while on, or shortly after, the cessation of the initial therapy (Cancer Genome Atlas Research Network 2011) with minimal impact of advanced treatment options on overall survival (McAlpine, J. N. *et al.* 2012, Maniar, P. k. *et al.* 2013). Therefore, better understanding of the molecular diversity of tumors that fall in the HGSOC class is essential for refining their management.

1. 4. 3 The dualistic model of epithelial ovarian cancer (Molecular and histopathological)

The complex molecular pathways and the widely variable morphological types of primary ovarian cancers have prompted Shih and colleagues to propose a dualistic model for ovarian carcinogenesis that, in general, includes type I and type II ovarian cancers (Shih, I. M. and Kurman, R. J. 2004, Kurman, R. J. and Shih, I.-M. 2016). Currently, the proposed model of classification incorporates the evolving molecular genetic background to the already existing histopathologic classification (Kurman, R. J. and Shih, I.-M. 2016). In general, the aim of the dualistic model of classification of ovarian cancer is to better understand carcinogenesis in ovarian cancer, detect drivers and identify potential targets for targeted therapy in each subtype. However, due to the existence of variants within each group, targeted therapy has been only partially applicable in clinical practice (Palmirotta, R. *et al.* 2017).

Type I epithelial ovarian cancers generally incorporates the less aggressive forms of ovarian cancers and these include endometrioid, clear cell, low grade serous, and mucinous carcinomas and less commonly, seromucinous carcinomas (designated mixed Müllerian tumors) and malignant Brenner tumors (Kurman, R. J. and Shih, I.-

M. 2016). Type II carcinomas are mostly composed of HGSOC and less commonly carcinosarcoma and undifferentiated carcinoma (Kurman, R. J. and Shih, I.-M. 2016).

Both type I and type II epithelial ovarian cancers are attracting huge interest in their investigation with the aim to assign molecular profiles to each phenotypic subtype (Palmirotta, R. *et al.* 2017). Recently, a large number of molecular and histopathologic studies were published that have significantly advanced our understanding of the origin and development of these tumors (Kurman, R. J. and Shih, I.-M. 2016). Accordingly, Kurman and Shih have recently published a revised new model of ovarian carcinogenesis that, in short, proposes four major tissue types as the origin for type I ovarian cancer; endometriosis, fallopian tube, germ cells and transitional cells. Type II, in comparison, is anticipated to originate from fallopian tubes and their fimbria only (Kurman, R. J. and Shih, I.-M. 2016).

The predominant histopathological features and the molecular characteristics in relation to histology and gene expression of type II (HGSOC) are discussed below.

1.4.4 Histopathological features of type II epithelial ovarian cancer (HGSOC)

The prototype for this group is HGSOC, which constitute more than 70% of all ovarian cancers (Crum, C. P. *et al.* 2007). Tumors belong to type II group are high grade by definition, aggressive, rapidly progressive and tend to metastasize early (Maniar, P. k. *et al.* 2013). Complex papillary architecture, high degree of nuclear atypia and necrosis are characteristic findings in this group (Rosai, J. 2011, Maniar, P. k. *et al.* 2013).

These tumors appear to originate mainly from tubal epithelium either by intraepithelial carcinoma accompanied by shedding of cells into the ovary (Crum, C. P. *et al.* 2007, Nik, N. N. *et al.* 2014) or by implantation of normal epithelium from the fallopian tubes to form inclusion cysts which subsequently give rise to serous carcinoma (Kurman, R. J. 2010).

Type II group also encompasses carcinosarcoma (malignant mixed müllerian tumor) that composes, as the name implies, of two distinct components; carcinoma and sarcoma intermixed together (Jin, Z. *et al.* 2003). Carcinosarcoma is largely agreed to be HGSOC that has undergone sarcomatoid differentiation (Jin, Z. *et al.* 2003). This is suggested by the shared molecular genetic aberrations with HGSOC in terms of *TP53* mutation (Abeln, E. C. *et al.* 1997) and by the frequent expression of epithelial markers by the seemingly sarcomatous cells (Maniar, P. k. *et al.* 2013). Furthermore, the few studies that has focused on this group carcinogenesis revealed, as in the case of

HGSOC, serous tubal intraepithelial carcinoma (STIC) as their precursor (Gagner, J.-P. and Mittal, K. 2005, Seidman, J. D. *et al.* 2011).

Type II ovarian tumors also incorporates undifferentiated carcinoma that are generally uncommon and is sometimes considered an undifferentiated variant of HGSOC, though, they lack the characteristic features of HGSOC and their gene expression profile is assumed to be closer to mesenchymal than to epithelial cells (Kurman, R. J. *et al.* 2014) .

1.4.5 The molecular features of type II ovarian cancer (HGSOC)

The genomic analysis of HGSOC performed by TCGA study revealed that the spectrum of mutations in this cancer is unexpectedly simple and is uniquely dissimilar to those of other solid tumors, as well as to those of other histological types of ovarian cancer (Cancer Genome Atlas Research Network 2011). The results of TCGA study were later verified in another genome-wide study (Patch, A. M. *et al.* 2015). The distinctive molecular characteristics of each subtype of ovarian cancer is commonly attributed to the different etiology and lineage in each group and can, therefore, provide opportunities for improving patients' outcomes through subtype centered therapeutic approaches (Cancer Genome Atlas Research Network 2011). Overall, four molecular subtypes of HGSOC were previously described by Tothill *et al* and subsequently verified by TCGA study, based on the gene expression in the clusters, namely: mesenchymal (C1), immunoreactive (C2), differentiated (C4) and proliferative (C5) (Tothill, R. W. *et al.* 2008, Cancer Genome Atlas Research Network 2011). The four molecular types exhibit different micro-environmental characteristics including immune cells and stromal cells (Tothill, R. W. *et al.* 2008, Bowtell, D. D. *et al.* 2015). Although, theses subtypes are not currently integrated in clinical practice, each subtype is known to associate with different clinical outcomes (Bowtell, D. D. *et al.* 2015). Expectedly, the most favorable outcomes reported to associate with immunoreactive subtype (Helland, Å. *et al.* 2011, Konecny, G. E. *et al.* 2014) and relevantly, immunoreactive subtype were found to associate with *BRCA* disruption (George, J. *et al.* 2013).

According to TCGA, the molecular profile of HGSOC has a low frequency of mutations involving oncogenes and tumor suppressor genes apart, predominantly *TP53*, *BRCA1* and *BRCA2* mutations (Cancer Genome Atlas Research Network 2011, Bowtell, D. D. *et al.* 2015) with another six genes that are mutated at very low frequency,

RB1, *NF1*, *FAT3*, *CSMD3*, *GABRA6* and *CDK12* (Cancer Genome Atlas Research Network 2011). Conversely, HGSOC exhibited a high frequency of genomic structural variation with focal DNA copy number alterations in the forms of gains and losses (The Cancer Genome Atlas Research, N. 2008, Cancer Genome Atlas Research Network 2011, Bowtell, D. D. *et al.* 2015). Therefore, chromosomal instability is a general characteristic of HGSOC and the cancer is a (C-class) malignancy (Ciriello, G. *et al.* 2013, Bowtell, D. D. *et al.* 2015). Of the frequent gene copy numbers alterations in HGSOC, *CCNE1* amplification was particularly common and is attracting an increasing interest (Cancer Genome Atlas Research Network 2011). *CCNE1* is commonly perceived as a driver oncogene of a distinct subset of HGSOC that is Platinum resistant and has poor outcome (Etemadmoghadam, D. *et al.* 2010, Nakayama, N. *et al.* 2010, Cancer Genome Atlas Research Network 2011, Karst, A. M. *et al.* 2014). Overall, the frequent copy number alterations in ovarian cancer have been ascribed to genomic instability that associates with frequent mutations and promotor methylation of DNA repair genes, including *BRCA1* and *BRCA2* members of the homologous recombination (HR) pathway (Cancer Genome Atlas Research Network 2011). Curiously, *BRCA1* and *BRCA2* mutations are strongly perceived to be exclusive of *CCNE1* amplification due to the proposed synthetic lethality between both aberrations (Cancer Genome Atlas Research Network 2011, Etemadmoghadam, D. *et al.* 2013) (Figure 1.3).

The earliest described molecular event in HGSOC is *TP53* mutations, which have been shown to be the initiating event in early carcinogenesis (Kindelberger, D. W. *et al.* 2007, Bowtell, D. D. 2010). Fittingly, *TP53* is the most commonly and significantly mutated gene in HGSOC and is therefore almost always a feature of this cancer subtype (Ahmed, A. A. *et al.* 2010, Cancer Genome Atlas Research Network 2011, Bowtell, D. D. *et al.* 2015) and of its proposed precursor, STIC (Yemelyanova, A. *et al.* 2011, Bowtell, D. D. *et al.* 2015).

The frequency of *TP53* mutation in HGSOC in TCGA study was initially reported to be 96% (Cancer Genome Atlas Research Network 2011). Later, it has been found that, except for one case, all the cases that were labelled as *TP53* non-mutated HGSOC were misclassified as high grade (Kurman, R. J. and Shih, I.-M. 2016). Therefore, *TP53* mutation is characteristic of HGSOC and is considered a significant tool in

differentiating HGSOC from low grade and other ovarian cancer subtypes (Kurman, R. J. and Shih, I.-M. 2016).

The second most commonly mutated genes in HGSOC are *BRCA1* and *BRCA2* that are known to be risk factors of HGSOC (Cancer Genome Atlas Research Network 2011). The overall absolute ovarian cancer risk is estimated to be 34% with *BRCA1* and 11% with *BRCA2* mutation compared to less than 1% in wild - type population (Risch, H. A. *et al.* 2006, Rebbeck, T. R. *et al.* 2015). Both *BRCA1* and *BRCA2* are members of the HR pathway, the pathway responsible for the repair of double-stranded DNA breaks that might accumulate during cell replication through the cell cycle (Takata, M. *et al.* 1998). Defective HR pathway was understood to be mainly the result of *BRCA1* and *BRCA2* germline and somatic mutations in addition to epigenetic silencing of *BRCA1*, all were found to be highly sensitive to PARP inhibitors (Turner, N. *et al.* 2004, Farmer, H. *et al.* 2005, Fong, P. C. *et al.* 2009, Veeck, J. *et al.* 2010, Yap, T. A. *et al.* 2010). However, a study conducted by TCGA (Cancer Genome Atlas Research Network 2011) that was later reinforced by others, (Rafnar, T. *et al.* 2011, Walsh, T. *et al.* 2011, Patch, A. M. *et al.* 2015) revealed that HR defects extended up to around 50% of cases of HGSOC (Mukhopadhyay, A. *et al.* 2010, Cancer Genome Atlas Research Network 2011, Bowtell, D. D. *et al.* 2015), of which only 20-25% was attributed to germline and somatic mutations of *BRCA1* and *BRCA2.* A further 11-15% was due to epigenetic silencing of *BRCA1* gene (Cancer Genome Atlas Research Network 2011, Patch, A. M. *et al.* 2015) . The rest were due to genomic alterations in other HR pathway genes, including *EMSY* amplification or mutation, *PTEN* mutation or focal deletion, *ATM* or *ATR*, *CHEK2* germline mutations, *PRIP1* germline mutation and *RAD51C* germline and somatic mutations. As intact HR is crucial for maintaining genomic stability, defective HR is commonly associated with genomic instability (Youds, J. L. *et al.* 2006, Moynahan, M. E. and Jasin, M. 2010). Genomic instability is a term applied to the ability of a cancer cell's to undergo chromosomal rearrangements that eventually result in either activating oncogene amplification or fusion or tumour suppressor genes inactivation involving oncogenic anti-apoptotic, pro- proliferative and drug-resistance genes (Cahill, D. P. *et al.* 1999, Tan, D. S. P. *et al.* 2008). Cases harbouring such mutations are collectively referred to as the HR deficient group of HGSOC and is typically platinum sensitive and a promising target for PARP inhibitors (Bowtell, D. D. *et al.* 2015, O'Cearbhaill, R. E. 2018, Jiang, X. *et al.* 2019).

The remaining half of HGSOC is designated HR proficient and is the sum of up to 30% *CCNE1* amplified cases and 20% of other molecular aberrations (Cancer Genome Atlas Research Network 2011, Patch, A. M. *et al.* 2015). As indicated earlier, *CCNE1* amplification is reported to be mutually exclusive of *BRCA* mutation due to the proposed synthetic lethality between both aberrations (Cancer Genome Atlas Research Network 2011, Etemadmoghadam, D. *et al.* 2013).

1.4.6 The origin of HGSOC and the role of driver genes

The origin of HGSOC is still in debate despite the clinical burden of HGSOC and the extensive research. The lack of full understanding of HGSOC oncogenesis have halted developing strategies for reducing their risk (Collins et al., 2011). Equally, failure to determine the precise site of origin of ovarian cancer has limited the development of screening methods and early detection (Crum, C. P. *et al.* 2007). Therefore, research has focused on identifying a reliable model that explains ovarian cancer origin.

A significant progress in our understanding of HGSOC carcinogenesis is the accumulating evidence which suggest that HGSOC starts in the fallopian tube secretory epithelial cells as precancerous lesions designated "P53 signatures" (Lee, Y. *et al.* 2007). P53 signature represent twelve or more sequential fallopian tube secretory epithelial cells that harbour benign morphology in H&E-stained sections but show intense nuclear staining for p53 by immunohistochemistry (Lee, Y. *et al.* 2007). Many p53 signatures share identical somatic *TP53* mutations with coexisting STIC and HGSOCs, suggesting that all three entities have a common origin that can progress into ovarian cancer (Lee, Y. *et al.* 2007). Moreover, STIC lesions and HGSOC are strongly suggested to have a clonal relationship. This is because STIC lesions have been found in the fallopian tubes of more than half of the cases of HGSOC and have been reported to share common histological features, gene mutations and molecular profiles with HGSOC (Lee, Y. *et al.* 2007, Dubeau, L. and Drapkin, R. 2013, Nik, N. N. *et al.* 2014). Notably, there are various compelling molecular , pathological, epidemiological and mouse model evidence that indicate that the secretory epithelial cells of the distal fallopian tube are the likely precursors of a significant proportion of HGSOCs, in support of the fallopian tube model (Bowtell, D. D. *et al.* 2015).

First, the molecular genetic evidence that involve the frequent co-occurrence of tubal intraepithelial carcinomas with pelvic (ovarian, fallopian tube and peritoneal) and the identical *TP53* mutations in the majority of STIC lesions neighbouring HGSOC

(Kindelberger, D. W. *et al.* 2007, Kuhn, E. *et al.* 2012). In addition to the fact that the molecular signature of early tubal lesions is almost identical to those of advanced tubal and ovarian lesions (Crum, C. P. *et al.* 2007).

The second group of evidence is pathological and incorporates higher frequency of dysplastic changes coupled with irregularities of cell cycle and apoptosis related proteins in women predisposed to developing ovarian cancer (Piek, J. M. J. *et al.* 2001, Anglesio, M. S. *et al.* 2013).

Thirdly is the epidemiological evidence that encompass a significantly lower ovarian cancer rate in women with previous salpingectomy (Falconer, H. *et al.* 2015). Relatedly, tubal ligation have been proven to associate with a significantly reduced ovarian cancer rate in general population (Cibula, D. *et al.* 2011) and in *BRCA* mutation carriers (Antoniou, A. C. *et al.* 2009, Collins, I. M. *et al.* 2011).

Lastly, the mouse model evidence through the demonstration that high-grade serous tumors can originate in fallopian tubal secretory epithelial cells and the determination of STIC as the precursor lesion of HGSOC and peritoneal carcinomas (Kim, J. *et al.* 2012, Perets, R. *et al.* 2013).

The molecular events that lead to tumorigenesis in the fallopian tube model has been particularly problematic and challenging (Kuhn, E. *et al.* 2016). This is because molecular studies rely on genome-wide assays, which typically require a substantial amount of lesion cells (Kuhn, E. *et al.* 2016). Notably, many putative precursors, together with the majority, if not all, STIC lesions harbour *TP53* mutations (Kindelberger, D. W. *et al.* 2007, Lee, Y. *et al.* 2007, Karst, A. M. *et al.* 2014). However, when unaided, p53 mutation has been proven not adequate to transform fallopian tube secretory epithelial cells and initiate ovarian tumorigenesis (Karst, A. M. *et al.* 2011, Perets, R. *et al.* 2013, Karst, A. M. *et al.* 2014). Consequently, research have focused on additional molecular events that might cooperate with p53 mutation in ovarian carcinogenesis, yet, our understanding of such events is still limited.

In *vitro* and in *vivo* studies have proposed a few genes as co- initiators, together with aberrant p53, including *PTEN* deletion, *H-Ras*^{v12} mutation, gain of *c-Myc* as well as *CCNE1* amplification (Cully, M. *et al.* 2006, Kim, J. *et al.* 2012) (Karst, A. M. *et al.* 2011). These are genes that transformed immortalized fallopian tube secretory epithelial cells or even resulted in HGSOC development in *vivo*. *PTEN* is a tumor suppressor gene that act as a phosphatase inhibitor of the PI3K pathway mediated cell

proliferation (Cully, M. *et al.* 2006, Kim, J. *et al.* 2012). Targeted deletion of *PTEN* has been proven to initiate HGSOC in mouse models (Kim, J. *et al.* 2012). According to The Cancer Genome Atlas, *PTEN* alterations affects more than 7% of HGSOC (Madore, J. *et al.* 2010, Roh, M. H. *et al.* 2010, Cancer Genome Atlas Research Network 2011, Hanrahan, A. J. *et al.* 2012, Martins, F. C. *et al.* 2014) and *PTEN* loss is described as a common event in HGSOC that defines a subgroup with significantly unfavourable outcome (Martins, F. C. *et al.* 2014). Other suggested co - initiators are *H-Ras*^{V12}, though their mutation is not a HGSOC characteristic, and the oncogenic *c-Myc* gene (Karst, A. M. *et al.* 2011). Gain of the 8q24 region carrying *c-Myc* is the most frequently gained region in HGSOC, reported to occur in more than half of the cases and commonly associate with high-level *c-Myc* gene amplification (Nowee, M. *et al.* 2007, Haverty, P. M. *et al.* 2009, Gorringe, K. L. 2010, Nakayama, N. *et al.* 2010, Karst, A. M. *et al.* 2011, Karst, A. M. *et al.* 2014, Kuhn, E. *et al.* 2016).

Although theses substantial data suggest that HGSOC originate mainly in the fallopian tubes (Levanon, K. *et al.* 2010, Auersperg, N. 2011, McCluggage, W. G. 2011, Eppig, J. T. *et al.* 2012, Nik, N. N. *et al.* 2014), some HGSOCs seem to arise without fallopian tube involvement. Correspondingly, some HGSOC experimental mouse models seem to evolve directly from fallopian tube precursors (Perets, R. *et al.* 2013)(Bowtell, D. D., Bohm, S. et al. 2015), while others are likely to originate primarily in the ovaries (Bowtell, D. D. *et al.* 2015, Kim, J. *et al.* 2015). The ovarian surface epithelium model was first described by Fathalla in 1971 (Fathalla, M. F. 1971) and hypothesizes that ovulation play a role in ovarian carcinogenesis 'incessant-ovulation hypothesis' (Fathalla, M. F. 1971). In summary, this model proposes the formation of inclusion cyst from the ovarian surface epithelium (Fathalla, M. F. 1971, Klotz, D. M. and Wimberger, P. 2017). Repeated rupture of the surface of the ovary during ovulation, followed by repair, can lead to increased proliferation in the ovulation-linked pro inflammatory environment (Fathalla, M. F. 1971, Klotz, D. M. and Wimberger, P. 2017). Moreover, ovulation itself is proposed to induce the expression of markers of stem cells in the ovarian surface epithelium and these stem cells are one of the proposed precursors of HGSOC (Ng, A. *et al.* 2014, Karnezis, A. N. *et al.* 2016). Tumorigenesis is enhanced by deregulation of the pluripotency of stem cells harboured by the ovarian surface epithelium and/ or inclusion cyst (Bowen, N. J. *et al.* 2009, Klotz, D. M. and Wimberger, P. 2017). Next, post ovulation auto healing process increases the chances

of double stranded DNA damage repair error due to disruptive homologous recombination repair, for example in patients with *BRCA1* or *BRCA2* mutations (Klotz, D. M. and Wimberger, P. 2017). Furthermore, ovulation induces genotoxic stress (Klotz, D. M. and Wimberger, P. 2017). Consequently, a buildup of DNA double strand breaks that can evade repair (Klotz, D. M. and Wimberger, P. 2017), together with the resulting deleterious mutations lead to malignant transformation of ovarian surface epithelium cells and give rise to HGSOC (Klotz, D. M. and Wimberger, P. 2017). The ovarian model, similar to the tubal model,

As with the tubal model, *BRCA1* and *BRCA2* mutations increases the risk of ovarian cancer arising from ovarian surface epithelium via HR response defect (Miki, Y. *et al.* 1994, Moynahan, M. E. *et al.* 1999, Moynahan, M. E. *et al.* 2001). Additionally, and similarly to the tubal model , focal accumulation of p53 protein have been documented in both ovarian surface epithelial cells within the cortical inclusion cysts of the ovaries for women at risk or with high serous ovarian cancer (Lee, Y. *et al.* 2007). Furthermore, and in support of the ovarian model, the seemingly lower risk of HGSOC in women receiving the ovulation regulating estrogen containing contraceptive pills (Fathalla, M. F. 1971). The protective effect of contraceptive pills is significantly duration dependent (Hankinson, S. E. *et al.* 1992, Pike, M. C. *et al.* 2004, Collaborative Group on Epidemiological Studies of Ovarian, C. 2008) and seems to be variable in women with germline *BRCA1* or *BRCA2* mutations (Narod, S. A. *et al.* 1998, Modan, B. *et al.* 2001, Antoniou, A. C. *et al.* 2009, Karnezis, A. N. *et al.* 2016). Moreover, the cancer preventive effect of contraceptive pills, previously linked to ovulation disruption (Fathalla, M. F. 1971), has recently been attributed to their chemo- protective effect (Karnezis, A. N. *et al.* 2016). This chemo- protective effect is suggested to be either due to the pills direct effect on the secretory cells of the fallopian tube that normally express estrogen and progesterone (Karnezis, A. N. *et al.* 2016). Otherwise, contraceptive pills indirectly protect the fallopian tube fimbria via shielding them from the monthly genotoxic effect of the ovulation- released follicular fluid (Bahar-Shany, K. *et al.* 2014, Karnezis, A. N. *et al.* 2016). Consequently, contraceptive pills can prevent the ovulation- associated damage induced metaplasia of the of ovarian surface epithelium; thus linking the ‘incessant-ovulation hypothesis” to the fallopian tube epithelium hypothesis (Karnezis, A. N. *et al.* 2016).

1. 4. 7 The role of cyclin E1 deregulation in HGSOC

There is a mounting evidence that deregulated cyclin E1 is as an early event in the development of HGSOC, at least in the amplified subset (Karst, A. M. *et al.* 2014, Bowtell, D. D. *et al.* 2015). Moreover, *CCNE1* amplified HGSOC is increasingly recognised as a distinct subset with unfavourable outcome that is potentially targetable. This part of literature review will therefore address the hypotheses of origin of ovarian cancer, including the role of cyclin E1 in their evolution. It also highlights the frequency, mechanisms and impact of cyclin E deregulation in HGSOC compared other molecular aberrations including *BRCA* mutations.

1.4.7.1 Cyclin E1 as a driver gene for early HGSOC

The cyclin E1 encoding gene, *CCNE1*, is another suggested cooperator in ovarian carcinogenesis that is proposed to implement their oncogenic effect via acting with aberrant p53 (Lee, Y. *et al.* 2007, Sehdev, A. S. *et al.* 2010). Amplification of *CCNE1* is increasingly perceived to be the driver of a distinct subset of HGSOC, together with their proposed earlier precursors, STIC lesions (Nakayama, N. *et al.* 2010, Karst, A. M. *et al.* 2014, Kuhn, E. *et al.* 2016). Notably, cyclin E1 is documented to provoke malignant features in untransformed secretory epithelial cells under certain circumstances (Karst, A. M. *et al.* 2014). Moreover, Kuhn and colleagues have recently shown that induced expression of cyclin E1 resulted in increased frequency of centrosome amplification, a sign of genomic instability, in the fallopian tube secretory epithelial cells (Kuhn, E. *et al.* 2016) . Correspondingly, they have reported higher incidence of centrosome amplification in HGSOC compared to STIC lesions (Kuhn, E. *et al.* 2016). These findings are in agreement with the notion that STIC is a precursor lesion that proceeds high-grade serous carcinomas, at least in some cases (Kuhn, E. *et al.* 2016).

Significantly, cyclin E1 has been reported to be expressed in early putative lesions in fallopian tubes (Lee, Y. *et al.* 2007, Karst, A. M. *et al.* 2014) as well as in the majority of STIC lesions though with variable level of expression (Sehdev, A. S. *et al.* 2010) but not in the adjacent normal fallopian tube epithelium (Karst, A. M. *et al.* 2014, Kuhn, E. *et al.* 2016). Fittingly, similar frequency of *CCNE1* gain/amplification has been reported in STIC lesions and in early-stage (stage I–II) , according to The International Federation of Gynecology and Obstetrics (FIGO), fallopian tube carcinomas as well as in HGSOC (Snijders, A. M. *et al.* 2003, Nowee, M. *et al.* 2007, Sehdev, A. S. *et al.*

2010, Siu, K. T. *et al.* 2012, Karst, A. M. *et al.* 2014, Kuhn, E. *et al.* 2016). *CCNE1* amplification is frequently associated with high level of cyclin E1 expression, though, high expression of cyclin E1 has also been reported without associated *CCNE1* amplification (Nakayama, N. *et al.* 2010, Siu, K. T. *et al.* 2012, Karst, A. M. *et al.* 2014, Noske, A. *et al.* 2015).

Importantly, the regulatory function of cyclin E1 on cell cycle is in part under *p53* control (Minella, A. C. *et al.* 2007). For instance, when p53 is intact, excess cyclin E1 is reported to induce series of events with a net effect of p53 mediated attenuation of cyclin E1 action via p53 stabilization and activation (Minella, A. C. *et al.* 2007). Activated p53 acts either by upregulating an inhibitor of cyclin E1/CDK2 ($p21^{CIP1}$), which inhibits CDK2 activity and facilitates cell-cycle arrest (Karst, A. M. *et al.* 2014). Otherwise, if p21 is inactivated, p53 rely on other mediators for inducing apoptosis in order to attenuate the effects of cyclin E1 (Karst, A. M. *et al.* 2014). These findings imply that when cyclin E1 is upregulated, p53 mutation or inactivation is crucial for cells to survive (Minella, A. C. *et al.* 2007). Consequently, with both aberrations been reported in early precursors, it is not unreasonable to speculate a cooperation between p53 dysfunction and aberrant cyclin E1 in ovarian cancer carcinogenesis (Karst, A. M. *et al.* 2014).

Recently, Karst and colleague have generated a model of cyclin E1 mediated transformation using primary human fallopian tube secretory epithelial cells (Karst, A. M. *et al.* 2014). Immortalization of the cells was achieved via inducing a form of p53 mutation identical to those found in in both p53 signatures and STIC lesions (Kindelberger, D. W. *et al.* 2007, Lee, Y. *et al.* 2007, Karst, A. M. *et al.* 2014). Karst *et al* have shown via a series of *in vitro* assays that persistently high cyclin E1 expression resulted in increased proliferation of p53- mutated fallopian tube secretory epithelial cells (Karst, A. M. *et al.* 2014). Moreover, those distorted cells exhibited malignant features including the ability to grow in clonal patterns and others (Karst, A. M. *et al.* 2014). Nonetheless, the resulting phenotype was less aggressive than those previously reported (Karst, A. M. *et al.* 2011, Kim, J. *et al.* 2012) implying that additional genetic events are needed for full transformation (Karst, A. M. *et al.* 2014). Additional genetic events, identified by an siRNA knockdown screen, that are likely to cooperate with cyclin E1 in inducing full transformation of immortalized cells involve co-amplification of *TPX2*, the cleavage of cyclin E1 to a low molecular weight form or

the co - expression of proteins such as the chromatin remodeling protein remodeling and spacing factor 1 (Rsf-1) (Bagheri-Yarmand, R. *et al.* 2010, Etemadmoghadam, D. *et al.* 2010, Sheu, J. J.-C. *et al.* 2013, Karst, A. M. *et al.* 2014).

Additionally, Karst and colleagues have shown cyclin E1 expression in 33% of STIC lesions and that cyclin E1 expression in fallopian tube secretory epithelial cells was associated with DNA damage and altered the expression of DNA damage repair genes i.e. down regulation of others such as the negative cell-cycle regulators or upregulation of certain genes such as *BRCA1* (Karst, A. M. *et al.* 2014). Upregulation of DNA repair genes conceivably enables cells to cope with cyclin E1 deregulation linked replication stress (Karst, A. M. *et al.* 2014).

In summary, these findings suggest that *CCNE1* deregulation initiates uncontrolled growth of fallopian tube secretory epithelial cells harbouring somatic *TP53* defects and result in DNA damage by inducing replication stress. Replication stress generates chromosomal instability and promotes carcinogenesis (Karst, A. M. *et al.* 2014).

While mounting evidence support the notion that cyclin E1 deregulation is an early event, the driver of deregulated cyclin E1 expression in early precursors is not fully explained (Sehdev, A. S. *et al.* 2010, Karst, A. M. *et al.* 2014). One possibility is *CCNE1* amplification. Additional mechanisms that might be responsible for driving high cyclin E1 expression include, oncogene induced transcription or disrupted periodic ubiquitin mediated degradation (Siu, K. T. *et al.* 2012).

1.4.7.2 The significance of cyclin E1 deregulation in HGSOC based on prevalence and clinico-pathologic association

Over the years, many studies have examined the expression and/or amplification of cyclin E1 in various epithelial ovarian cancer cohorts and the findings were assessed against the clinical and pathological parameters of the tumor. Cyclin E1 overexpression by itself is reported to promote tumor growth via increasing the proliferative activity and decreasing the apoptotic activity. However, the frequency and clinico-pathological impact of deregulated cyclin E1 in epithelial ovarian cancer, including HGSOC, remains controversial. For instance the reported frequency of cyclin E1 overexpression in ovarian cancer ranged between 6% in one study (Schraml, P. *et al.* 2003) to about 70% in another (Sui, L. *et al.* 2001). Similarly, the frequency of *CCNE1* gene amplification has been reported in 8% of the cases in one study (Schraml, P. *et al.* 2003) and in up to 37% in others (Nakayama, K. *et al.* 2007, Noske, A. *et al.* 2015). Equally,

conflicting results have been reported in terms of association between cyclin E1 expression and amplification where both aberrations been reported concurrently or separately (Nakayama, N. *et al.* 2010, Karst, A. M. *et al.* 2014, Noske, A. *et al.* 2015). Furthermore, literature findings have been inconsistent in terms of both or either overexpressed cyclin E1 and *CCNE1* amplification correlation with clinico-pathologic parameters including patient's age, tumor size or residual tumor, grade, histological type, stage as well as their impact on patients' clinical outcome. A summary that provides the variable literature findings is provided in Tables 1-1 & 1-2. Another example of literature disagreement is the many studies that have reported cyclin E1 overexpression to be predominately prevalent in serous subsets (Mayr, D. *et al.* 2006, Rosen, D. G. *et al.* 2006, Noske, A. *et al.* 2015) while others reported either significantly higher incidence in non-serous types (Marone, M. *et al.* 1998) or no significant difference between serous and non-serous subsets at all (Farely J., S. L. M., Darcy K.M. ,et al 2003).

Importantly, the majority of the reviewed literature has reported a negative impact of deregulated cyclin E1 (amplification and/or expression) on patient's outcome (Rosenberg, E. *et al.* 2001, Sui, L. *et al.* 2001, Farely J., S. L. M., Darcy K.M. ,et al 2003, Mayr, D. *et al.* 2006, Rosen, D. G. *et al.* 2006, Etemadmoghadam, D. *et al.* 2009, Etemadmoghadam, D. *et al.* 2010, Karst, A. M. *et al.* 2014). Several others reported no impact on survival (Heeran, M. C. *et al.* 2012, Noske, A. *et al.* 2015). Nakayama reported an adverse impact of amplification but not overexpression (Nakayama, N. *et al.* 2010) while Rosenberg reported negative impact of overexpression rather than amplification and only one paper reported positive impact on outcome (Pils, D. *et al.* 2014).

Notably, the amplification of *CCNE1* gene, or its harboring locus (19q12) is reported is one of the few reported aberrations that are strongly implicated in platinum treatment failure (Etemadmoghadam, D. *et al.* 2009, Patch, A. M. *et al.* 2015). However, a co-localised gene with *CCNE1* on 19q12 locus, *URI1*, amplified in 10% of cases and is found to be implicated in platinum resistance and adverse outcome in HGSOC patients (Theurillat, J. P. *et al.* 2011). URI1 protein function is to inactivate an inhibitor of survival signalling (Theurillat, J. P. *et al.* 2011). Interestingly, both *CCNE1* and *URI1* were independently found to be "addicting" oncogenes that are amplified and overexpressed in ovarian cancer cell-lines and human ovarian cancer (Nakayama, N. *et*

al. 2010, Theurillat, J. P. *et al.* 2011). However, siRNA knockdown of all the genes within the 19q12 amplicon, regardless of their amplification status, revealed *CCNE1* as the only gene within the amplicon that reduced ovarian cancer cell survival (Etemadmoghadam, D. *et al.* 2009). Therefore, *CCNE1* amplification status is commonly perceived to be potentially helpful in predicting patient outcome and Platinum response.

In summary, there is controversy regarding the significance of cyclin E1 in ovarian cancer, especially in the serous subset. Reasons for this could include the cohort selection, the technique used in assessing the expression and amplification and the variable methods of cut offs determination. Therefore, we have used a well characterised cohort of serous ovarian cancer all of high grade and stage III to IV from patients enrolled in the Australian Ovarian Cancer Study. We have also used pre-validated automated assays to assess *CCNE1* amplification and cyclin E1 expression. Lastly the cut-offs used in determining amplification and over expression status was optimised against each other and in association with outcome.

Table 1-1: Literature findings of the prevalence and the clinico-pathological correlation of cyclin E1 expression in ovarian cancer

Author/year	Cohort	Techniques	Cut-Off	(Hi/all)%	Impact on outcome	Tumor size	Grade	Stage	Other associations
(Courjal, F. et al. 1996)	OC	Slot-blot(RNA expression)	Signal:GAPDH>twice the ratio in the normal control	(4/22)18%	NR	NR	NR	NR	NR
(Marone, M. et al. 1998)	47 primary OC, 8 Met & 19 recurrent disease	RT-PCR(RNA expression)	3-folds > normal OSE cell lines HIO-118 and HIO-135	(22/74)29.5%	NR	NR	No correlation	No correlation	Not with age, serous < non-serous & associates with CDK2 expression
(Sui, L. et al. 2001)	33 benign	IHC	>50%	(3/33)9.1%	Shorter OS	No correlation	No correlation	Higher	Associated with ascites Not associated with age

	23 borderline			(11/23)47.8%					
	47 malignant EOC			(33/47)70.2%					
(Rosenberg, E. et al. 2001)	Serous EOC	IHC	>50%	(10/34)29%	Shorter OS	No correlation	No correlation	No correlation	Not associated with age
	Non serous EOC			(16/19)84%	No impact				
(Farley, J. et al. 2003)	Sub-optimally de-bulked EOC stage III and IV	IHC	>40% cyclin E1 positive	(63/139)45%	Shorter OS	No correlation	No correlation	No correlation	Not associated with age, race, cell type

(Schraml, P. et al. 2003)	Serous OC	IHC	moderate staining in ≥ 80% and strong staining in ≥ 20% of tumour cell nuclei	(3/47)6.4%	NR	NR	NR	NR	Associates with amplification
(Mayr, D. et al. 2006)	OC	IHC	High expression	(21/204)10.3%	Shorter OS	NR	Higher	NR	Associates with amplification & histologic type
(Rosen, D. G. et al. 2006)	Primary EOC	IHC	>10%	(256/405)63.2 %(including 218/315serous)70%	Shorter OS	Greater residual tumor	Higher	Higher	Higher age & serous type
(Nakayama, N. et al. 2010)	45serous,10 mucinous,10 clear cell,23 endometrioid carcinomas	IHC	Median of modified H score (70) range (0-353)	(43/85)50.6%	No impact on disease free survival and OS	Greater residual tumor	No correlation	Higher	Associate with amplification

(Etemadmoghadam, D. et al. 2010)	43 advanced stage HGSOC and 52 Platinum resistant	qPCR (mRNA)	median signal value + [0.5 x MAD]	(36/95*)37.9%	Reduced PFS	No correlation	No correlation	No correlation	NR
(Heeran, M. C. et al. 2012)	EOC	IHC	≥10%	(306/493)62%	Reduced OS in Serous OC in UVA but not MVA	NR	Higher	Higher	Not associated with age
(Pils, D. et al. 2014)	172 FIGO II/III/IV serous EOC tissues	RT-qPCR	NR	NR	Positive impact on OS	NR	NR	NR	Associates with *CCNE1* amplification
(Karst, A. M. et al. 2014)	HGSOC stage III-IV	IHC	3 or 4>50%	(38/138)27.5%	NR	NR	NR	NR	Associates with amplification
	TCGA HGSOC	RPPA	Z>1.5	(30/412)7.1%	Reduced OS	NR	NR	NR	Associates with amplification

		mRNA	Z score>1.5	(90/489)18.4%	Reduced OS	NR	NR	NR	Associates with amplification
		TCGA data (Cerami, E. et al. 2012, Gao, S. et al. 2013)	NR	(106/489) 21/7%	Reduced OS	NR	NR	NR	Associates with expression
(Noske, A. et al. 2015)	LG &HG EOC	IHC	Median H score 140	(72/138)52.2%	NR	NR	NR	NR	HG EOC and in the clear cell and serous subtypes and amplification
(Sapoznik, S. *et al.* 2017)	HGSOC	IHC	>10% positive cells	(27/40)67.5%	No impact on OS	NR	NR	NR	Does not predict taxane-platinum chemoresistance

OC: ovarian cancer, NR: not reported, Met: metastatic, OSE: ovarian surface epithelium, IHC: immunohistochemistry, EOC: epithelial ovarian cancer, OS: overall survival, PFS: progression free survival, MAD: median absolute deviation, UVA: univariate analysis, MVA: multivariate analysis, TCGA: The Cancer Genome Atlas, RPPA: Reverse phase protein lysate, LG: low grade, HG: high grade.

Table 1-2: Literature findings of the prevalence and the clinico-pathological correlation of *CCNE1* amplification in ovarian cancer

Author/year	Cohort	Techniques	Cut-Off	(Amp/all)%	Impact on outcome	Tumor size	Grade	Stage	Other associations
(Courjal, F. *et al.* 1996)	OC	SB_(DNA amplification)	Signal/GAPDH >twice the ratio in the normal control	(31/237)12.5%	NR	NR	No correlation	No correlation	NR
(Marone, M. *et al.* 1998)	4 benign, 41 primary, 13 Met &4 recurrent tumors	SB-hybridization	2- to 11-folds	(13/62)21%	NR	NR	NR	NR	NR
(Schraml, P. *et al.* 2003)	Serous OC	FISH	> 10 or clusters of 5 gene signals, cyclin E1/Cep17 ratio>3 in ≥ 5%	(3/40)8%	NR	NR	NR	NR	Expression

(Mayr, D. *et al.* 2006)	Serous OC	CGH	Normalisation with normal tissue	(12/12)100%	NR	NR	NR	NR	Expression
	All			(17/26)65%					
(Nakayama, K. *et al.* 2007)	HGSOC	FISH	>3	(30/83)36.1%	NR	NR	NR	NR	Co-amplifies with *AKT2*, *Not ch3* &chr12p13
(Etemadmoghadam, D. *et al.* 2009)	Platinum resistant	qPCR	>0.3	(14/52)27%	Shorter OS	NR	NR	NR	NR
	Platinum responsive			(3/33)10%					

(Nakayama, N. *et al.* 2010)	45 serous, 10 mucinous, 10 clear cell, 23 endometrioid	ISH	*CCNE1* /chro2 or 19 2:1	(18/88)20.4%	Shorter DFS& OS	Greater residual tumor	No correlation	Higher	Expression
(Etemadmoghadam, D. *et al.* 2010)	43 advanced stage HGSOC & 52 Platinum resistant	qPCR (Copy number)	≥2 (8 copies)	(6/95)6.3%	Shorter PFS &OS	No correlation	No correlation	No correlation	20q11 gain(TPX2)&younger age
(Pils, D. *et al.* 2014)	FIGO II/III/IV serous EOC tissues	qPCR (Copy number)	>4 copies	(14/172)8%	No impact on OS	NR	Higher	No correlation	Older age & expression
(Karst, A. M. *et al.* 2014)	HGSOC	FISH	*CCNE1*:control ≥ 3	(23/87)26.4%	NR	NR	NR	NR	Expression
	HGSOC	TCGA data(Cerami, E. *et al.* 2012, Gao, S. *et al.* 2013)	NR	(106/489)21.7 %	Shorter OS	NR	NR	NR	Expression

(Noske, A. *et al.* 2015)	Primary EOC	FISH	CCNE1:CEN 19p ≥2,polysomy ≥4 copies in 40% cells	(30/134)21.7%	No impact on survival	NR	Higher	Higher	Older age & expression
	Primary EOC	Dual ISH	19q12/INSR was found being equal to or greater 2.0	(49/134)36.6%	No impact on survival	No correlation	No correlation	Higher	Older age and expression
(Stronach, E. A. *et al.* 2018)	HGSOC(SCOTROC4 clinical trial)	SNP assay	CNV>2.4	(39/178)22%	Worse PFS	NR	NR	NR	HR proficient

Amp: amplified, SB: Southern blot, OC: ovarian cancer, NR: not reported, FISH: Fluorescence in situ hybridiasation, CGH: Comparative Genomic Hybridization, Met: metastatic, OS: overall survival, PFS: progression free survival, DFS, disease free survival, UVA: univariate analysis, MVA: multivariate analysis, TCGA: the Cancer Genome Atlas, EOC: epithelial ovarian cancer, ISH: in situ hybridiasation, SNP:single nucleotide pleomorphic, CNV: Copy number variation

1.5 Cyclin E1 deregulation in breast cancer

1.5.1 Epidemiology of breast cancer

Breast cancer is the most common type of cancer affecting women worldwide, accounting for up to 22% of all cases (Cancer Genome Atlas Research Network 2012, Valentin, M. D. *et al.* 2012, Gru, A. A. and Allred, D. C. 2013). In 2017, breast cancer is estimated to be the most commonly diagnosed cancer in females (17,586 cases), and the 2nd leading cause of cancer death in females Australia (Australian Institute of Health and Welfare 2017). The disease is the most common cancer in each of the age groups 25-49, 50-64 and 65 and older, while rarely present in women younger than 25 (Australian Institute of Health and Welfare 2017). The risk of developing breast cancer is estimated to be 1 in 8 by the age of 85 (Australian Institute of Health and Welfare 2017). While the incidence of breast cancer continues to be high, the overall mortality has decreased due to both early detection via national screening programs and advances in therapeutic tools (Rakha, E. A. *et al.* 2008).The main risk factors for breast cancer include family history of breast carcinoma with 2 or 3 times higher risk in women who have a first-degree relative with breast cancer compared to general population (Skolnick, M. H. and Cannon-Albright, L. A. 1992). Other risk factors include reproductive history, hormonal factors, alcohol consumption, obesity and others (Australian Institute of Health and Welfare 2017). Genetic susceptibility is another important risk factor and is detailed below.

1.5.2 The genetic susceptibility for breast cancer

A substantial advance in the study of breast cancer has been achieved by the finding of the two high-penetrance susceptibility genes *BRCA1* and *BRCA2* (Collins, L. C. 2018). Germline mutations of these genes are associated with an increased risk for development of breast cancer, in addition to other tumors especially ovarian cancer (Wooster, R. and Weber, B. L. 2003). Initially, *BRCA1* and *BRCA2* mutations were believed to be responsible for a high proportion of familial breast carcinomas (Collins, L. C. 2018). Familial breast cancer is estimated to constitute 5%–10% of all breast cancers (Wooster, R. and Weber, B. L. 2003). However, *BRCA1* and *BRCA2* are currently known to account for only 16% of familial breast cancer (Collins, L. C. 2018). Mutations of these genes are present in approximately 2% of the Ashkenazi Jewish population; the risk among *BRCA1* and *BRCA2* mutations carrier is estimated to be 70%–80% by the age of 75 (Narod, S. A. and Foulkes, W. D. 2004, Collins, L. C. 2018).

If testing for those mutation is positive, the lifetime risk of breast cancer should be closely observed and/or reduced by bilateral prophylactic mastectomy (Robson, M. and Offit, K. 2007).

The protein encoded by *BRCA1* is involved in DDR through HR and cell cycle checkpoint control (Tan, D. S. P. *et al.* 2008, Collins, L. C. 2018). It also plays a role in ubiquitylation, chromatin remodeling, and DNA decatenation (Tan, D. S. P. *et al.* 2008, Collins, L. C. 2018). The function of *BRCA2*-encoded protein includes DNA repair, cytokinesis, and meiosis (Tan, D. S. P. *et al.* 2008, Collins, L. C. 2018). Therefore both *BRCA1* and *BRCA2* are essential for the integrity of the DNA double-strand breaks repair via HR (Collins, L. C. 2018). Loss of DNA repair function in the *BRCA* associated cancers is the key concept—for the novel targeted therapy PARP inhibitors that block repair of DNA damage via alternate pathways, process that has been named "synthetic lethality" (Ashworth, A. 2008, Fong, P. C. *et al.* 2009).

In addition to *BRCA1* and *BRCA2*, other genes such as *CHEK2*, *CDH1*, *RAD50*, and *PALB2* have been reported to associate with a low to moderate increased risk for the development of breast cancer (Sheikh, A. *et al.* 2015).

1.5.3 The classification of breast cancer

Breast cancer is a heterogeneous disease that includes various entities on the basis biological, histological, molecular and clinical behavior (Natrajan, R. *et al.* 2012, Valentin, M. D. *et al.* 2012).

1.5.3.1 The histologic classification of breast cancer

The histologic classification of breast cancer incorporates two main elements, first is invasiveness, where non-invasive pre-malignant tumors are confined to the ducto-lobular system, designated carcinoma in situ, while malignant tumous which have invaded the surrounding stroma are entitled invasive carcinoma (Collins, L. C. 2018). The second determinant is whether the tumor is of ductal or lobular type, i.e. arises from or involves a duct or lobule respectively (Collins, L. C. 2018). From the prognostic point of view the tumor invasiveness far outweighs that of tumor classification into ductal vs lobular (Collins, L. C. 2018).

1.5.3.2 The therapeutic groups of breast cancer

The classification of breast cancer incorporates in addition to the histopathologic classification, the identification of three main predictive biomarkers, ER, progesterone receptor (PR), and human epidermal growth factor receptor type 2 (HER2) statuses

(Weigelt, B. *et al.* 2010). Based on these currently available predictive biomarkers, there are three major therapeutic groups (van 't Veer, L. J. *et al.* 2002, Parise, C. A. *et al.* 2009). These are the *estrogen receptor positive (ER^+)* group which benefit from oestrogen antagonists, Tamoxifin accounting for 70% of cases (Hanna, W. M. *et al.* 2014). The *HER2 amplified/overexpressing group* accounts for up to 20% of breast cancer cases and usually associated with more progressive disease and reduced survival (Hanna, W. M. *et al.* 2014). HER2 amplification /overexpression is evaluable by both HER2 copy number (ISH) and/or HER2 protein expression (IHC) and targetable by anti HER2 agents including Trastuzumab.

The third group is the *triple negative breast cancer (TNBC) group*, of which about 75% are basal -like breast cancer (BLBC) (Rakha, E. A. *et al.* 2008). Together TNBC and BLBC account for 15%-20% of breast cancer cases (Hong, R. *et al.* 2014). By definition TNBC tumors lack expression of both estrogen and progesterone receptors as well as the expression and/or amplification of HER2, whereas BLBC overlaps with cases that may express ER, PR and / or HER2. Patients with these subgroups typically lack the conventional therapeutic targets and usually face poorer outcome and higher rate of recurrence within 1-3 years after treatment (van 't Veer, L. J. *et al.* 2002, Cancer Genome Atlas Research Network 2012, Gazinska, P. *et al.* 2013, Herold, C. I. and Anders, C. K. 2013, Hong, R. *et al.* 2014). The studies of *BRCA1* associated cancers have revealed higher proportion of tumors with the basal-like gene expression profile (Rakha, E. A. *et al.* 2008).

The defining feature of basal like breast cancer is that it is positive for basal cytokeratins (eg CK5/6) and or EGFR and/or c-KIT (Nielsen, T. O. *et al.* 2004) and are mostly *BRCA1* related (about 60%) (Rakha, E. A. *et al.* 2008). These tumors are usually of high grade with high mitotic activity, have unique growth patterns of pushing margins and confluent necrosis (Tan, D. S. P. *et al.* 2008, Collins, L. C. 2018). Importantly, cyclin E1 overexpression and gene upregulation are common features of these subsets (Yehiely, F. *et al.* 2006, Voduc, D. *et al.* 2008, Valentin, M. D. *et al.* 2012). As for ovarian cancer, the impact of cyclin E1 overexpression and/or gene amplification on prognosis is controversial. Given that BLBC are usually of high grade, p53 associated, and *BRCA1* linked TCGA as has identified them as a HGSOC-like subset (Cancer Genome Atlas Research Network 2012). However, unlike HGSOC, *BRCA1* mutation and cyclin E1 overexpression commonly co-occur in BLBC suggesting that either

CCNE1 amplification is not mutually exclusive and therefore both cancer types are different or that cyclin E1 expression in BLBC, esspecially *BRCA1* mutant, is due to mechanisms other than *CCNE1* amplification.

1.5.4 The significance of cyclin E1 deregulation in breast cancer

Over the years, there are more than 500 articles that have studied *CCNE1* amplification and /or cyclin E expression (protein or mRNA) in various cancer types (Zhao, H. *et al.* 2018). While this reflects the significance of cyclin E1, it also demonstrates the difficulties in reviewing all the research that has focused on cyclin E1. We have summarised the findings of the most promiment of these publications relating to breast cancer in Tables 1-3 & 1-4.

As in the case of HGSOC, the several studies that have examined the expression of cyclin E1 in breast cancer revealed inconsistent results in terms of prevalence, impact on outcome and association with tumor characteristics such as subtype, grade, stage, nodal or receptor status. For instance, the prevalence of cyclin E1 overexpression ranged from around 25% (Nielsen, N. H. *et al.* 1998) to as high as 65% (Peters, M. G. *et al.* 2004). This inconsistency is expected as various techniques and reagents were used for cyclin E1 expression assessment in addition to the broad range of the cut offs that have been used to determine high versus low expressers. In one study, the use of western blotting and IHC techniques to assess cyclin E1 expression revealed a 37% discrepancy in the prevalence of high expressers despite using the same antibody to assess the same patient's samples (Keyomarsi, K. *et al.* 2002). The associations with other clinico-pathologic parameters such as tumor size, grade, stage, patients' age and Ki67 status is also highly varied (Tables 3 & 4).

In terms of outcome, research findings varied significantly with the majority of studies reported an adverse impact of cyclin E1 overexpression on patient outcomes (Nielsen, N. H. *et al.* 1996, Porter, P. L. *et al.* 1997, Nielsen, N. H. *et al.* 1998, Keyomarsi, K. *et al.* 2002, Kühling, H. *et al.* 2003, Foulkes, W. D. *et al.* 2004, Callagy, G. *et al.* 2005, Potemski, P. *et al.* 2005, Potemski, P. *et al.* 2006, Berglund, P. *et al.* 2008, Voduc, D. *et al.* 2008, Scaltriti, M. *et al.* 2011, Lundgren, C. *et al.* 2015, Fredholm, H. *et al.* 2017) while fewer studies reported no correlation of high cyclin E1 with patients' outcome (Callagy, G. *et al.* 2005, Potemski, P. *et al.* 2006, Luhtala, S. *et al.* 2016) and only one reported a positive impact of high cyclin E1 expression (Peters, M. G. *et al.* 2004). Notably, in breast cancer, cyclin E1 overexpression is commonly attributed to

mechanisms other than gene amplification, for example disrupted degradation (Moelans, C. B. *et al.* 2014). In one study, the majority (73%) of *CCNE1* amplified cases were reported to have associated high cyclin E1 expression while only small percentage (17%) of high expressers were amplified (Callagy, G. *et al.* 2005).

Only a few studies have directly assessed *CCNE1* amplification in breast cancer (Table 3) and these generally revealed no association with patients' outcome (Luhtala, S. *et al.* 2016) except for Zhao *et al* that has recently reported an adverse impact of CCNE amplification in TNBC though in a small cohort (Zhao, Z. M. *et al.* 2019). Overall, *CCNE1* gene amplification is less frequent in breast cancer compared to ovarian cancer (Cancer Genome Atlas Research Network 2012). However, *CCNE1* amplification, as well as cyclin E1 protein expression, are reportedly more common in ER negative groups with the amplification of cyclin E1 harboring locus (19q12) reported in 15% of the cases (Nielsen, N. H. *et al.* 1996, Kühling, H. *et al.* 2003, Callagy, G. *et al.* 2005, Potemski, P. *et al.* 2006, Adelaide, J. *et al.* 2007, Berglund, P. *et al.* 2008, Voduc, D. *et al.* 2008, Natrajan, R. *et al.* 2009, Scaltriti, M. *et al.* 2011, Luhtala, S. *et al.* 2016).

As previously explained, the TNBC subset significantly overlaps with the BLBC subset (Rakha, E. A. *et al.* 2008, Cancer Genome Atlas Research Network 2012). Cyclin E1 is overexpressed in about 45% of BLBC cases (Yehiely, F. *et al.* 2006, Voduc, D. *et al.* 2008) while the gene is up regulated in 5-8% of cases (Cancer Genome Atlas Research Network 2012, Natrajan, R. *et al.* 2012).

In patients with HER2 positive breast cancer, *CCNE1* amplification has been implicated in Trastuzumab resistance, unfavorable outcome and low progression free survival (Scaltriti, M. *et al.* 2011). Scaltriti *et al* observed that blocking cyclin E1 activity in *CCNE1* amplified-Trastuzumab resistant cells by *CCNE1* gene knockdown or CDK2 inhibitors, resulted in significant decline in proliferation and increased apoptosis both in *vitro* and in *vivo* (Scaltriti, M. *et al.* 2011). Nevertheless, Peters et al previously reported no association of cyclin E1 overexpression with HER2 positivity (Peters, M. G. *et al.* 2004) and Luhtala et al have recently reported no impact of cyclin E1 overexpression on patients' outcome in HER2 positive group (Luhtala, S. *et al.* 2016).

Overall, the reported relationship between cyclin E1 expression and amplification in breast cancer, and their effect on outcome, varies considerably in published reports. We aimed to address these inconsistencies by assessing a carefully selected cohort and used the same pre -validated automated assays to assess *CCNE1* amplification and cyclin E1

expression in the ovarian cancer cohort. Lastly the cut-offs used in determining amplification and over expression status was optimised with the best association with outcome used the statistical significance, minimum p and the reported prevalence in BLBC.

1.6 *BRCA1-CCNE1* association in HGSOC and BLBC

Alterations in the *RB1/CCNE1* pathway, that is responsible for cell cycle regulation, including *CCNE1* amplification, *RB1* loss and *RBBP8* gain, occur in about 30% of HGSOC cases, and are almost always exclusive of *BRCA1/2* inactivation that are normally responsible for DNA repair (Cancer Genome Atlas Research Network 2011, Ciriello, G. *et al.* 2012, Etemadmoghadam, D. *et al.* 2013). The known role of both aberrations in promoting genomic instability and cancer development provides a plausible explanation for the mutual exclusivity between them as both aberrations are synthetically lethal and there is no selective advantage of having both of them (Caldon, C. E. and Musgrove, E. 2010, Roy, R. *et al.* 2011, Etemadmoghadam, D. *et al.* 2013). Later, the mutual exclusivity was reported to extend to cases that harbor epigenetic inactivation of *BRCA1/2* and defects in HR pathway "BRCAness" supporting the suggested synthetic lethality between both genomic alterations (Patch, A. M. *et al.* 2015). The synthetic lethality is hypothesized to be due to the crucial requirement of an intact DNA repair response to repair the genomic aberrations that are associated with *CCNE1* amplification (Bartek, J. and Lukas, J. 2003). The intact HR is strongly perceived as the cause of platinum resistance in *CCNE1*-amplified tumors (Patch, A. M. *et al.* 2015). Consequently it is speculated that *CCNE1*-amplified tumors are unlikely to respond to *BRCA1/2* inactivation targeted therapies such as PARP inhibitors (Etemadmoghadam, D. *et al.* 2013).

In breast cancer, it is estimated that about three quarters of *BRCA1* associated breast cancers are basal like (Waddell, N. *et al.* 2010). According to TCGA, this group commonly shares numerous features with HGSOC (Cancer Genome Atlas Research Network 2012). Those include *BRCA1* inactivation, *CCNE1* gene amplification, high frequency of *TP53* mutation and others (Cancer Genome Atlas Research Network 2012).

Table 1-3: Literature findings of the prevalence and impact of cyclin E1 expression in breast cancer

Author/year	Cohort	Techniques	Cut-Off	(Hi/all)%	Impact on outcome	Tumor size	Grade	stage	Receptor	Other associations
(Nielsen, N. H. *et al.* 1996)	Stage -IV	WB	≥ 0.5 high	(34/114)29.8 %	Higher risk of death and relapse	Higher	NR	No correlation	ER Neg.	NR
(Porter, P. L. *et al.* 1997)	20-44y old women	IHC	4-6 out of 0-6	(96/278)34.5 %	Worse survival in all & node negative	NR	NR	NR	NR	Ki67 correlation
(Nielsen, N. H. *et al.* 1998)	(same patients in Nielson 1996)	IHC	Intensity 3 plus	(18/74)24.30 %	Significantly impaired prognosis	NR	NR	NR	NR	Ki67 and with WB detected cyclin E1
(Kim, H. K. *et al.* 2001)	BC	IHC	>50%	(52/128)41%	NR	NR	NR	NR	NR	NR

(Keyomarsi, K. *et al.* 2002)	BC	WB	> cyclin E in normal breast epithelium	LMW cyclin E1(106/395)26.8	Shorter survival in UVA and MVA	NR	NR	Higher	NR	NR
				FL cyclin E1(125/395)31.6	Worse survival in UVA but not MVA					
				Total cyclin E1(127/395)32.2	Shorter survival in UVA &MVA					
		IHC	IHC score total 0-10, >2 high	(136/256)53.1	Shorter survival in UVA & MVA	NR	NR	NR	NR	NR
(Kühling, H. *et al.* 2003)	Node negative invasive BC stage I and II	IHC	Median	(132/237)55.7	Predicts metastasis	No correlation	NR	NR	NR	NR

(Peters, M. G. *et al.* 2004)	Untreated invasive BC	IHC	10%	(36/56)64.3	Longer RFS	NR	NR	Higher in lower stage	NR	NR
(Foulkes, W. D. *et al.* 2004)	Ashkenazi Jewish, age <65 years primary non-metastatic BC	IHC	Pos. vs Neg.	Ck5/6 –(Non basal) (22/150)14.7 % (4 Ck5/6+(basal)1/92)44.6%	Shorter survival	NR	NR	NR	NR	NR
(Potemski, P. *et al.* 2005)	IDC-NOS treated by total mastectomy & axillary clearance	IHC	Cyclin E labelling indexes ***≥ 2%	Ck5/6 –(Non basal) (52/106)49.1 % Ck5/6+(basal) (57/70)81.4	Shorter survival	NR	NR	NR	NR	NR

(Callagy, G. *et al.* 2005)		IHC	25%	24.70%	Unrelated to outcome	NR	NR	NR	NR	NR
(Callagy, G. *et al.* 2005)	Node Neg., no adjuvant chemotherapy	RT-qPCR	Median	(317/635)49.9%	Poor survival in stroma enriched CCNE1 high	NR	NR	NR	NR	NR
(Callagy, G. *et al.* 2005)	IDC untreated (136),tamoxifen only treated (69)	RT-qPCR	Median	(103/205)50 %	Worse RFS	NR	NR	NR	NR	NR
(Potemski, P. *et al.* 2006)	IDC-NOS	IHC	>2%	(74/124)59.7 %	Adverse in the node positive group	NR	NR	NR	ER/PR Neg.	Younger age
(Potemski, P. *et al.* 2006)	IDC	Rt-qPCR	>1.0	(50/124)40.3 %	No impact on prognosis	NR	NR	NR	NR	NR

(Voduc, D. *et al.* 2008)	BC	IHC	10%	(147/319)46 %	Predicts poor prognosis in UVA not MVA	NR	Higher	NR	ER Neg./HER2 Neg.	Young age, basal biomarkers EGFR and cytokeratin 5/6
(Berglund, P. *et al.* 2008)	Premenopausal, stage II tamoxifen treated	IHC	>10%	(145/385)40 %	Worse RFS in untreated BC with infiltrative growth	Higher	Higher	NR	ER/PR Neg.	Pushing margin growth pattern in
(Scaltriti, M. *et al.* 2011)	HER2 amplified breast tumors	IHC	H score>30	(19/55)35%	Low PFS , worse outcome	NR	NR	NR	ER Neg.	Lower clinical benefit from Trastzumab
(Lundgren, C. *et al.* 2015)	Node Neg. no adjuvant chemotherapy	IHC	≈20%	(181/372)48.7%	Shorter survival	NR	NR	NR	NR	NR
(Luhtala, S. *et al.* 2016)	HER2 positive, 87 Trastuzmab	IHC	≥50 %	(74/202)36.6 %	No prognostic significance on RFS	Higher	Higher	NR	NR	NR

	treated , 106 not treated									
(Fredholm, H. *et al.* 2017)	Population-based cohort.	IHC	>10%	(435/857)50.8%	Poor outcome	NR	NR	NR	NR	NR

WB: western blot, NR: not reported, ER: estrogen receptor, PR: progesterone receptor, IHC: immunohistochemistry, BC: breast cancer, LMW: low molecular weight, FL: full length, UVA: univariate analysis, MVA: multivariate analysis, RFS: relapse free survival, POS: positive, Neg: negative, IDC: invasive ductal carcinoma, NOS: not otherwise specified, PAD: publically available data, RMA: Robust multi-array averages.

Table 1-4: Literature findings of the prevalence and impact of *CCNE1* amplification in breast cancer

Author/year	Cohort	Techniques	Cut-Off	(Amp/all)%	Impact on outcome	Tumor size	Grade	Stage	Receptor	Other associations
(Callagy, G. et al. 2005)	Node Neg.	Array-CGH.	1.2	(12/52)23.1%	Higher in early BC specific death	NR	NR	NR	NR	NR
(Callagy, G. et al. 2005)	Population based	FISH	>1.5	(12/197)6%	No correlation	NR	Higher	NR	ER Neg.	NR
(Scaltriti, M. et al. 2011)	HER2 amplified breast tumors	FISH	Cyclin E/chromosome 19>1.5	(18/55) 35%	Worse clinical benefit from Trastuzumab & PFS	NR	NR	NR	NR	Trastazumab resistance

(Natrajan, R. et al. 2012)	All BC cases	Array-CGH	>0.45	(16/313) 5.1%	NR	NR	Grade III	NR	ER Neg./HER2 Neg.	NR
	Grade III BC			(16/188) 7.8%						
(Zhao, Z. M. et al. 2019)	TNBC priamary and Met	Targeted sequencing, microarray analysis	≥6	(6/55)10.9%	Shorter OS, no impact on PFS	NR	NR	Higher	TNBC	Absent *BRCA* mutation
	METABRIC(TNBC)			(27/299) 9%	NR	NR	NR	NR		NR
	METABRIC(non-TNBC)			(27/1605)						NR

	TCGA(TNBC)			(11/111)10%						Absent *BRCA* mutation
	TCGA(non-TNBC)			(33/962)3.5%						Absent *BRCA* mutation

Amp: amplified, CGH: Comparative Genomic Hybridization, Neg: negative, BC: breast cancer, NR: not reported, FISH: Fluorescence in situ hybridiasation, ER: estrogen receptor, PFS: progression free survival, TNBC: triple negative breast cancer, TCGA: the Cancer Genome Atlas Data, OS: overall survival, PFS: progression free survival.

As cyclin E1 overexpression is a common feature of BLBC, a perplexing question is whether, like HGSOC, *BRCA1* inactivation is mutually exclusive from *CCNE1* gene amplification in the BLBC subset. If this is confirmed, it contraindicates the potential therapeutic targeting of *BRCA1* loss in *CCNE1* amplified BLBC cases. If this is not true, then there is a paradox that needs to be meticulously addressed, as these aberrations are thought to be synthetically lethal (Etemadmoghadam, D. *et al.* 2013). There are conflicting reports on the co-occurrence of cyclin E1 gene amplification and protein expression with *BRCA1* inactivation, Honrado reported absence of *CCNE1* amplification in *BRCA1* associated cases and linked that to the overall low incidence of *CCNE1* amplification in breast cancer (Honrado, E. *et al.* 2005). In addition, Zhao and colleagues have recently reported the absence of *BRCA1* mutation in all *CCNE1* amplified TNBC cases (Zhao, Z. M. *et al.* 2019). However, Chappuis et al found that high cyclin E1 is characteristic of *BRCA1* inactivated breast cancer (Chappuis, P. O. *et al.* 2005).

To explore the relationship between *BRCA1* gene inactivation, *CCNE1* gene amplification and cyclin E1 protein expression, we have assessed a cohort of familial breast cancer using samples from patients enrolled in the KConFab study that is enriched for *BRCA1/2* mutation. We assessed samples for *CCNE1* amplification, cyclin E1 expression in addition to the expression of cyclin E1 degradation associated proteins.

1.7 Proposed targeted therapies for cyclin E1 overexpression/ amplification in HGSOC and BLBC

Women with *CCNE1* amplified HGSOC represent a group with unfavorable outcomes who are in urgent unmet clinical need for targeted therapies. Similarly, patients with BLBC that has proven to show great resemblance to HGSOC, are usually of young age and have poor prognosis.

The currently proposed therapeutic approaches for *CCNE1* amplified ovarian, and other cancers, are based on our improved understanding of the molecular events that characterise the *CCNE1* amplified cancers. For instance, *CCNE1* amplified ovarian cancer cells are reported by many studies to be addicted to the presence of cyclin E1 expression in support of the notion that *CCNE1* is a driver oncogene that can initiate ovarian cancer (Etemadmoghadam, D. *et al.* 2010, Torti, D. and Trusolino, L. 2011, Yang, L. *et al.* 2015). Fittingly, many studies have reported that in *CCNE1* amplified

cells, depletion of *CCNE1* expression via knockdown or suppression of cyclin E1 activity via CDK2 inhibitors lead to the suppression of ovarian cancer and breast cancer cell proliferation (Nakayama, N. *et al.* 2010, Natrajan, R. *et al.* 2012, Etemadmoghadam, D. *et al.* 2013, Yang, L. *et al.* 2015). Therefore, the use of small molecule inhibitors of CDK2 (CDK inhibitors) has emerged as a potential cytostatic therapeutic tool for *CCNE1* amplified tumors (Yang, L. *et al.* 2015). However, the pharmacological inhibition of cyclin E1 has been reported to be less effective than gene knockdown either because of the non-specificity of inhibitors such as Dinaciclib or due to the kinase independent activities of *CCNE1* in amplified HGSOC (Geng, Y. *et al.* 2007).

Dinaciclib, a potent CDK2 inhibitor in *CCNE1*-amplified HGSOC cell lines, is one of the most clinically advanced CDK2 inhibitors that has recently been commenced in preclinical and clinical trials (Mita, M. M. *et al.* 2014, Stephenson, J. J. *et al.* 2014, Asghar, U. *et al.* 2015). The drug has revealed apoptotic and anti-cancerous effect in human cancer cell lines and solid tumors of patients including in phase I trials (Mita, M. M. *et al.* 2014, Stephenson, J. J. *et al.* 2014). However, as a monotherapy dinaciclib did not show effect when used in phase II trial in non-small cell lung cancer and only showed a trivial effect in those with advanced breast cancer (Mita, M. M. *et al.* 2014, Stephenson, J. J. *et al.* 2014). Resistance to CDK inhibitors is reported to be one of the possible mechanisms of treatment failure and the resistance has been attributed to factors like CDK2 upregulation, increased *AKT1* copy number and up-regulation of genes in the AKT pathway and polyploidy (Etemadmoghadam, D. *et al.* 2013). Using a combinatorial drug screen, Au-Yeung *et al* has identified an Akt-specific inhibitor (GSK2110183), as a drug that can synergies with dinaciclib in targeting CDK inhibitor-resistant cell lines as well as *in vitro* and *in vivo* models of *CCNE1*-amplified HGSOC (Au-Yeung, G. *et al.* 2018). The synergistic impact of dinaciclib and AKT inhibitors is partially explainable by the frequent co amplification of both *AKT* and *CCNE1* genes (Liang, J. *et al.* 2002). However, an analogous effect was reported in cells that had *CCNE1* but not *AKT* amplification, namely FUOV1 (Barretina, J. *et al.* 2012, Au-Yeung, G. *et al.* 2018) implying other mechanisms for such synergistic impact between the two targeted therapies.

The principle of using CDK2 inhibitors is to target cyclin E1 kinase related activity. However, the recently suggested kinase independent role of cyclin E1 in driving cell proliferation in hepatic cancer cells (Geng, Y. *et al.* 2018) questions the efficacy of CDK2 inhibitor as a therapeutic approach. An alternative is a recently developed strategy that targets selective protein stability (Winter, G. E. *et al.* 2015), thus giving the opportunity to design compounds that selectively target cyclin E1 for degradation (Geng, Y. *et al.* 2018). Another potential therapeutic strategy for cyclin E1 overexpression, especially when linked to disrupted degradation, is via the use of compounds that enhance cyclin E1 degradation in cyclin E1 overexpressing cells. For instance, protease inhibitors that specifically target deubiquitinases such as USP28 (D'Arcy, P. *et al.* 2015, Wang, X. *et al.* 2018). Both of these alternative approaches are dependent on confirming the reliance of HGSOC and BLBC subset on cyclin E1 protein expression.

Another proposed therapeutic options are designed to tackle the ability of the genomically unstable *CCNE1* amplified cells to survive. For example, via the use of members of proteasome inhibitors called Bortezomib, as a *BRCA1* targeting agent (Gu, Y. *et al.* 2014). This proposed therapeutic tool is based on the reliance of *CCNE1* amplified cells on *BRCA1*, as a DNA damage repair tool, for their survival and the proposed synthetic lethality between both *CCNE1* amplification and *BRCA1* inactivation (Etemadmoghadam, D. *et al.* 2013). As in the case of CDK inhibitors, Bortezomib had a minimal effect when used as monotherapy in phase II clinical trial for recurrent ovarian cancer cases (Aghajanian, C. *et al.* 2009) but improved activity when combined with Platinum therapy (Kobrinsky, B. *et al.* 2013, Wiedemeyer, W. R. *et al.* 2014). Other potential therapeutic agents include those that target members of DNA repair, other than BRCA1, such as Chk1 and/or Wee1 inhibitors as the genomically unstable *CCNE1* amplified cells are likely to rely on the ATM-ATR-Chk1-Wee1 pathway for their survival (Matheson, C. J. *et al.* 2016, Geenen, J. J. J. and Schellens, J. H. M. 2017) (Figure1-3).

Given the controversy regarding the prevalence and mechanisms of Cyclin E1 dysregulation in cancer, the unmet clinical need driven by lack of effective benefit from standard of care treatment and several potential targeted treatment options, this thesis aims to better characterize the prevalence of these aberrations using large well – defined cohorts and pre-validated assays and cut offs, investigate potential mechanisms

involved in cyclin E1 overexpression in non-*CCNE1* amplified cases and to explore the similarities and differences in cyclin E1 associated HGSOC and BLBC.

1.8 Aims

The overall aim of this thesis is to better characterise the prevalence, pathogenesis, clinical significance of and therapeutic options for HGSOC and BLBC with 19q12 (*CCNE1*) amplification and / or cyclin E1 overexpression.

Hypotheses:

1. Impaired proteosomal degradation of cyclin E1 is responsible for high cyclin E1 expression in HGSOC and BLBC without 19q12 (*CCNE1*) amplification.
2. 19q12 (*CCNE1*) amplification results in genomic instability due to replication stress, requires HR deficiency for cell survival, and is associated with a poorer clinical outcome and responsiveness to CDK2 and / or CHEK1 – Wee1 inhibitors.
3. Impaired proteosomal degradation of cyclin E1 results from altered FBXW7, USP28 or cyclin E1 phosphorylation site and does not cause replication stress or genomic instability, does not require HR deficiency for cell survival and is associated with better clinical outcomes and responsiveness to proteosomal inhibitors.
4. Differences in the proportion of patients with cyclin E1 over - expression due to 19q12 (*CCNE1*) amplification versus impaired proteosomal degradation explains the differences in mutual exclusivity with BRCA1/2 mutation between HGSOC and BLBC.

In chapter 3, the specific aims relating to HGSOC are as follows:

1. Assess the prevalence of 19q12 amplification using a pre validated automated tissue based assays that utilise dual ISH probe in a well characterised cohort from patients enrolled in the AOCS who have stage III or IV HGSOC.
2. To assess the expression level of the two co localised genes on the 19q12 locus cyclin E1 and URI1 and their correlation with 19q12 amplification status and with each other.
3. Assess the prevalence and correlation, in HGSOC, of cyclin E1 degradation associated proteins, FBXW7 and USP28, with excess cyclin E1 expression in the presence and absence of 19q12 (*CCNE1*) amplification with outcome.

4. Assess the clinical impact of cyclin E1 overexpression in the presence and absence of 19q12 (*CCNE1*) amplification in HGSOC in terms of both overall survival and progression free survival.
5. Determine whether cyclin E1 overexpression, in the presence and absence of 19q12 (*CCNE1*) amplification, is mutually exclusive from *BRCA1* and *BRCA2* in HGSOC in the AOCS cohort.

In chapter 4, the specific aims relating to BRCA/cyclin E1 correlation are as follows:

1. To identify the prevalence of cyclin E1 overexpression assessed by IHC and 19q12 amplification assessed by ISH and their correlation with each other in breast cancer cases based on the *BRCA* status from patients enrolled in the KConFab study.
2. Assess the clinical impact of cyclin E1 overexpression and 19q12 (*CCNE1*) amplification in *BRCA1/2* mutant and *BRCA1/2* wild type samples in terms of both OS and PFS.
3. Assess the prevalence and correlation of cyclin E1 degradation associated proteins, FBXW7, USP28, and cyclin E1 T62 with excess cyclin E1 expression in *BRCA1/2* mutant and *BRCA1/2* wild type samples
4. Determine prevalence of cyclin E1 overexpression in *BRCA* mutated cancers in presence and absence of 19q12 (*CCNE1*) amplification, as determine whether these are mutually exclusive from *BRCA1* and *BRCA2* in the BLBC and NBLBC subsets.

In chapter 5, this thesis systematically compares and contrasts HGSOC and BLBC with respect to cyclin E1 dysregulation and *BRCA1/2* inactivation. The specific sub-aims are:

1. To assess the prevalence of cyclin E1 expression and 19q12 amplification in BLBC versus NBLBC, their correlation to each other and their impact on survival.
2. To identify similarities between HGSOC and BLBC subsets from patients' enrolled in the AOCS and KConFab, respectively, as well as from TCGA in terms of cyclin E1 level of expression and 19q12 amplification.
3. To identify whether the prevalence and impact of *CCNE1* amplification and impaired cyclin E1 degradation as potential drivers of cyclin E1 overexpression in BLBC is similar to that observed in HGSOC subsets.
4. To identify whether *BRCA*-cyclin E1 association in BLBC matches that observed in HGSOC subsets.

In chapter 6, this thesis will discuss the implications of our findings and present a mechanistic model to explain how aetiological differences in cyclin E1 overexpression due to *CCNE1* amplification versus impaired protesomoal degradation, in HGSOC and BLBC, results in different pathogenic mechanisms, clinical outcomes and therapeutic options and accordingly proposes that these are, distinct disease subsets.

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Chapter 2: Methods

2.1 Tissue sample selection

2.1.1 Ovarian cancer cohort

A set of seven tissue microarrays (TMAs) of primary epithelial ovarian carcinomas that have been created by the Australian Ovarian Cancer Study (AOCS) were used in this project. Heamatoxylin and Eosin stained sections of AOCS TMAs were assessed for histopathological evaluation. Sections were then assessed by tissue - based assays for 19q12 (*CCNE1*/ *URI1*) amplification and for the expression of cyclin E1 and URI1, as well as for the assessment of the expression of cyclin E1 degradation associated proteins, FBXW7 and USP28, in ovarian cancer.

These TMAs included at least duplicate cores of 357 formalin fixed paraffin embedded tissue (FFPET) of de-identified cases of epithelial ovarian cancers. The World Health Organization criteria was applied for the histological classification of ovarian cancers (Kurman RJ, C. M., Herrington CS, Young RH. 2014) and revealed 341 cases as HGSOC. Cancers other than serous and, those of low or moderate grade serous carcinomas were excluded from this study (n = 16). HGSOC samples were assessed in terms of sufficient tumor tissue and staining quality for cyclin E1 IHC and ISH, of which 262 cases were assessable.

FIGO/American Joint Committee on Cancer staging system was used for tumor staging (Sweeney, K. J. *et al.* 1998). Nine cases (3%) were stage IA-C, 15 (6%) were stage IIA-C, 149 (74%) were stage IIIA-C, 37 (14%) were stage IV and the remaining 7 (3%) were of unknown stage. *CCNE1* copy number had been previously measured for 67 cases by quantitative PCR (Etemadmoghadam, D. *et al.* 2010).For most of the cases prognostic data was available. These include progression free survival (PFS) and overall survival (OS), both measured from the time of diagnosis. Details of other clinical and pathological data are provided in Table 2-1.

Table 2-1: The clinical and pathological characteristics of AOCS cohort

Parameter		Number of cases	% of cases
Age (years)			
	Mean	60.1	
	Range	30.2 - 80	
Primary Site			
	Ovary	197	(75%)
	Peritoneum	54	(21%)
	Fallopian tube	11	(4%)
Subtype			
	Serous	253	(97%)
	Carcinoma (NOS)	9	(3%)
Grade			
	1	0	(0%)
	2	72	(27%)
	3	190	(73%)
Stage			
	IA-C	9	(3%)
	IIA-C	15	(6%)
	IIIA-C	194	(74%)
	IV	37	(14%)
	Unknown	7	(3%)
Residual Disease			
	No macroscopic	65	(25%)
	≤1 cm	84	(32%)
	>1 cm	88	(34%)
	Tumor not resected	10	(4%)
	Unknown	15	(6%)
Germline *BRCA1*/2 Status			
	BRCA1 mutant	41	(15.6%)
	BRCA2 mutant	25	(9.5%)
	Wild type	170	(64.9%)

	Untested	26	(10%)
Progression-free survival			
	Events	222	(85%)
	Median months	14.20	
Overall survival			
	Events	192	(73%)
	Median months	40.14	
Total Cases		262	

2.1.2 Breast cancer cohort

A set of four TMAs representing 308 cases of primary breast cancer were obtained from the Kathleen Cunningham Foundation Consortium for research into Familial Breast cancer (KConFab) (http://www.KConFab.org), of which 237 cases were assessable in terms of sufficient tumour tissue and staining quality for IHC and ISH. The World Health Organization criteria were used to histologically classify breast cancers as infiltrating duct carcinoma not otherwise specified (NOS), N=190 (80.2%), carcinoma NOS N=17 (7.2%), lobular carcinoma NOS N=10 (4.2%), infiltrating duct and lobular carcinoma N=8 (3.4%), medullary carcinoma NOS N=5 (2.1%) and others N=7 (2.9%). Estrogen and progesterone receptor, HER2 status, and basal markers (ck5, ck14 and EGFR) were available from research data provided by KConFab. Cases were classified as BLBC if the tumour tissue was CK5/14 and /or EGFR positive and non-basal like breast cancer (NBLBC) where the tumour tissue was both CK5/14 and EGFR negative. Tumours were further classified based on estrogen receptor, progesterone receptor and HER2 status. Details of other clinical and pathological data are provided in Table 2-2.

Table 2-2: The clinical and pathological characteristics of KconFab cohort

Parameter		**Number of cases**	**% of cases**
Age (years)			
	Mean	43.8	
	Range	19 - 77	
Histological Subtype			
	Infiltrating duct carcinoma, NOS	190	80.2
	Lobular carcinoma, NOS	10	4.2
	Carcinoma NOS	17	7.2
	Infiltrating duct and lobular carcinoma	8	3.4
	Medullary carcinoma, NOS	5	2.1
	Others	7	2.9
Grade			
	1	24	10.1
	2	67	28..3
	3	123	51.9
	No Grade	23	9.7
Nodal status			
	N0	102	43
	N1 (1-3)	37	15.6

	N2 (4-9)	13	5.5
	N3 (10 or >)	6	2.5
	Not known	79	33.3
Familial breast cancer genes status			
	BRCA1	103	43.5
	BRCA2	48	20.3
	CHEK2	2	0.8
	PALB2	4	1.7
	Wildtype	80	33.8
BLBC (Ck5/14 and /or EGFR) positive (75)			
	ER/PR Positive, HER2 Negative	6	8
	ER/PR Positive, HER2 Positive	3	4
	ER/PR Negative, HER2 Positive	13	
	Triple Negative	51	
	Not tested	2	
NBLBC (Ck5/14 and /or EGFR) negative (143)			
	ER/PR Positive, HER2 Negative	80	
	ER/PR Positive, HER2 Positive	30	
	ER/PR Negative, HER2 Positive	5	
	Triple Negative	24	
	Not tested	4	
Progression-free survival (all cases)			
	Events	64	
	Median months	161.06	
Progression-free survival (BLBC)			
	Events	22	
	Median months	153.4	
Overall survival (all cases)			
	Events	60	
	Median months	165.17	
Overall survival (BLBC)			
	Events	21	
	Median months	160.8	

2.1.3 Ethics approvals

Ethics board approval was obtained as per the National Statement on Ethical Conduct in Human Research (NHMRC) for patient recruitment, sample collection and research studies at each of University of Melbourne HREC (1238391), Peter MacCallum Cancer center HREC (1/60), Australian Ovarian Cancer Study, the KConFab and Melbourne Health (2015.075).

Written informed consent was obtained from all participants for participation in research studies.

2.2 19q12/INSR ISH Automated Assay

2.2.1 Rationale

The rationale for using ISH technique rather than other methods is mainly the accuracy and precision of quantitating gene copy number in individual cells using a small amount of tissue (only 50 cells required). Furthermore, automated staining methods make dual ISH one of the most robust techniques for determining gene amplification status.

2.2.2 19q12/INSR ISH probe

ISH staining was performed using a repeat-free DNA probe developed by Ventana (Ventana Medical Systems, Tucson, Arizona) that is capable of detecting single gene copies in formalin-fixed paraffin embedded tissue samples. The probe spans approximately 560 kb of chromosome 19q12 that contains the coding sequences of *CCNE1* and *URII* (Noske, A. *et al.* 2015). The reported homology within the alpha-satellite sequences of chromosomes 1, 5 and 19 has previously created difficulties in the specific identification of chromosome 19 (Noske, A. *et al.* 2015, Noske A, B. S., Valtcheva N,Wagner U, Zhong Q, Bellini E, Fink D, . Obermann E. C, Moch H, Wild P.J 2016). Therefore, a second probe free of repetitive DNA sequences was developed

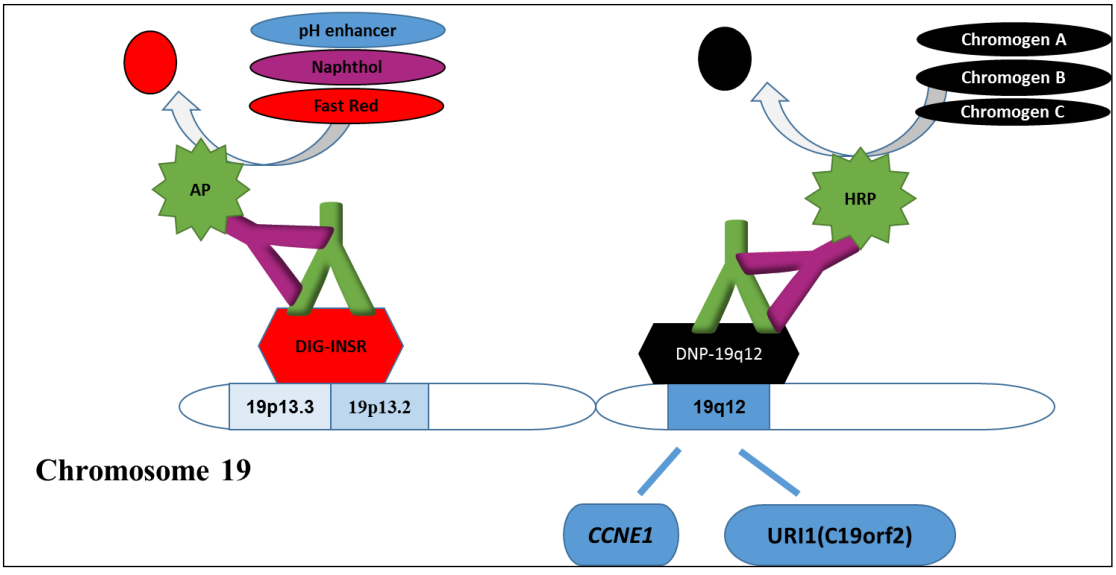

Figure 2-1: Schematic diagram of chromosome 19 highlighting the positions of the dual ISH probes (19q12 and INSR) and the dual chromogenic detection; adapted from techniques of signals (adapted from Noske *et al* 2015 and Inform HER2 Dual ISH DNA Probe cocktail assay training lesson video, lesson 1.

by Ventana to enable chromosome 19 copy number evaluation (Noske, A. *et al.* 2015). The second probe hybridizes to a span of approximately 600 kb within the long arm of chromosome 19, 19p13.2 and 19p13.3 which includes the coding sequences for insulin receptor, INSR (Noske, A. *et al.* 2015). The 19q12 probe is labeled with dinitrophenol (DNP) to detect the number of gene copies by silver *in situ* hybridization to sections from FFPET. The INSR probe is labeled with digoxin (Dig) that can be detected by Fast Red (Figure 2- 1).

2.2.3 Automated ISH staining protocol

Using the Ventana Bench Mark Ultra automated staining platform, the AOCS and KConFab TMAs were stained as follows: four micron sections from formalin-fixed, paraffin-embedded (FFPE) TMAs were baked at 63°C for 20 minutes. Sections were then deparaffinised at 69°C for 16 minutes. Next, sections were treated with cell conditioning 2 (CC2) at 82°C for four cycles of 12 minutes each, followed by ISH protease 3 (Ref 780-4149) treatment for 24 minutes. The next step was 19q12 DNP and INSR DIG probes co-incubation for 8 minutes, followed by co-denaturation at 80° C and then 19q12 DNP and INSR DIG probes were co hybridized at 47°C for 6 hours and washed for 3 cycles at 68°C for 8 minutes. These steps were followed by detection of DNP and DIG signals by Ventana UltraView^{TM} SISH DNP (Ref 760-098) and Ventana UltraView^{TM} RED ISH DIG (Ref 760-505) detection kits respectively, followed by counterstaining with haematoxylin II (Ref 790-2208) and bluing reagent (Ref 760-2037).

2.2.4 Assessment of 19q12/INSR ISH stained sections

First, the presence of tumour tissue was confirmed with Haematoxylin and Eosin staining for each TMA core. Samples were then evaluated for acceptable staining including the presence of appropriate signals in normal cells, adequate signal strength, and low background.

The Ventana dual colour 19q12 DNP/INSR DIG ISH assay uses the same chemistry, detection reagents and automated staining platform as the Ventana INFORM™ *HER2* Dual ISH DNA Probe Cocktail Assay (Grogan, T. M. *et al.* 2010), and therefore has similar analytical performance characteristics and was optimised for use on the Ventana ULTRA™ platform. A section of ovarian cancer known to be *CCNE1* amplified was included in each staining cycle as a positive control. Additionally, and as per Ventana recommendations, staining of non-neoplastic cells whether stromal or inflammatory was used as an internal positive control. The sections were reviewed to identify tumor tissue with acceptable staining for both 19q12 (silver signal) and INSR (red signal). The number of red and silver signals were assessed in the nuclei of each of 50 cells per TMA core divided as 17, 17 and 16 cells distributed over three different high power The scoring was done blindly to the clinical and pathological history of patients. Following scoring of 50 cells the average of 19q12 copy number (silver signals) was divided by the average of INSR copy number (red signals) to establish the fields (Figure 2-2). 19q12/INSR ISH ratio. For cases with more than one assessable core, the average between replicates was used.

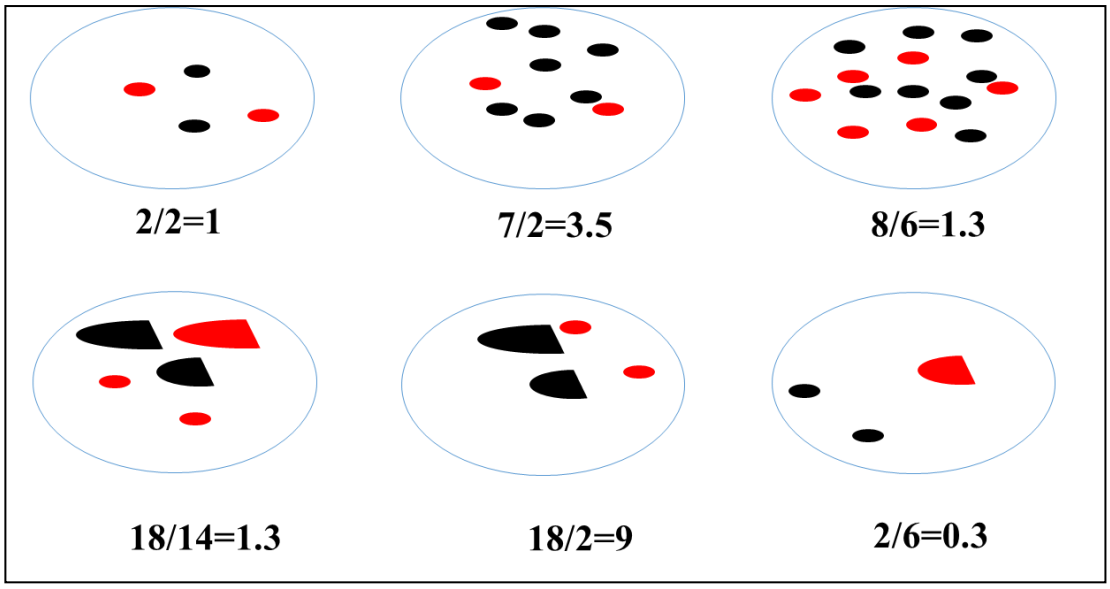

Figure 2-2: Schematic examples of dual ISH signals scoring; black 19q12 (*CCNE1)*, Red: INSR, small size dots=one copy, medium size dots=cluster of 6 copies, large size dots =cluster

2.3 Detection of cyclin E1, URI1, FBXW7, USP28, and cyclin E1 T62 protein expression by IHC

The expression of each of cyclin E1, URI1, FBXW7, USP28 and cyclin E1 T62 was assessed by optimised automated IHC assay. The optimised automated IHC protocol used the Ventana Bench Mark Ultra automated staining platform and the Optiview detection kit for all the antibodies. The protocol was adjusted according to the optimal staining conditions detailed in Table 2-3. The assay in summary included the following: four micron sections from the TMAs of the studied cohort were deparaffinised by heating to 72°C then treated with cell conditioning 1 or 2 at 100°C for certain duration. Afterwards, sections were blocked by pre-primary peroxidase inhibitor (if applicable) followed by application of pre-diluted antibody at 36°C and incubation for the recommended incubation time. Sections were then counterstained with haematoxylin II and bluing reagent. A positive control for each assay was included in each of the staining cycles. The expression of each protein was interpreted as brown nuclear, nuclear membrane or cytoplasmic stain. For cases with more than one assessable core, the average between replicates was used.

2.4 Detection of cyclin E1 expression by IHC

2.4.1 Rationale

The 19q12/INSR dual ISH probe scans *CCNE1* as well as the neighboring gene, *URI1* within the same locus. For this reason, protein expression from the *CCNE1* gene was assessed by cyclin E1 antibody and correlated to 19q12 amplification status.

2.4.2 Cyclin E1 antibodies

Two cyclin E1 antibodies were tested for their staining quality, robustness and reproducibility. The cyclin E1 Antibody (HE12): sc-247, is a mouse monoclonal IgG_{2b} (kappa light chain) provided at 200 µg/ml by Santa Cruz Biotechnology, California, and was successfully optimised. The second antibody, cyclin E1 HE12 (sp-147), Spring Bioscience, Inc, failed optimization and was therefore omitted.

The cyclin E1 Antibody (HE12): sc-247 antibody was optimized for use at a 1:100 concentration. Antibody Diluent (catalogue number 251 – 018, Ventana Medical Systems, Inc.), which is a buffered, proteinaceous solution used to dilute rabbit and mouse antibodies optimized for use on VENTANA automated staining machines, was used for antibody solution preparation.

Table 2-3: Summary of the automated staining protocols for the used antibodies panel

Protein assay	Cohorts studied	CC type	CC duration (min)	PPP	AB used	AB Concentration	AB incubation (min)	Signal location	Positive control
Cyclin E1	AOCS & KConFab	CC1	56	Yes	Cyclin E1 (HE12) Sc-247	1:100	16	Nucleus	Ovarian Cancer
URI1	AOCS	CC1	56	Yes	URI (1–21, VENTANA)	Pre diluted	16	Cytoplasm	Ovarian Cancer
FBXW7	AOCS & KConFab	CC1	32	No	FBXW7-(Sp-237)	1:25	32	Nucleus, nuclear membrane	Cervix
USP28	AOCS & KConFab	CC1	32	No	USP28 rabbit polyclonal (HPA006778)	1:50	28	Nucleus	Colorectal Cancer
Cyclin E1 T62	KConFab	CC2	32	Yes	Cyclin E1 T62 rabbit polyclonal (4136)	1:50	40	Nucleus	Ovarian Cancer

CC: cell conditioning, AB: antibody, min: minutes, CC1: cell conditioning, CC2: cell conditioning 2, PPP: pre-primary peroxidase

2.5 Detection of URI1 Expression by IHC

2.5.1 Rationale

The 19q12/INSR dual ISH probe scans *CCNE1* as well as neighboring gene, *URI1* within the same locus. *URI1* protein expression was assessed by URI1 antibody and correlated to 19q12 amplification status.

2.5.2 URI1 antibody

Pre diluted rabbit monoclonal antibody specific for URI1 (1–21, VENTANA) was tested for its staining quality, robustness and reproducibility by the Ventana Medical system and at the University of Melbourne. Sections from AOCS TMAs were stained for URI1 expression. The positive control of *CCNE1* amplified ovarian cancer was included in the staining cycle. The expression of URI1 was interpreted as brown cytoplasmic stain.

2.6. Detection of FBXW7 expression by IHC

2.6.1 FBXW7 antibodies

Three FBXW7 antibodies were tested for their staining quality, robustness and reproducibility. These are the FBXW7 (Sp-237), a rabbit monoclonal antibody, California, that was obtained from Spring Bioscience, Inc. California, and was successfully optimised and validated for use. The other two antibodies that failed optimisation and/ or validation were FBXW7 mouse monoclonal antibody (M02), clone 3D1 (H00055294-M02), IgG_{2a} (kappa), Abnova, Taiwan, and FBXW7 mouse monoclonal antibody [3D1] (ab128062), IgG_{2a} (kappa), Abcam, Cambridge, UK.

2.62 Automated FBXW7 IHC staining protocol optimization

The automated FBXW7 IHC assay was optimized following trials of different protocols using different antibody concentrations (1:100, 1:25), incubation periods (16, 32 minutes). FBXW7- (Sp-237) antibody was optimised for use at a 1:25 concentration. Antibody Diluent (catalogue number 251 – 018, Ventana Medical Systems, Inc.), was used for antibody solution preparation. Sections from both AOCS and KConFab cohorts TMAs were assessed for FBXW7 expression by IHC staining using the automated optimised FBXW7 IHC assay. The positive control of cervix was included in the staining cycle. The expression of FBXW7 was interpreted as brown nuclear and nuclear membrane stain.

2.6.3 FBXW7 antibody specificity validation

The specificity of the FBXW7 rabbit monoclonal, (SP-237) (Spring Bioscience, CA antibody was tested by assessing FBXW7 expression using the optimised automated IHC assay to stain sections from cell blocks of both FBXW7 and FBXW7$^{-/-}$ HCT116 human colorectal cancer cell lines.

2.6.3.1 FBXW7 wildtype and FBXW7 -/- HCT116 cell blocks

Human colon cancer cells HCT 116 cell line was a gift from Dr Fred Hollande (Melbourne University) and HCT116 FBXW7$^{-/-}$ cell line was a gift from Dr. Bert Vogelstein (Rajagopalan, H. *et al.* 2004) via Dr. John Mariadson (Olivia Newton John Cancer Centre, Melbourne).

In a laminar flow cell culture hood and using sterile techniques, HCT116 and FBXW7 -/- HCT116 cells were thawed by the addition of 9 ml of warmed antibiotic free McCoy's 5A with L-Glutamine media to each (Life Technologies, California, and USA) with 10% fetal bovine serum in a 15 ml tube. The tube was centrifuged at 1500 round per minute (rpm) for 5 minutes. The supernatant was discarded, the pellet resuspended in 10 ml of fresh media, placed in a T75 flask and incubated in a humidified incubator at 37 °C and 5% CO2 for 7 days. Cells were washed with phosphate-buffered saline (PBS), treated with 1 ml trypsin, a proteolytic enzyme which dissociates adherent cells by breaking down proteins that enable the cells to adhere to their culture flask, for 2-3 minutes. Next, 9ml of the culture media was added and aspirated into 15ml tube and centrifuged at 1800 rpm for 5 minutes. The supernatant drained and the freshly cultivated cell pellets was fixed in 4% paraformaldehyde for 2 hours. Next, cells in formalin were then centrifuged at 1800 rpm for 5 minutes, the supernatant discarded and the cell pellet was placed in a liquefied (microwave heated for 1-2 seconds) nutrient agar. The pellet-containing agar was left to solidify, dislodged by formalin, placed in a pre-labelled tissue cassette and then embedded in paraffin blocks. Sections from FBXW7 wildtype and FBXW7$^{-/-}$ HCT116 cell block were then cut from FFPE cell pellets and stained for FBXW7 using the optimised automated staining protocol.

Sections from FBXW7$^{-/-}$ HCT116 cell block had negative FBXW7 expression compared to sections from FBXW7 wildtype HCT116 cell block that had moderate or high expression confirming the specificity of the optimised FBXW7 antibody assay (Figure 2-3). Next, the protocol was validated and used to stain sections form both AOCS and KConFab Cohorts TMAs.

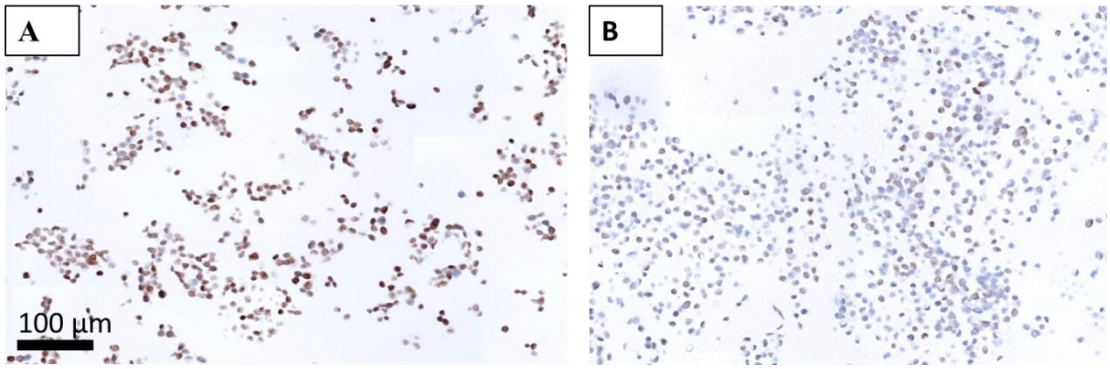

Figure 2-3: FBXW7 IHC assay specificity validation; FBXW7 IHC staining of 4 micron section of FFPE cell blocks of **A:** FBXW7 wildtype and **B:** FBXW7$^{-/-}$ HCT-116 cell-lines. Fixation time 2hours, Antibody used FBXW7 Sp237 in 1:25 concentration, cell conditioning 1 for 32 minutes and incubation time for 32 minutes.

2.7 Detection of USP28 expression by IHC

2.7.1 USP28 antibody

A rabbit polyclonal USP28 antibody (HPA006778, Sigma Aldrich) was tested for its staining quality, robustness and reproducibility: and was successfully optimised and validated for use at a 1: 50 concentration. Antibody Diluent (catalogue number 251 – 018, Ventana Medical Systems, Inc.).

2.7.2 Automated USP28 IHC staining protocol optimization

The automated USP28 IHC assay was optimized following trials of different protocols using different antibody concentrations (1:150, 1:100, 1:50), incubation periods (4, 16, 24, 28, 32 minutes). The assay was optimised using the antibody at 1:50 concentration and 28 minutes incubation period. Sections from both AOCS and KConFab cohorts TMAs were assessed for USP28 expression by IHC using the automated optimised USP28 antibody IHC assay. The positive control of colorectal cancer was included in the staining cycle. The expression of cyclin E1 was interpreted as brown nuclear stain.

2.7.3 USP28 antibody specificity validation

The specificity of the USP28 rabbit polyclonal (HPA006778) (Sigma Aldrich) antibody was assessed by comparing the expression of USP28 knocked down MCF7 cell lines compared to untreated cell lines by both qPCR and IHC staining of sections from FFPE cell blocks of both USP28 knocked down and untreated MCF7 cell lines.

2.7.3.1 DharmaFECT™ siRNA transfection protocol

Cell Plating: MCF7, human breast adenocarcinoma, HTB-22 was obtained from the American Type Culture Collection (ATCC, Virginia, United States). According to DharmaFECT™ siRNA transfection protocol, the optimal cell density required for MCF7 cells in 96 wells plate is 1 x 10^4 per well. In order to estimate the required seeding density in 12 wells plate, the seeding density was multiplied by the surface area of 12 wells plate and divided by the surface area of 96 wells plate 0.3 cm^2/well, and is equal to 13 x 10^4 per well.

MCF7 cells were revived by the addition of 9 ml of warmed antibiotic free DMEM media (Life Technologies, California, and USA) with 10% fetal bovine serum in a 15 ml tube in a laminar flow cell culture hood and using sterile techniques. The tube was centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, the pellet resuspended in 10 ml of fresh media, placed in a T75 flask and incubated in a humidified incubator at 37 °C degree and 5% CO2 for 3-5 days.

In order to obtain the required cell density, cells were washed with PBS, treated with 1 ml trypsin, for 2-3 minutes. This was followed by the addition of 1ml of the DMEM culture media, of which 10µL was aspirated into Hemocytometer and cells were counted under an inverted microscope. Cells were plated at 2 different densities in two 12 wells plate , 5 x10^4 and 10 x 10^4 in 700 µL antibiotic free DMEM media+10% fetal bovine serum per well of a 12-well plate for qPCR and cell block. Next cells plates were incubated in humidified incubator at 37°C with 5 % CO_2 overnight.

siRNA Transfection Reagents: Transfection of MCF7 cells was performed using ON-TARGET plus SMARTpool 5 nmol L-006076-00-0005, Human USP28 (57646) and DharmaFECT Transfection reagent (Millennium Science Pty Ltd, Vic., Au), *Silencer*™ Negative Control No. 1 siRNA 100µl/50µm, AM4611 (Thermofischer Scientific, Massachusetts, United States) and the recommended protocol.

Solution preparation: all steps were performed in a laminar flow cell culture hood using sterile techniques. siRNA resuspension protocol was used for siRNA solution

preparation as following: tubes containing Silencer™ Negative Control No. 1 siRNA and ON-TARGET plus SMARTpool, Human USP28 siRNA were briefly centrifuged to ensure that the siRNA pellets are collected at the bottom of their containing tubes. Next, Negative control and USP28 siRNA containing tubes were re-suspended in 900µl and 1000 µl of sterile RNase-free water respectively for the desired final concentration of 5µm. Solutions were then gently pipetted up and down 5 times. Next, securely sealed solution tubes were then placed mixer/shaker for 30 minutes at room temperature. siRNA containing tubes were next briefly centrifuged to ensure that the solution is collected at the bottom of the tube and then aliquoted into smaller volumes to limit the number of freeze-thaw cycles and stored at -20 °C.

The USP28 siRNA (Tube 1) and the DharmaFECT transfection reagent (Tube 2) were diluted in separate tubes with serum-free DMEM medium as 1 tube for each well as below:

- Tube 1: 100 µL volume of the siRNA was prepared in serum-free medium by adding 5 µL of 5 µM siRNA to 95 µL of serum free DMEM medium.
- Tube 2: 100 µL volume of diluted DharmaFECT transfection reagent was prepared in serum-free DMEM medium. The DharmaFECT reagent amount used varied between 1 and 9 µL (Table 1) as per the recommended volume of DharmaFECT reagent based on cell line type and seeding density, the higher the density the higher the volume of DharmaFECT transfection reagent.

The contents of each tube were gently mixed by pipetting carefully up and down and then incubated for 5 minutes at room temperature. The contents of Tube 1 were added to Tube 2, for a total volume of 200 µL, mixed by pipetting carefully up and down and incubated for 20 minutes at room temperature.

Cell transfection: each experiment included untreated cells, USP28 siRNA and negative control siRNA in triplicate.

The viability of cells in cell plates was confirmed under an inverted microscope. The culture medium was then removed from the wells of the 12 well plate. Cells were transfected with the 200 mix of the corresponding transfecting reagent and siRNA and the volume was completed to 1000 µl via the addition of 800 µl of the antibiotic free DMEM only medium to each well. Lastly, cells were incubated in a humidified incubator at 37°C in 5% CO2 for 24 hours, after which the media was removed from each well and discarded and a 1 ml/per well of antibiotic free complete medium

(DMEM+10% fetal bovine serum) was added in order to reduce cytotoxicity. The following day cells were scraped and placed with their media into 2 pre-labeled corresponding Eppendorf tubes per well, for qPCR and cell block. Tubes were then centrifuged at 3000 g for 3 minutes, the supernatant was discarded and the pellets were frozen at-80 °C for later use accordingly.

2.7.3.2 Quantitative Real-Time PCR (RT-qPCR)

qPCR primers: USP28 forward primer 5′-GCA GCC CTG GAT CTA TTA AAG G-3′and reverse primer 5′- GAA TGC GTC CTC TAG CCA AT-3′ and GAPDH forward primer 5′-CTC TCT GCT CCT CCT GTT C -3′, and reverse primer 5′-GCG CCC AAT ACG ACC AA-3′.

RNA extraction: The total RNA was next isolated from both freshly cultivated USP28 wildtype and USP28- knocked down MCF7 cell lines using high pure RNA isolation kit (Roche, NSW, AU) as per the below protocol. The protocol was repeated for each of the USP28 wildtype and USP28- knocked down MCF7 cell lines. Cells were re-suspended in 200 µl PBS to which 400 µl lysis/binding buffer was added and mixed via vortex for 15 seconds. For each of the two cell lines and in order to transfer samples to a high pure filter tube, one high pure filter tube was inserted into one collection tube and the entire sample was pipetted into the upper reservoir of the filter tube (max. 700 µl). The entire high pure filter tube assembly was then inserted into a standard table-top centrifuge and centrifuged at 8,000 × g for 15 seconds. After centrifugation, the filter tube was removed from the collection tube; the supernatant was discarded, and the filter tube and the used collection tube were re combined. After re-inserting the filter Tube and for each sample, 90 µl DNase I incubation buffer was pipetted into a sterile reaction tube to which 10 µl DNase I was added and mixed. The solution was next pipetted onto the glass fiber fleece in the upper reservoir of the filter tube and incubated for 15 minutes at room temperature (15 to 25°C). Next, the first wash was done by the addition of 500 µl wash buffer I to the upper reservoir of the filter tube assembly and centrifuged at 8,000 × g for 15 seconds. The supernatant was then discarded and the filter tube and the used collection tube were re combined. The second wash involved the addition of 500 µl wash buffer II to the upper reservoir of the filter tube assembly and centrifuged at 8,000 × g for 15 seconds. The supernatant was then discarded and the filter tube and the used collection tube were re combined. last wash was done by adding 200 µl wash buffer II to the upper reservoir of the filter tube assembly and

centrifuge at maximum speed (approx. 13,000 × g) for approximately 2 minutes to remove any residual wash buffer. The extra centrifugation time was recommended by Roche to ensure removal of residual wash buffer. Next, the collection tube was discarded and the filter tube was inserted into a clean, sterile 1.5 ml micro-centrifuge tube. The last step involved the addition of 50 – 100 µl elution buffer to the upper reservoir of the filter tube in order to elute the RNA. The tube assembly was then centrifuged at 8,000 × g for one minute. The eluted, purified RNA was then stored at –80°C for later analysis.

cDNA Synthesis: Extracted RNA was reversely transcribed by SensiFAST™ cDNA Synthesis Kit (Bioline, NSW, AU) and the recommended protocol. qRT-PCR was used to determine mRNA expression using SensiFAST SYBR® No-ROX Kit (Bioline, NSW, AU). The mastermix was prepared on ice and all solutions were mixed by Vortex and centrifuged briefly before use. Extracted RNA was measured by Nano dropping. The mastermix was prepared on ice and all solutions were mixed by Vortex and centrifuged briefly before use. Extracted RNA was quantitated by on a Nanodrop (insert company). Next, the mastermix was prepared as following, Total RNA (up to 0.5 µg) n µl + 2 µl of 5x TransAmp Buffer + 0.5 µl Reverse Transcriptase + DNase/RNase free-water up to 10 µl. The mastermix was mixed gently by pipetting. The thermal cycler was setup as following: 25 °C for 10 minutes (primer annealing), 42 °C for 15 minutes (reverse transcription), 85 °C for 5 minutes (inactivation) and 4 °C hold (or chill on ice).

The RT-qPCR was run on a Lightcycler ^{R} 480 instrument, Roche as following: A new experiment under monocolor hydrolysis probe and a reaction volume of 10µl were selected. Three cycles were then added that included the following: 1st pre-incubation (polymerase activation) at 95°C for 2 minutes. The 2nd step was amplification and included: denaturation at 95°C for 30 seconds, annealing at 60°C for 10 seconds and extension at 72°C for 10 seconds. The third step is cooling 40°C continuous and melting curve selected. The data was next saved for analysis in AbsQuant, wells were selected, calculated and then exported to excel sheet for DNA copy number assessment.

Assessment of DNA copy numbers: The reference gene used was GAPDH. The relative level of gene expression was determined by 2^- ΔΔCt (Sample (ΔCt) – Control (ΔCt)). Experiment was repeated in duplicates and revealed 3.5 fold higher expression

of USP28 in USP28 wildtype cell lines compared to USP28 knocked out cell lines (Figure2-4 (c)).

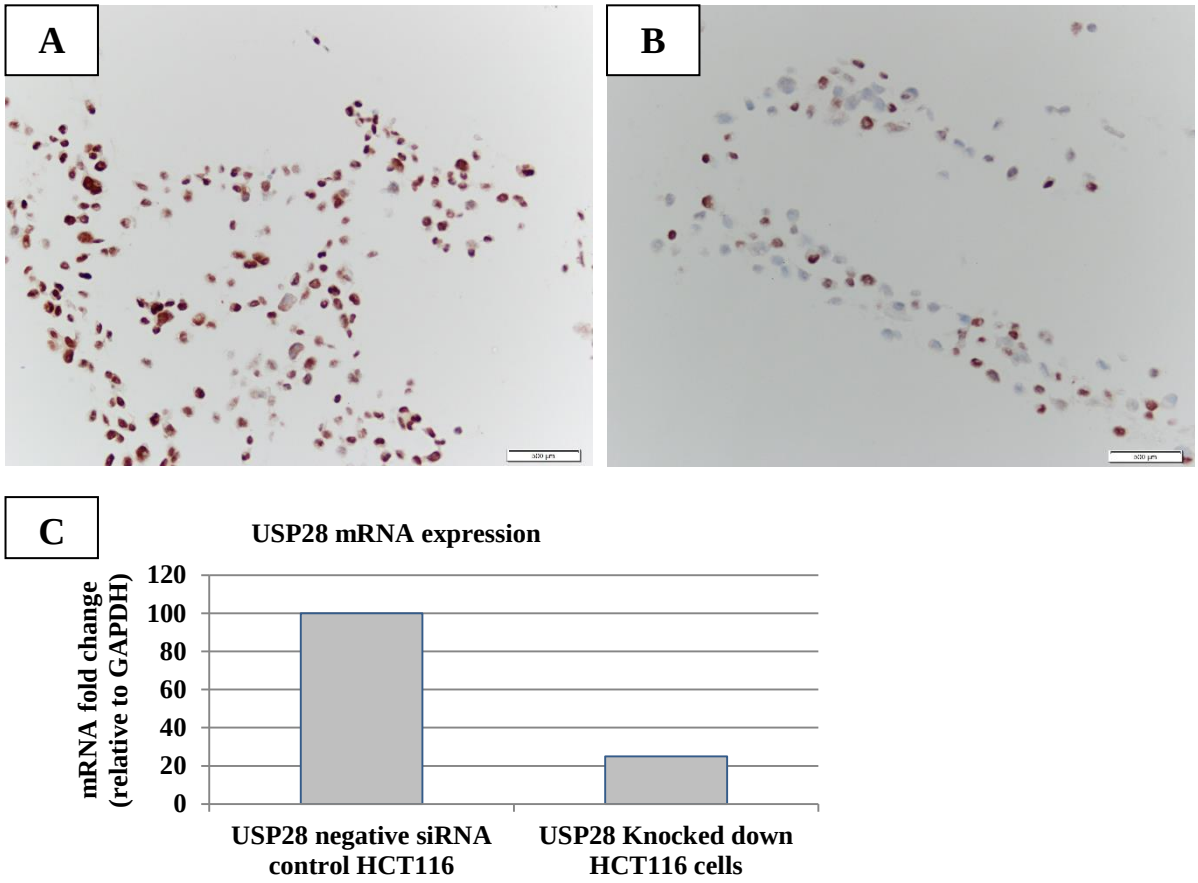

Figure 2-4: USP28 IHC assay specificity validation: USP28 IHC staining of 4 micron section of FFPE cell blocks of **A:** untreated and **B :**USP28 siRNA knocked down MCF7 cell-lines; fixation time 2 hours, the antibody used USP28 rabbit polyclonal (HPA006778) in 1:50 concentration, cell conditioning 1 for 32 minutes and incubation time for 28 minutes. **C:** Bar graph showing fold changes in USP28 mRNA expression in untreated and USP28 knocked down cells (RT- qPCR)**.**

2.7.3.3 Untreated and USP28 siRNA knocked down MCF7 Cell Blocks

Cell pellets from both untreated and USP28 siRNA treated cell lines were re-suspended in 1 ml of culture media and were added to 9ml of the culture media in 15ml tube and centrifuged at 1800 rpm for 5 minutes. The supernatant was drained and the freshly cultivated cell pellets were fixed in 4% para formaldehyde for 2 hours. Next, cells in formalin were centrifuged at 1800 rpm for 5 minutes, the supernatant discarded and the cell pellet was placed in a liquefied (microwave heated for 1-2 seconds) nutrient agar. The pellet-containing agar was left to solidify, dislodged by formalin, placed in a pre-labelled

tissue cassette and then embedded in paraffin blocks. Sections from untreated and Knocked down MCF7 cell block were then cut from FFPE cell pellets and stained for USP28 using the optimised automated staining protocol.

2.8 Detection of Phospho-Cyclin E1 (T62) Expression by IHC

2.8.1 Rationale

FBXW7 mediated degradation of cyclin E1 requires cyclin E1 phosphorylation at multiple sites of which only T62 phosphorylation site is assessable by IHC.

2.8.2 Phospho-Cyclin E1 (T62) Antibody

A rabbit polyclonal cyclin E1 T62 antibody (Catalogue number 4136) was obtained from Cell Signaling Technology, Massachusetts, United States. Cyclin E1 T62 epitope is a phospho epitope that can easily be lost in FFPET tissue in non-ideal fixation conditions. Therefore, cyclin E1 T62 antibody was tested for its stability and robustness within various fixation times. In addition, cyclin E1 T62 antibody was tested for its staining quality and reproducibility and specificity.

2.8.3 Automated cyclin E1 T62 IHC staining protocol optimization

The automated cyclin E1 T62 IHC assay was optimized following trials of different protocols using different antibody concentrations (1:100, 1:50), incubation periods (24, 40 minutes) and cell conditioning 2 (24,32,64 minutes). The assay was optimised using the antibody at 1:50 and incubation for 40 minutes. Sections from TMAs of the KConFab cohort were assessed for cyclin E1 T62 expression by IHC using the optimised automate assay. Positive control from *CCNE1* amplified ovarian cancer was included in the staining cycle. The expression of cyclin E1 T62 was interpreted as brown nuclear stain.

2.8.4 Cyclin E1 T62 antibody validation

In order to confirm the robustness and the stability of cyclin E1 T62 antibody detection of protein under different fixation conditions we used paraffin embedded cell blocks of HCT116 colorectal cancer cell lines that were **v**ariably fixed in formalin at variable conditions. These cell blocks were a kind gift from Dr. Danielle Ferraro. Different fixation conditions included both variable time to fixation (TTF), between pellet stage and 4% PFA addition; and variable time of fixation (TOF), the duration for which the pellet was fixed in 4% PFA. Sections from all variably FFPE cell blocks were stained using the optimized protocol and revealed acceptable robustness of detection (Figure 2-5 A).

In order to assess the specificity of cyclin E1 T62 antibody, MDA-MB-436 cells were treated with cyclin E1 siRNA and confirmed for cyclin E1 knockdown by western blot. Knocked down and untreated MDA-MB-436 cells were prepared and embedded in cell blocks by Ms Christine Lee of Dr Liz Caldon's laboratory (Replication and Genome Stability Group, Garvan Institute, Sydney). Next, sections from FFPE blocks of each of the cyclin E1 knocked down and untreated cells were stained with the optimized protocol and revealed aligned protein expression to those seen in western blot (Figure2-5B). As those experiments revealed the stability, reliability and specificity of cyclin E1 T62 antibody, the antibody was next used to stain sections from TMAs of samples of patients enrolled in the KConFab cohort.

2.9 Assessment of cyclin E1, URI1, FBXW7, USP28 and cyclin E1 T62 IHC stained sections

First, the presence of tumor tissue was confirmed with Haematoxylin and Eosin stained slides for each TMA core. Samples were then evaluated for acceptable staining including the presence of appropriate staining quality and low background. Next, sections were scored according to a semi-quantitative scoring systems called H score, which takes into consideration the staining intensity of the nuclei in conjunction with the percentage of cells that stained positive. It is measured as follows:

3 x % of strongly staining nuclei + 2 x % of moderately staining nuclei + % of weakly staining nuclei, to give a range of 0 to 300 (McCarty, K. S. *et al.* 1986). For cases with more than one assessable core, the average H score between replicates was used.

2.10 Statistics

All statistics were performed on Prism software. Non parametric Spearman correlation was used to assess correlations between variables. Fisher exact test was used for the contingency analysis. T test was used to assess the difference between means of expression as a nonparametric variable. Kaplan Meier curve and Log-rank Mantel Cox test were used to assess the difference between survival curves and the impact of the studied biomarkers on overall survival and Progression free survival.

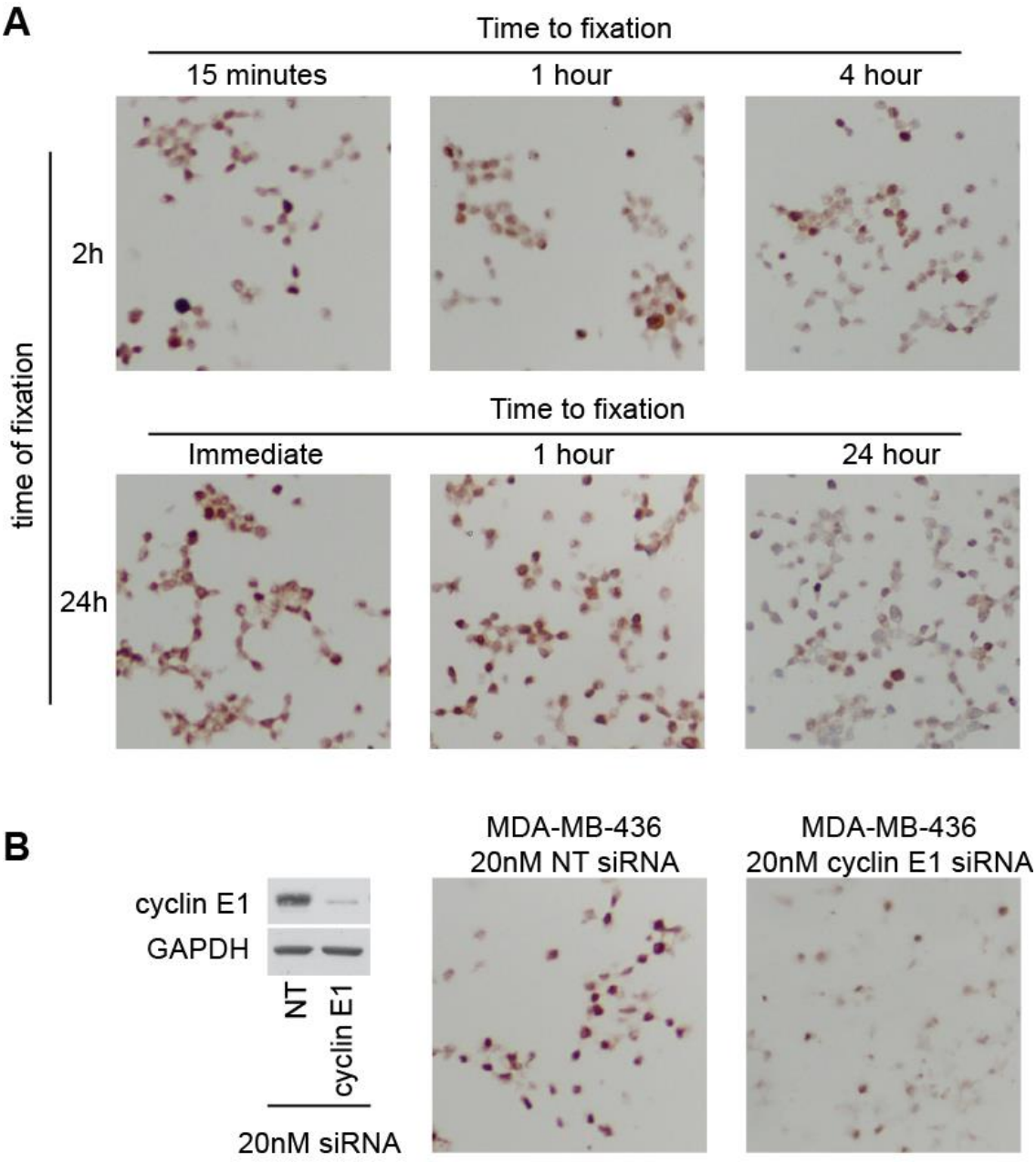

Figure 2-6: cyclin E1 T62 antibody validation **A:** Stability at different times to and of fixations in 4% formaldehyde **B:** Specificity validation: WB confirming the knockdown of cyclin E1 by siRNA in MDA-MB-436 cell lines (left), cyclin E1 T62 IHC staining of sections from FFPE cell blocks of untreated (middle) and cyclin E1 siRNA knocked down (right) MDA-MB-436 cell lines, fixation time prior to embedding in paraffin is 2 hours, the antibody used cyclin E1 T62 rabbit polyclonal (4136) in 1:50 concentration, cell conditioning 2 for 32 minutes and incubation time for 40 minutes.

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Chapter 3: The significance of cyclin E1 deregulation in HGSOC

Preface

The aim of this chapter was to investigate the prevalence and the clinical significance of cyclin E1 overexpression in HGSOC by assessing their impact in the presence and absence of amplification in a cohort of HGSOC from patients enrolled in the AOCS. This chapter is published in the Gynaecologic Oncology Journal, as per the following reference:

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19q12 amplified and non-amplified subsets of high grade serous ovarian cancer with overexpression of cyclin E1 differ in their molecular drivers and clinical outcomes

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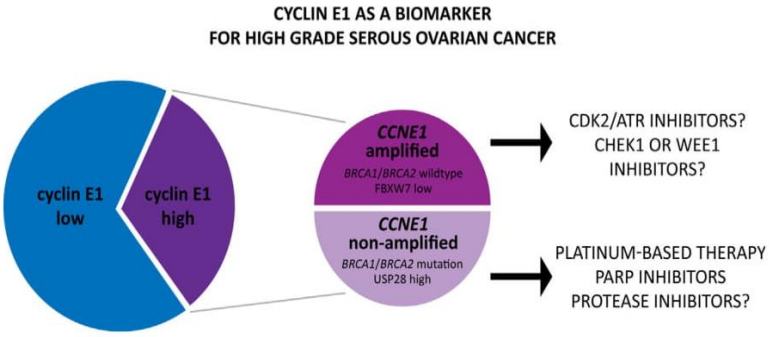
HIGHLIGHTS

- Cyclin E1^{hi} /non-amplified HGSOC can be either *BRCA1/2* wildtype or mutated.
- Cyclin E1^{hi} /non-amplified HGSOC have better outcomes than Cyclin E1^{hi} /amplified cases.
- High USP28 expression is a likely driver of high cyclin E1 in non-amplified HGSOC.
- Low FBXW7 expression is likely to augment high cyclin E1 in amplified HGSOC.
- Chromosomal instability is significantly higher in cyclin E1^{hi} /amplified cases.

GRAPHICAL ABSTRACT



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ABSTRACT

Objectives. Readily apparent cyclin E1 expression occurs in 50% of HGSOC, but only half are linked to 19q12 locus amplification. The amplified/cyclin E1^{hi} subset has intact *BRCA1/2*, unfavorable outcome, and is potentially therapeutically targetable. We studied whether non-amplified/cyclin E1^{hi} HGSOC has similar characteristics. We also assessed the expression of cyclin E1 degradation-associated proteins, FBXW7 and USP28, as potential drivers of high cyclin E1 expression in both subsets.

Methods. 262 HGSOC cases were analyzed by in situ hybridization for 19q12 locus amplification and immunohistochemistry for cyclin E1, URI1 (another protein encoded by the 19q12 locus), FBXW7 and USP28 expression. Tumors were classified by 19q12 amplification status and correlated to cyclin E1 and URI1 expression, *BRCA1/2* germline mutation, FBXW7 and USP28 expression, and clinical outcomes. Additionally, we assessed the relative genomic instability of amplified/cyclin E1^{hi} and non-amplified/cyclin E1^{hi} groups of HGSOC datasets from The Cancer Genome Atlas.

Results. Of the 82 cyclin E1^{hi} cases, 43 (52%) were amplified and 39 (48%) were non-amplified. Unlike amplified tumors, non-amplified/cyclin E1^{hi} tumor status was not mutually exclusive with *gBRCA1/2* mutation. The

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non-amplified/cyclin E1^{hi} group had significantly increased USP28, while the amplified/cyclin E1^{hi} cancers had significantly lower FBXW7 expression consistent with a role for both in stabilizing cyclin E1. Notably, only the amplified/cyclin E1^{hi} subset was associated with genomic instability and had a worse outcome than non-amplified/cyclin E1^{hi} group.

Conclusions. Amplified/cyclin E1^{hi} and non-amplified/cyclin E1^{hi} tumors have different pathological and biological characteristics and clinical outcomes indicating that they are separate subsets of cyclin E1^{hi} HGSOC.

1. Introduction

Amplification of *CCNE1* is one of very few molecular therapeutic targets in high grade serous ovarian cancer (HGSOC), occurring in 15–30% of HGSOC cases [1,2]. This subset typically has intact homologous recombination repair pathways (HR), as *CCNE1* amplification is mutually exclusive with germline *BRCA1* or *BRCA2* mutation [2]. *CCNE1*-amplified tumors respond poorly to platinum therapy and have limited responsiveness to PARP inhibitors [2,3], which has focused efforts on developing targeted therapy for this particular subgroup [4].

CCNE1 is located on chromosome 19q12, within a 0.2 Mb cluster of five tightly co-regulated genes, including *C19orf2* (*URI1*) [5]. Cyclin E1, the cell cycle protein encoded by *CCNE1*, is a master regulator of progression through G_1/S phase and centrosome duplication. Overexpression of cyclin E1 drives cell cycle progression and correlates with platinum-resistance [5,6]. In ovarian cancer cells, suppression of *CCNE1* results in G_1 arrest, reduced cell viability and apoptosis but only when cells are 19q12 amplified. This suggests that cyclin E1 is the main driver oncogene of 19q12 amplification [5], although there is evidence that URI1-amplified ovarian cancer cells can be dependent on URI1 function for their survival [7].

High cyclin E1 expression occurs in up to 50% of HGSOC cases [8]. In both HGSOC and other tumor types it is apparent that high expression can occur in the absence of 19q12 amplification [8–11]. To date the studies on high cyclin E1 expression as a molecular subtype of HGSOC have been almost exclusively in the context of *CCNE1* amplification, and it is not known whether tumors with cyclin E1^{hi} in the absence of 19q12 amplification have the same clinical and biological features. Understanding the subsets of cyclin E1 over expressing tumors that may be targeted is important as clinical trials advance [12], especially given that in vitro studies suggest a requirement for *CCNE1* amplification for therapeutic efficacy [5,13].

Apart from amplification, disruption of degradation is one of the major mechanisms driving high cyclin E1 protein in cancer. In normal cell cycles the cyclin E1 protein is proteosomally degraded during the cell cycle [14]. The FBXW7 subunit of the SCF^{FBXW7} ubiquitin ligase complex binds and ubiquitinates phosphorylated cyclin E1 during S phase, marking it for degradation [15,16]. Loss-of-function *FBXW7* mutations lead to cyclin E1 stabilization but are infrequent in HGSOC [17]. However, FBXW7 can be inactivated by other mechanisms such as antagonism by the deubiquitinase USP28. This occurs through two mechanisms, loss of USP28 leads to FBXW7 autoubiquitination and degradation, or high USP28 will deubiquitinate and stabilize FBXW7-target proteins, including cyclin E1 [18–20].

In this study we address the prevalence of high cyclin E1 expression in HGSOC and its relationship to 19q12 amplification, *BRCA1/2* status, genomic stability and patient outcome. We address for the first time the expression of cyclin E1 degradation-associated proteins in relation to cyclin E1 expression with or without *CCNE1* amplification. These investigations reveal that amplified/cyclin E1^{hi} and non-amplified/cyclin E1^{hi} are non-equivalent subsets of HGSOC, with distinct associations to *BRCA* status, FBXW7 and USP28 proteins, genomic instability and patient outcome.

2. Methods

2.1. Tumor samples

Seven tissue microarrays (TMA) representing 341 cases of HGSOC were provided by the Australian Ovarian Cancer Study (AOCS), of which 262 cases were assessable in terms of sufficient tumor tissue and staining quality for immunohistochemistry (IHC) and in situ hybridization (ISH). The World Health Organization criteria were used to histologically classify ovarian cancers as HGSOC [21]. The International Federation of Gynecology and Obstetrics (FIGO)/American Joint Committee on Cancer staging system was used for tumor staging [22]. Ethics board approval was obtained at all institutions for patient recruitment, sample collection and research studies. Written informed consent was obtained from all participants for participation in research studies.

2.2. Dual-colour ISH assay for detection of the 19q12 locus amplification status

A pre-diluted ready to use 19q12 DNP ISH probe that covers the coding sequences of the *CCNE1* and *URI1* genes [9] and an insulin receptor (INSR) DIG ISH probe a surrogate reference for diploid copy number located on chromosome 19p13.2, were provided by Ventana Medical Systems (Tucson, AZ, USA). The assay was optimized for use on the Ventana ULTRA™ platform. Assessable cases were those with interpretable black (19q12) and red (INSR) signals in normal and malignant cells, at least 50 assessable malignant cells, and minimal background staining. Amplification status at the 19q12 locus was determined by dividing the average 19q12 copy number by the average INSR copy number for 50 tumor cells per core. Tumors with a 19q12: INSR ratio ≥ 3 and/or 19q12 locus copy number ≥ 6 were considered amplified (assessment criteria and staining technique are detailed in Supplementary methods, and Supplementary Fig. S1A–F).

2.3. Cyclin E1, URI1, FBXW7 and USP28 Immunohistochemistry

Cyclin E1 (clone sc-247-HE12) (Santa Cruz Biotechnology, CA) 1:100, and URI1 (clone 1–21) (Ventana) mouse and rabbit monoclonal antibodies (pre-diluted), FBXW7 rabbit monoclonal 1:25, which detects the three isoforms α,β and γ (SP-237) (Spring Bioscience, CA) and the USP28 rabbit polyclonal (HPA006778) 1:50 (Sigma Aldrich) antibodies were optimized using the Ventana Bench Mark ULTRA™ automated staining platform and the Optiview™ Detection kit. Cyclin E1, FBXW7 and USP28, were assessed based on nuclear staining whereas URI1 expression was assessed by cytoplasmic staining. Positive controls were included in each cycle from HGSOC for cyclin E1 and URI1, uterine cervix for FBXW7, and colorectal cancer for USP28. The expression of each of the proteins was assessed using a 0 to 3+ intensity score. A semi quantitative H score was obtained by adding 3 times the percentage of strongly staining (3+) cells plus 2 times the percentage of moderately staining (2+) cells plus 1 times the percentage of weakly staining (1+) cells, giving a range of 0 to 300. FBXW7 staining was almost exclusive of the nuclear membrane. Heterogeneous expression was captured using the semi-quantitative H score (Supplementary Methods, Supplementary Fig. S1H–I).

For both ISH and IHC, the tumors were scored by a trained and qualified observer blinded to each of the IHC results, *BRCA1/2* status and clinical outcomes. Categorization of ambiguous cases was confirmed by a pathologist.

2.4. Statistics

Statistical analysis was performed using Prism Software™ version 7. We determined the frequency and correlation of *19q12* amplification and cyclin E1 expression across our cohort using protein and copy number assessments from consecutive sections of TMAs. We developed optimized cut-offs for amplified versus non-amplified and high versus low expressers using Receiver Operator Curve (ROC) that reflected the highest sensitivity and specificity of gene/protein expression correlation (Supplementary methods, Supplementary Fig. S1F–G) as well as the best correlation with outcome. Kaplan Meier curves were used to plot the progression free survival (PFS) and overall survival (OS).

3. Results

3.1. Patient demographics

Clinical data from 262 HGSOC cases from patients enrolled in the AOCS is provided in Table 1. The age of the patients ranged between 30.2 and 80 years, with a median age of 60.1 years. The median PFS from the time of diagnosis was 14.20 months, and the median OS was 40.14 months. One case was lacking PFS and OS data. Twenty six cases were untested for *BRCA1/2* (10%) and 236 cases had documented germ-line *BRCA1* and *BRCA2* status, 41 (15.6%) with *BRCA1* mutation, 25 (9.5%) with *BRCA2* mutation and 170 (64.9%) being wild type for both.

Table 1
AOCS cohort patients' demographics

Patient characteristics	Number of cases	% of cases
Age (years)		
Mean	60.1	
Range	30.2–80	
Primary site		
Ovary	197	(75%)
Peritoneum	54	(21%)
Fallopian tube	11	(4%)
Subtype		
Serous	253	(97%)
Carcinoma (NOS)	9	(3%)
Grade		
1	0	(0%)
2	72	(27%)
3	190	(73%)
Stage		
IA-C	9	(3%)
IIA-C	15	(6%)
IIIA-C	194	(74%)
IV	37	(14%)
Unknown	7	(3%)
Residual disease		
No macroscopic	65	(25%)
≤1 cm	84	(32%)
>1 cm	88	(34%)
Tumor not resected	10	(4%)
Unknown	15	(6%)
Germline *BRCA1*/2 status		
BRCA1 mutant	41	(15.6%)
BRCA2 mutant	25	(9.5%)
Wild type	170	(64.9%)
Untested	26	(10%)
Progression-free survival		
Events	222	(85%)
Median months	14.20	
Overall survival		
Events	192	(73%)
Median months	40.14	
Total cases	262	

3.2. High cyclin E1 expression occurs independently of 19q12 amplification

19q12 (*CCNE1*) amplification and cyclin E1 expression were assessable in 262 cases. Overall, 20.2% (n = 53) of the cohort had 19q12 amplification, 13% (n = 34) had low copy number gain and the remaining 66.8% (n = 175) were disomic (Fig. 1A). Eighty one percent (43/53) of 19q12 amplified cases had high level cyclin E1 immunostaining. Only a moderate correlation was found between 19q12 (*CCNE1*) copy number and cyclin E1 expression ($r = 0.46$, $p < 0.0001$, Fig. 1A) because a third of the high expressers did not demonstrate any copy number gain of *CCNE1* and 12.1% (n = 10) had low copy number gain (Fig. 1B). Conversely 18.9% (n = 10) of amplified samples had low levels of cyclin E1 immunostaining. Therefore, while *CCNE1* amplification is consistently associated with cyclin E1 overexpression, almost half of tumors with high levels of cyclin E1 protein overexpression appears to be due to non-amplification-dependent mechanisms.

3.3. 19q12 amplification is homogenous within HGSOC samples

Clonal variation within solid cancers is common including subclonal involvement of driver mutations [23]. We determined the clonal status of *19q12* amplification to assist in validating it as a therapeutic target, and also assess whether tumors could be considered simply as amplified or non-amplified. Comparing locus copy number values in 153 cases with duplicate tissue cores revealed a highly significant correlation between the locus copy numbers ($r = 0.94$, $p < 0.0001$) (Fig. 1C). Next, we examined full-face tissue sections from multiple tumor samples of 5 amplified and 2 non-amplified cases with high cyclin E1 expression. Sections from each were stained with the 19q12 probe and the locus copy number was assessed in 50 cells in three representative regions from each tumor section. We found that amplification was consistently high (≥6 *19q12* copy number) in the full-face sections from those samples identified as having 19q12 amplified TMA cores, and that these tumors also had invariably high cyclin E1 expression (H score range: 160–270). An example of a 19q12 amplified cyclin $E1^{Hi}$ tumor is shown in Fig. 1 for IHC and ISH (Fig. 1D), TMA cores (Fig. 1E) and full-face sections (Fig. 1F), and four further cases in Supplementary Fig. S2A. Conversely, the two cases identified as non-amplified cyclin $E1^{Hi}$ from TMA cores had low amplification in all regions of the full-face tissue sections (<6 *19q12* copy number) and high H scores in all regions (H score range: 150–210) (Supplementary Fig. S2B). These findings are consistent with previous reports of *CCNE1* amplification being an early event in the genesis of HGSOC [24,25], and a prior observation of homogenous 19q12 amplification through an ovarian tumor [26].

3.4. Low FBXW7 and high USP28 may augment high cyclin E1 expression in amplified/cyclin $E1^{hi}$ and non-amplified/cyclin $E1^{hi}$ cases, respectively

To explore potential mechanisms underlying high cyclin E1 expression in non-amplified tumors, we investigated whether cyclin E1 overexpression was related to impaired proteasomal degradation of cyclin E1 due to loss of FBXW7 expression and/or change in USP28 expression. We correlated cyclin E1, FBXW7 and USP28 expression with 19q12 amplification status. Both FBXW7 and USP28 expression ranged between 0 and 300 and the median H scores of 40 and 110, respectively, were used as a cut-off for high and low expression levels. Exhaustion of tissue cores led to unequal numbers of assessable cases for each antibody.

Loss of FBXW7 leads to stabilization of cyclin E1, and, notably, 31% (n = 13/36) of the amplified high cyclin E1 expresser cases had no apparent FBXW7 expression (Fig. 2A). Examples of the expression levels of FBXW7 observed in different cases of HGSOC are shown in Fig. 2B. In tumors with high levels of cyclin E1 (n = 75), low FBXW7 was positively associated with 19q12 amplification (p = 0.010, Fisher Exact Test),

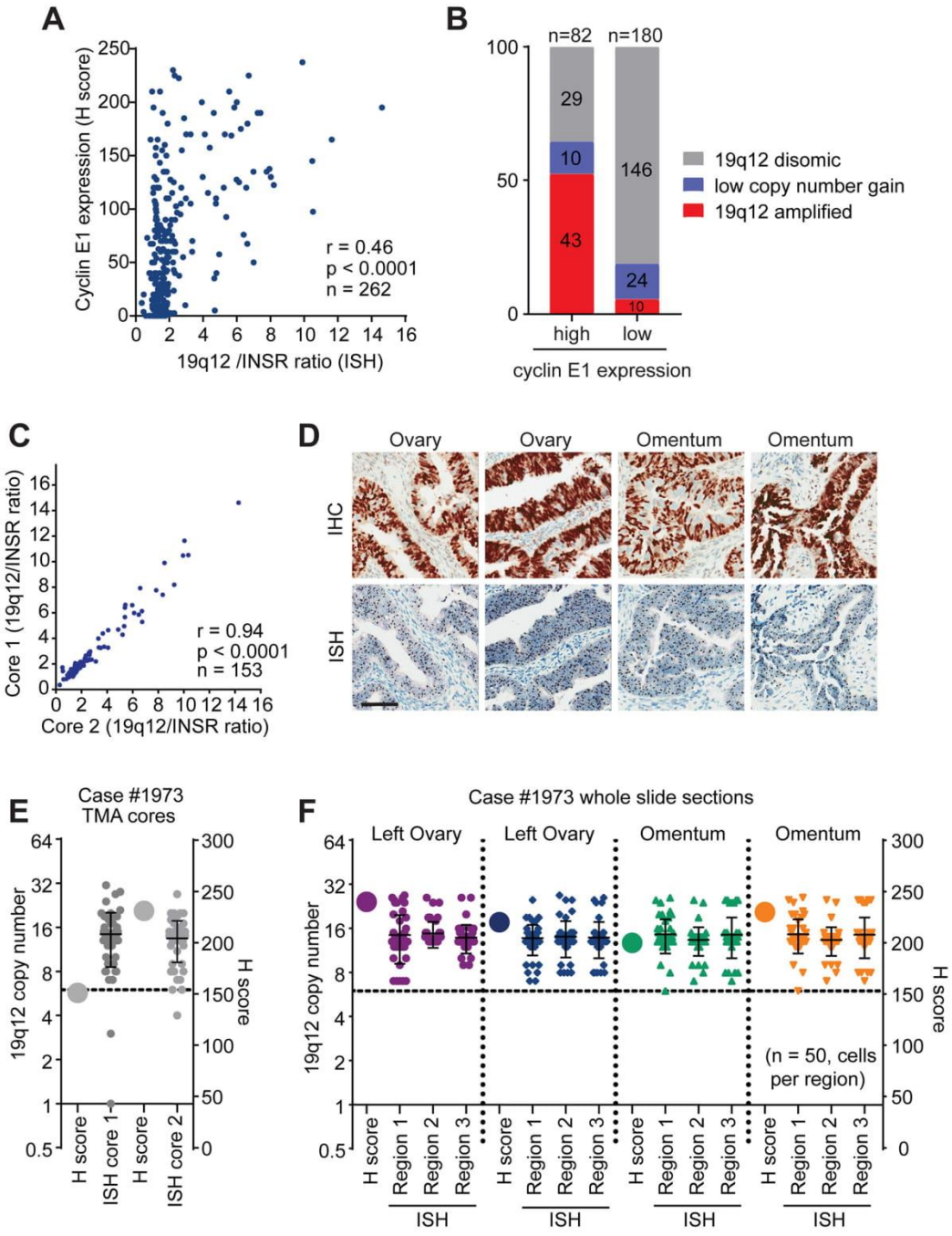

Fig. 1. 19q12 (CCNE1) amplification correlates with cyclin E1 expression and the amplification is homogenous. **A**: The distribution of cyclin E1 by 19q12: INSR ratio. **B**: Segregation of high versus low cyclin E1 expressers by 19q12 amplification status. **C**: Concordance of 19q12: INSR ratios between duplicate cores for 153 cases. r = Spearman coefficient, p = probability. **D**: Microscopic images of 19q12 ISH (lower) and cyclin E1 IHC (upper) on two cores from the left ovary and omentum from case #1973. Scale bar is 250 μm. **E**: 19q12 copy number and cyclin E1 H scores for TMAs from case #1973. **F**: 19q12 copy number and cyclin E1 H scores for different regions per block for multiple FFPE blocks/sites of representative case #1973 (Dotted line represents 19q12 copy number amplification threshold; n = 50 cells per region).

suggesting that *CCNE1* amplification also requires reduced cyclin E1 turnover to maintain high protein levels to exert its oncogenic effect. In contrast, no significant difference in FBXW7 expression was observed in the group with low cyclin E1 expression, regardless of 19q12 copy number (p = 0.745, n = 162) (Fig. 2C).

USP28 can induce cyclin E1 stabilization either through its absence, which leads to autoubiquitination and degradation of FBXW7, or through its overexpression, which leads to deubiquitination and stabilization of cyclin E1 [20]. We examined our cohort for loss of USP28 expression. Absence of USP28 protein was found in only 6 cases and is therefore unlikely to impact on FBXW7 or cyclin E1 expression.

The remainder of cases showed a wide range of USP28 staining, and there was a moderate but significant positive correlation between USP28 expression and cyclin E1 across these cases (Fig. 3A). Examples

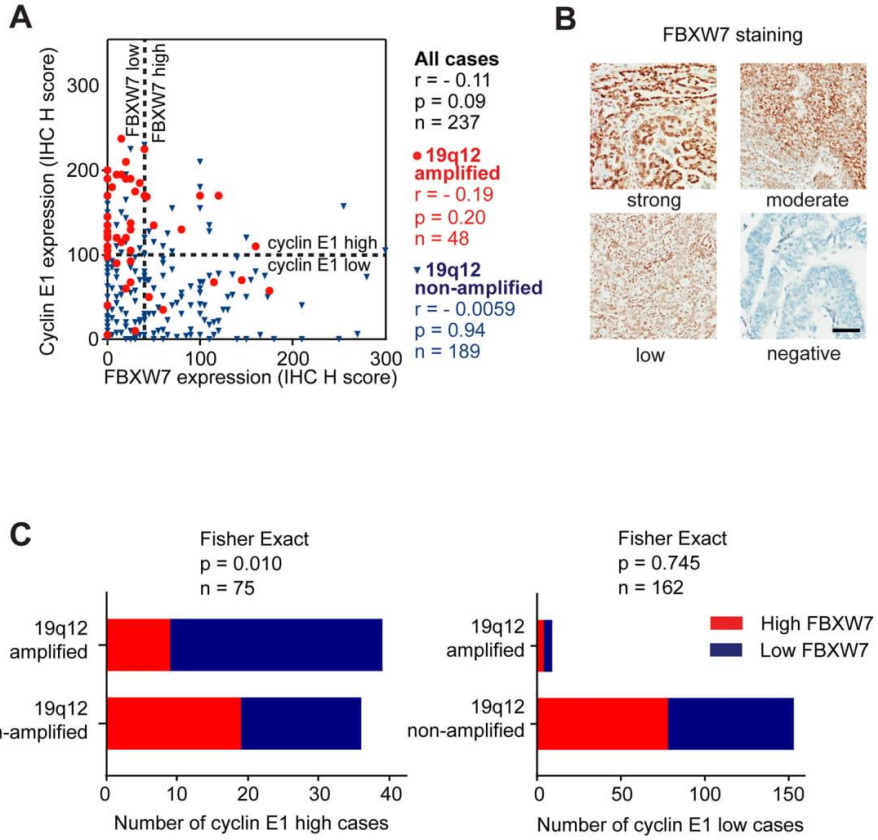

Fig. 2. FBXW7 expression is low or absent in amplified/cyclin E1^{hi} cases. **A**: Association of FBXW7 expression with cyclin E1 in all, amplified and non-amplified cases. Dashed lines on each axis represent the cutoff between high and low cyclin E1 (x-axis) and high and low FBXW7 expression (y-axis). **B**: Microscopic images of various expression of FBXW7 in different cases of HGSOC. Scale bar is100 μm. **C**: Association of FBXW7 high and low expression by 19q12 status and cyclin E1 high (left) and low (right) expression. p = probability (Fisher Exact test).

of USP28 expression are shown in Fig. 3B. Among tumors with high levels of cyclin E1 (n = 76), USP28 was significantly higher in non-amplified/cyclin E1^{hi} compared to amplified cases/cyclin E1^{hi} (p = 0.009, Fisher Exact Test). Similar to FBXW7, no significant difference in USP28 expression was observed in the group of low cyclin E1 expressers, whether amplified or not (p > 0.999, n = 165) (Fig. 3C).

We also examined TCGA ovarian cancer dataset for gene amplification and protein expression finding that amplification of USP28 had a non-significant trend to be mutually exclusive with *CCNE1* amplification (p = 0.14) (Fig. 3D). We analyzed HGSOC cases from the TCGA that were diploid at the 19q12 locus or displayed low level gain, and found a non-significant trend for high cyclin E1 protein expression to be associated with increased copy number of USP28 (p = 0.07, Mann-Whitney test; Fig. 3-E). Together with the findings in our cohort, these data suggest that reduced protein turnover may contribute to high cyclin E1 expression in the non-amplified cases, possibly by USP28-mediated functional deactivation of FBXW7.

3.5. BRCA1/2 germline mutations are significantly more prevalent in non-amplified/cyclin E1^{hi} cases than in amplified cases/cyclin E1^{hi}

We, and others [27,28] (Fig. 4A) had previously noted the mutual exclusivity of *CCNE1* amplification with mutation in either *BRCA1* or *BRCA2* (*BRCA1/2*), arising due to synthetic lethality associated with these events [29]. Mutual exclusivity therefore provides a measure by which we could indirectly evaluate the effect of cyclin E1 over expression in the absence of amplification. We related *BRCA1* and *BRCA2* germline mutation status to cyclin E1 protein and *19q12* locus copy number in 236 samples where complete information was available.

The overall distribution and correlation of cases by their germline *BRCA1* and *BRCA2* status versus their 19q12: INSR ratio and cyclin E1 protein expression (n = 236) is shown in Fig. 4B. Regardless of cyclin E1 expression, the amplified cohort had significantly fewer cases with *BRCA1/2* mutations than the non-amplified cohort (p = 0.005, Fisher's Exact Test) (Fig. 4C). When further subdivided by protein and copy number grouped by amplification, a significantly higher rate of *BRCA1/2* mutation was observed in non-amplified/cyclin E1^{hi} (9 out of 37 (24.3%) of cases) compared to amplified cases/cyclin E1^{hi} (2 out of 34 (5.9%) of cases) (p = 0.050, Fisher's Exact Test) (Fig. 4D). No significant difference in *BRCA1/2* mutation rate was observed in non-amplified/cyclin E1^{lo} compared to amplified/cyclin E1^{lo} (Fig. 4E). Thus, the mutual exclusivity between *CCNE1* amplification and *BRCA1/2* does not appear to extend to non-amplified/cyclin E1^{hi} tumors.

3.6. High expression of cyclin E1 is associated with poorer survival when accompanied by 19q12 amplification

As a further measure of the impact of cyclin E1 over expression in the absence of amplification, we evaluated patient outcome in amplified and non-amplified subjects. Subjects were divided into four groups according to locus copy number (amplified or non-amplified) and cyclin E1 expression status (high or low). Patients with amplified/cyclin E1^{hi}

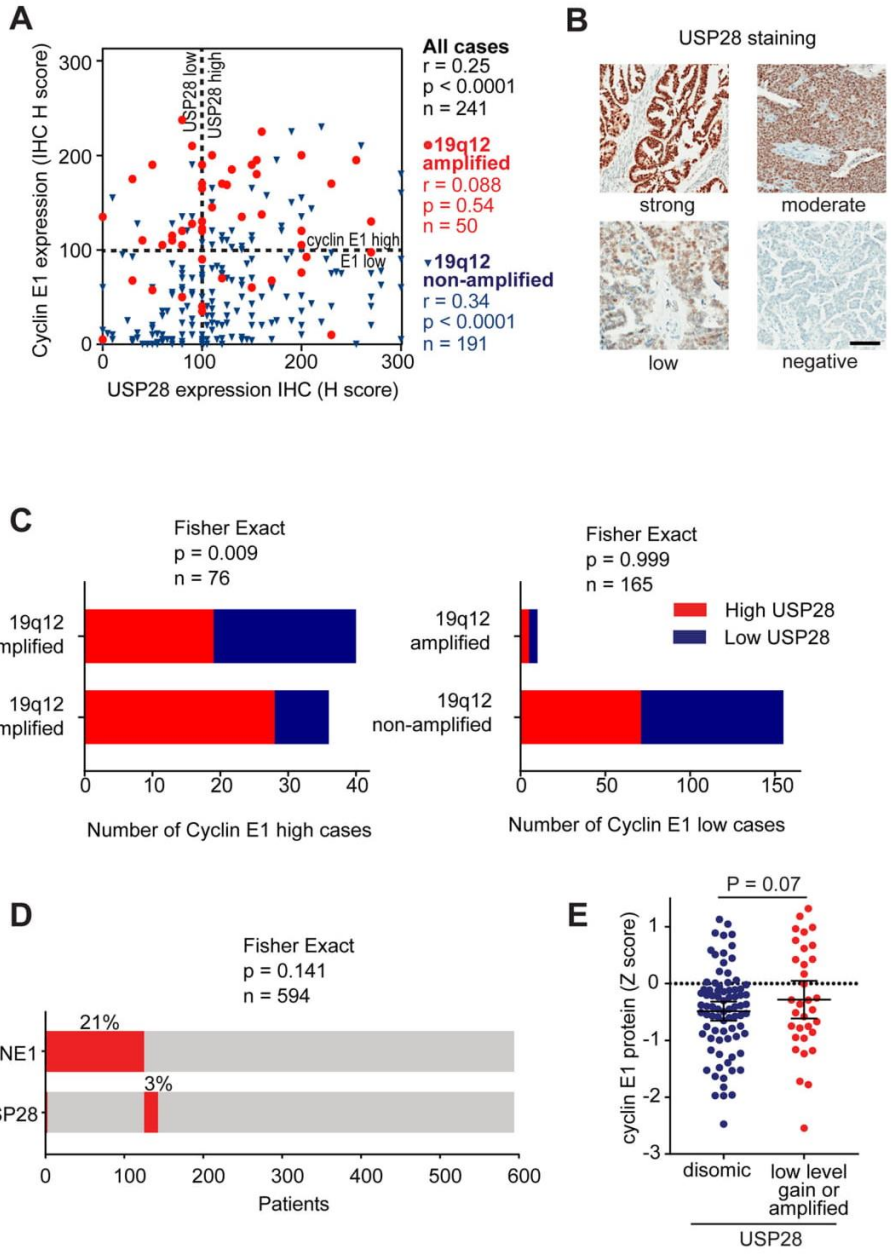

Fig. 3. USP28 expression is high in non-amplified/cyclin E1^{hi} cases. **A**: Association of USP28 expression with cyclin E1 in all, amplified and non-amplified cases. Dashed lines on each axis represent the cut-off between high and low cyclin E1 (x-axis) and high and low USP28 expression (y-axis) **B**: Microscopic images of various expression of USP28 in different cases of HGSOC. Scale bar is100 μm. **C**: Association of USP28 high and low expression by 19q12 status and cyclin E1 high (left) and low (right) expression. p = probability (Fisher Exact test) **D**: Cyclin E1 amplification tends to be mutually exclusive with *USP28* amplification in HGSOC from TCGA datasets. **E**. *USP28* copy number gain has a non-significant trend for association with high cyclin E1 protein expression in the HGSOC TCGA dataset, *P*-value for two-sided Mann-Whitney test, error bars indicate 95% confidence interval.

tumors had significantly worse OS when compared to the non-amplified/cyclin E1^{lo} group (median 28.57 vs. 45.6 months, HR 0.6, 95% CI, 0.43 to 0.91, p = 0.0202). Although amplified/cyclin E1^{hi} cases had shorter OS compared to non-amplified/cyclin E1^{hi} cases, the difference was not statistically significant (median 28.6 vs. 43.3 months, HR 0.7, 95% CI, 0.41 to 1.07, p = 0.298). Moreover, within the amplified subset, amplified/cyclin E1^{hi} had a shorter OS when compared to the amplified/cyclin E1^{lo} (median 28.57 vs. 66.34 months) however due to the small number of amplified low expressers, the study was not powered to give a statistical significance (p = 0.20). Additionally, for the non-amplified cases, non-amplified/cyclin E1^{hi} tumors had no significant impact on OS compared to non-amplified/cyclin E1^{lo} tumors (median 43.33 vs. 45.6 months, HR 0.9, 95% CI, 0.64 to 1.41, p = 0.40). However, when the four groups compared the OS was not significantly different (p = 0.0955). Neither locus copy number or expression status appeared to have an impact on PFS (Supplementary Fig. S3A). These findings imply that for cyclin E1, both gene amplification and high protein expression are associated with poor outcome and that the clinical

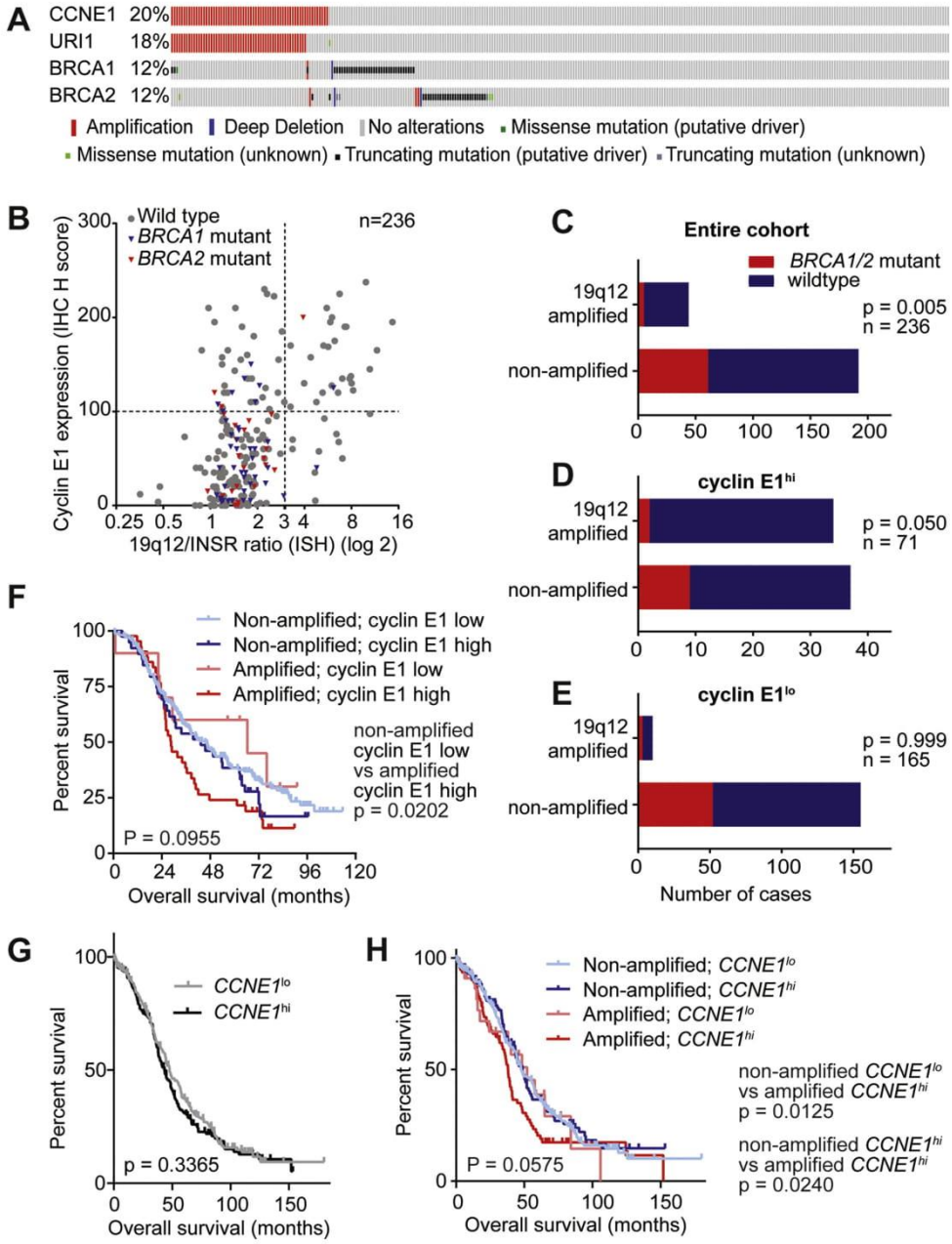

Fig. 4. Mutual exclusivity of 19q12 amplification, cyclin E1 expression and germline *BRCA1/2* mutation and the prognostic impact of 19q12 amplification and cyclin E1 expression. **A**: TCGA oncoprint from 316 HGSOC cases showing mutual exclusivity of *CCNE1* and *URI1* copy number alterations from *BRCA1* and *BRCA2* mutation. **B**: The distribution of *BRCA1/2* mutations against cyclin E1 (n = 236) IHC H scores and 19q12: INSR ISH ratio (Dashed lines represent high cyclin E1 expression threshold and 19q12: INSR amplification threshold). **C–E**: The correlation of *BRCA1/2* mutation status vs 19q12 amplification status in the entire cohort Cyclin E1^{hi}, Cyclin E1^{lo} cohorts. p = probability (Fisher Exact test). **F**: Kaplan Meier curves of overall survival for allocated subgroups of HGSOC, according to cyclin E1 expression status and amplification status. **G**: Kaplan Meier curves of overall survival for subgroups of HGSOC defined as *CCNE1* low (z-score < 0.7) and *CCNE1* mRNA high (z-score > 0.7) (n = 530). **H**: Kaplan Meier curves of overall survival according to amplification status and *CCNE1* mRNA (n = 530).

behaviour of tumors showing high expression of cyclin E1 without amplification is unlikely to be similar to those with amplification.

We performed a similar analysis using the TCGA ovarian cancer dataset, but instead comparing overall survival of subsets of patients defined by high or low *CCNE1* mRNA expression, and *CCNE1* gene amplification. The cutoff for high *CCNE1* expression was a z score of 0.7 based on the data distribution (Supplementary Fig. S3B), and the tumors were classified as amplified or non-amplified cases using the GISTIC prediction used by TCGA. Generally, the OS of *CCNE1*^{hi} patients was not significantly different from that of *CCNE1*^{lo} patients (P = 0.3365; Fig. 4G). When we further divided these patients into groups with tumors that were amplified or not amplified, we found that the OS was not significantly different when comparing the 4 groups (P = 0.0575). However, both patients with non-amplified/*CCNE1*^{lo} tumors and

patients with non-amplified/*CCNE1*^{hi} tumors had significantly better survival compared to those with amplified/*CCNE1*^{hi} tumors (P = 0.0125; P = 0.0240 respectively, Fig. 4H, Supplementary Fig. S3C).

3.7. High cyclin E1 is associated with an index of chromosomal instability (CIN25) in 19q12 amplified cancers

HGSOC is characterized by its high genomic instability and cyclin E1 is a known driver of genomic instability, and in particular, chromosomal instability (CIN) [30]. The mutual exclusivity between *BRCA1/2* mutation and *CCNE1* amplification is thought to be due synthetic lethality arising from the failure to repair the excessive genomic instability associated with high cyclin E1 expression [31]. We assessed how cyclin E1 expression relates to genomic instability in HGSOC, and whether there is a difference between amplified/cyclin E1^{hi} HGSOC and non-amplified/cyclin E1^{hi} HGSOC. To do this we used the CIN25 gene signature [32]. CIN25 is the sum expression of 25 genes that correlate most highly with functional aneuploidy in tumors, and it is a significant predictor of clinical outcome in cancer [32].

Gene and protein expression data were obtained from TCGA for the Ovarian Serous Cystadenocarcinoma dataset, and a CIN25 signature was determined (see Supplementary Methods). We found that overall cyclin E1 protein had only a slight correlation to the ovarian CIN25 signature (Pearson's correlation = 0.2164, p < 0.0001) (Fig. 5A). Tumors classified as 19q12 amplified by GISTIC had a moderate correlation with CIN25 (r = 0.4221, p = 0.0004) whereas non-amplified tumors only had a weak correlation (r = 0.1772, p = 0.0033). We then used Fisher Exact test to determine if there was a correlation between 19q12 amplification, cyclin E1 protein expression and CIN25. For this analysis, we classified tumors as cyclin E1^{Hi} if they had a Z score > 0 and high CIN25 for a Z score > 0, (Supplementary Fig. S3D). Overall, high cyclin E1 protein was significantly correlated with high CIN25 (p = 0.0028), but this relationship only remained significant for 19q12 amplified cancers (p = 0.0028) and not for non-amplified cancers (p = 0.0810). Thus, the expression of cyclin E1 protein has a stronger association with chromosomal

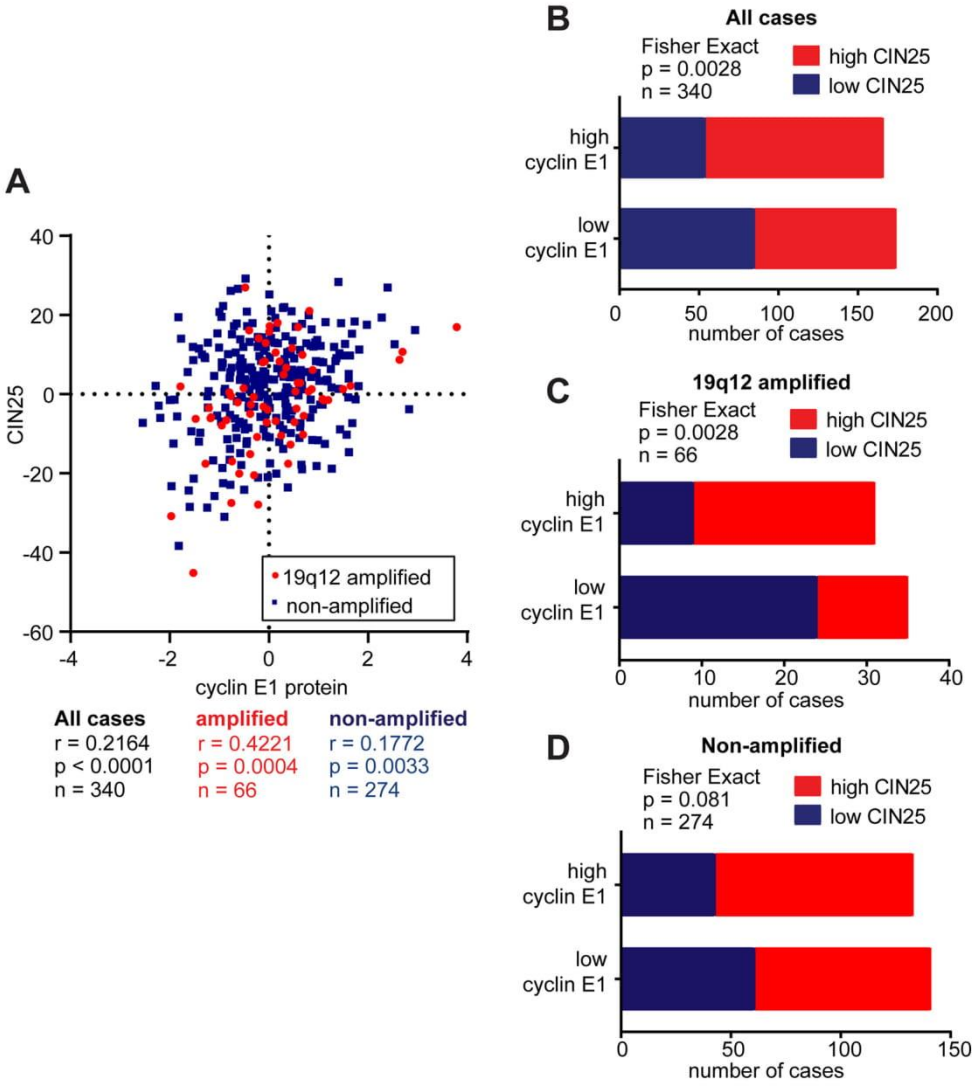

Fig. 5. High cyclin E1 is associated with an index of chromosomal instability (CIN25) in 19q12 amplified cancers **A**: Association between CIN25 and cyclin E1 expression across TCGA HGSOC cases. r = Pearson's coefficient, p = probability. **B**: High CIN25 (CIN25 > 0) is correlated with high cyclin E1 expression (cyclin E1 z-score > 0) across 340 TCGA HGSOC cases. **C&D**: High cyclin E1 is correlated with high CIN25 in *CCNE1* amplified cases (C), but not in *CCNE1* non-amplified cases (D). p = probability (Fisher Exact test).

instability in amplified/cyclin E1^{hi} cancers than in non-amplified tumors/cyclin E1^{hi}.

3.8. URI1, as another driver of 19q12 amplification

Given the previous suggested role of *URI1* in HGSOC [7] and its co-localization with *CCNE1*, we sought to investigate *URI1* as another driver for 19q12 amplification. We tested URI1 expression correlation to 19q12 amplification, cyclin E1 expression, clinical outcome and *BRCA1/2* status in both amplified and non-amplified subsets. URI1 data are provided in Supplementary Fig. S4. Similarly to cyclin E1, URI1 expression correlated to 19q12 amplification ($r = 0.232$ $p = 0.0005$) (Supplementary Fig. S4A), and representative URI1 IHC images are provided in Supplementary Fig. S4B. URI1 was high in the majority of 19q12 amplified cancers (39/46; 84.8%), and high URI1 expression was also noted in (80/176; 45.5%) of tumors without 19q12 amplification (Supplementary Fig. S4C).

Amplified/URI1^{hi} tumors also were depleted for germline *BRCA1/2* mutations. When grouped by amplification, a significantly higher rate of *BRCA1/2* mutation was noted in non-amplified/URI1^{hi} (19 out of 74 (25.7%) of cases) compared to amplified//URI1^{hi} cases (2 out of 34 (5.9%) of cases) ($p = 0.003$, Fisher's Exact) while no significant difference was noted in non-amplified/URI1^{lo} compared to amplified/URI1^{lo} (Supplementary Fig. S4D and E).

The majority of 19q12-amplified cases co-expressed both URI1 and cyclin E1. Similar to *19q12*-amplified tumors, amplified/URI1^{hi} patients had the worst outcome ($p = 0.0021$) (Supplementary Fig. S4F). The clinical impact of cases that were *URI1*- amplified but not *CCNE1*-amplified was not assessable as the dual ISH probe covers both *CCNE1* and *URI1* genes and the majority of amplified cases expressed both proteins. This finding is in agreement with TCGA data where out of 316 cases of HGSOC, none of the cases were *URI1* amplified but not *CCNE1* amplified, while only 9 out of 64 (14%) cases were *CCNE*1 but not *URI1* amplified (Fig. 4A). The relative contribution of *URI1* and *CCNE1* to the behavior of 19q12-amplified tumors is difficult to untangle due to the closely correlated nature of amplification of the two genes. A moderate correlation between cyclin E1 and URI1 protein expression irrespective of 19q12 amplification suggests that these proteins could act in concert to drive HGSOC growth and survival (Supplementary Fig. S4G).

4. Discussion

CCNE1 amplification currently defines the largest molecular subset of HR-proficient HGSOC. *BRCA1* and *BRCA2* HR-deficient HGSOC is the subject of much interest due to the therapeutic efficacy of PARP inhibitors, but no equivalent has been developed for HR-proficient HGSOC. CDK2 inhibitors are an attractive potential treatment for cyclin E1 overexpressing tumors and show promising efficacy in *CCNE1* amplified cell lines and xenografts [33,34], especially in combination with AKT inhibitors [4]. It is not known whether this approach will be limited to tumors with *CCNE1* amplification, and for this reason we have defined the characteristics of HGSOC cancers with high cyclin E1 in the absence of gene amplification.

Non-amplified/cyclin E1^{hi} tumors make up 33–47% of all cyclin E1^{hi} HGSOC, and three orthogonal findings suggest that non-amplified/cyclin E1^{hi} HGSOC has distinct molecular attributes. First, the almost complete mutual exclusivity of *CCNE1* amplification and germline *BRCA1/2* mutation does not extend to non-amplified/cyclin E1^{hi} tumors as non-amplified/cyclin E1^{hi} cancers more frequently exhibit *BRCA1* or *BRCA2* mutations. Second, evidence of CIN was more frequent in *CCNE1* amplified tumors. Finally, the clinical outcome of patients with 19q12 amplification was worse than those with cyclin E1 over expression in the absence of amplification.

The regulatory systems that control the degradation of cyclin E1 are often disrupted in cancer, and in HGSOC we find that different mechanisms may stabilize cyclin E1 depending on the amplification status of *CCNE1*. Low or absent FBXW7 expression is more common in 19q12 amplified/cyclin E1^{hi} cases, and high expression of its antagonist, USP28, is common to non-amplified cases. The low FBXW7 expression in amplified/cyclin E1^{hi} subset suggests that the proteosomal degradation of cyclin E1 needs to be impaired in order to sustain high cyclin E1 levels. Since somatic inactivating FBXW7 mutations are uncommon in ovarian cancer, absent or reduced FBXW7 expression may result from silencing of FBXW7, for instance, by promoter hypermethylation [17–19]. Notably, loss of either p53 or p21 allows persistent high levels of cyclin E1, resulting from FBXW7 loss, to continuously drive genome instability [6,35] and FBXW7 has been demonstrated to drive chromosomal instability via upregulation of cyclin E1 [36].

We speculate that persistently high level cyclin E1 expression, caused by *CCNE1* amplification, during S phase, potentially exacerbated by epigenetic silencing of FBXW7, results in oncogene – induced replication stress. This aberration generates chromosomal instability that leads to tumors with inherently poor clinical outcomes. Therefore, a successful replication stress response would be required to maintain replication competency and would therefore depend upon various intact DNA repair mechanisms (DDR), including the Ataxia-Telangiectasia mutated and Rad 3-related (ATR)-CHK1-WEE1 and HR pathways [37,38]. This presumably accounts for the mutual exclusivity of *CCNE1* amplification and *BRCA1* and *BRCA2* mutations and the consequent resistance of *CCNE1* amplified tumors to platinum chemotherapy. Accordingly, it is not unreasonable to speculate that the amplified subset is likely to respond to DDR inhibition through CHEK1 and/or Wee1 inhibitors.

In non-amplified//cyclin E1^{hi} cancers we observe high levels of USP28 rather than decreased FBXW7. We speculate that this is because these cells do not have the observed genomic instability that is associated with 19q12 amplification in our analysis of the correlation of cyclin E1 expression to CIN25 in the TCGA HGSOC cohort. In unstressed cells, USP28 forms a complex with FBXW7 and antagonizes substrate ubiquitination, however, after DNA damage this complex dissociates promoting FBXW7-dependent substrate degradation [20]. Accordingly, the co-expression of cyclin E1 and USP28 in the 19q12 non-amplified HGSOC suggests that USP28 overexpression maintains persistently high cyclin E1 levels during S phase in the absence of replication stress and DNA damage associated with *CCNE1* amplification.

In this study, we have shown, for the first time, significant biological and clinical differences between 19q12 amplified and non-amplified HGSOC cases that overexpress cyclin E1. These observations have implications for choice of therapy for each of these subsets. First, some non-amplified/cyclin E1^{hi} HGSOC have HR deficiencies that may make them responsive to platinum based therapies and/or PARP inhibitors. This may explain the failure of cyclin E1 expression to predict taxane-platinum chemoresistance, when not characterized by 19q12 amplification status [39]. Non-amplified cases could also potentially be treated with protease inhibitors that specifically target deubiquitinases such as USP28 [40], but this is dependent on showing that this HGSOC subset is reliant on cyclin E1 expression. By contrast, the amplified subset could feasibly respond to CHEK1 and/or Wee1 inhibitors as they are likely to become dependent upon the ATM-ATR-CHEK1-Wee1 pathway. These distinct therapeutic options depend on the molecular attributes of each cyclin E1^{hi} subset, and thus indicate caution in combining all cyclin E1^{hi} HGSOC patients into clinical trials without a careful consideration of *CCNE1* amplification status.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2018.08.039.

Conflict of interest statement

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Authors' contribution:

Study conception and design: P.W., D.B., D.E., G.A., D.A.; Acquisition of data: D.A., AOCS, N.D., C.E.C.; Analysis and interpretation of data: D.A., P.W., D.E., C.E.C., R.H.; Drafting of manuscript: D.A., P.W., C.E.C.; Critical revision: D.A., C.E.C., P.W., D.B., D.E.

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Supplementary methods and data

Assessment of 19q12 status

Four micron sections from formalin-fixed, paraffin-embedded (FFPE) TMAs were deparaffinised, treated with cell conditioning 2 (CC2) for four cycles of 12 minutes each, followed by ISH protease 3 for 24 min. After co-denaturation, 19q12 DNP and INSR DIG probes were hybridized at 47°C for 6 hours and washed for 3 cycles at 68 °C for 8 minutes. 19q12 DNP and INSR DIG signals were detected using VENTANA ultraView SISH DNP and VENTANA ultraView RED ISH DIG detection kits, respectively, and counterstained with hematoxylin II and bluing reagent. Slides were directly scored, but contrast of ISH images was adjusted with Photoshop to improve the printed resolution of the examples presented in figures. Included in these TMAs are 59 HGSOC cases with previously known *CCNE1* gene copy number derived by qPCR [1]. The presence of tumor tissue was confirmed with Haematoxylin and Eosin stained slides for each TMA core. Samples were assessed for acceptable staining and scored according to Ventana's dual ISH cocktail Assay interpretation Guide for *HER2* [2]. A section of ovarian cancer known to be *CCNE1* amplified was included in each staining cycle as a positive control. Additionally, and as per Ventana recommendations, staining of non-neoplastic cells whether stromal or inflammatory were used as an internal control (examples of signals scoring in Figure S1A). Tumours with a 19q12: INSR ratio ≥ 3 and / or 19q12 locus copy number ≥ 6 (n=53) were considered amplified while cases with 19q12: INSR ratio <3 and 19q12 locus copy number <6 were considered non-amplified (n=209). The non-amplified cases were further divided into disomic cases with 19q12 locus copy number <4 (n=175) and 4 – 6 (n=34), which were considered to have low frequency copy number gain (Supplementary Table 1). For cases with more than one core, the average between replicates was used. The distribution of average 19q12 and INSR copy numbers and the average 19q12: INSR ratios for each of the 262 subjects are shown in Figure S1B, S1C and S1D. The median 19q12 and INSR locus copy numbers and 19q12: INSR ratio for the entire cohort were 3 (range 0.86 to 21.7), 2 (range 0.71 to 8.93) and 1.63 (range 0.36 to 14.62), respectively.

Cut point determination for in situ hybridization.

Cut-off thresholds for 19q12 amplification were guided by the prevalence of polysomy, the expected prevalence of *CCNE1* amplification [3, 4], previous assay reports [4] and

earlier *CCNE1* copy number results generated by qPCR. ISH scores for 59 HGSOC cases were plotted against *CCNE1* copy number assessed by qPCR and a statistically significant correlation ($r = 0.6$, $p<0.0001$) was found, indicating a high degree of concordance across the linear range between the assays (Supplementary Figure S1E). The 19q12: INSR ISH ratio of 3 was also assessed by Receiver Operator Curve (ROC) analysis and was 95% sensitive and 51% specific for cyclin E1 ($p<0.0001$) and 91% sensitive and 65% specific for URI1 ($p=0.0009$) expression, respectively (Fig S1F). Using the 19q12: INSR ISH ratio, 81 (30.9 %) and 46 (17.6%) of cases had a ratio ≥2 and ≥3, respectively, which agrees with the reported frequency range for *CCNE1* amplification in HGSOC. Using these cut offs, 5 (21.7%), 8 (34.8%) and 10 (43.5%) of the 23 cases with an INSR ≥3.0 also had amplification, low locus copy number gain or were disomic, respectively, at 19q12 locus (Supplementary Table 1). The low locus copy number gain subgroup had the highest proportion of cases with an INSR ≥3.0 (8 of 37, 21.6%), compared to the amplified (5 of 50, 10%) and the disomic (10 of 175, 5.7%) groups (Supplementary Table 1).

H score and cut point determination for immunohistochemistry

A semi quantitative H score was assessed as the sum of strongly stained cells *3 plus the percentage of moderately stained cells *2 plus the percentage of weakly stained cells, giving a range of 0 to 300 [5, 6]. Based on 19q12 ISH results, optimised IHC cut-offs of 100 and 70 were established by ROC analysis to distinguish between low versus high cyclin E1 (80% sensitivity, 18% specificity, $p<0.0001$, $n=262$) and URI1 (89% sensitivity, 46%, specificity, $p<0.0001$, $n=222$) protein expression, respectively (Fig S1G). The distribution of mean H scores for cyclin E1 and URI1 are shown in Figure S1H and S1I, respectively. The median H scores were 60 (range: 0 - 237.5) for cyclin E1 ($n=262$), 75 (range: 0 - 285) for URI1 ($n=222$). Exhaustion of tissue cores led to unequal numbers of assessable cases for each antibody.

TCGA OVCA analysis

TCGA analyses were performed by accessing data via cBioPortal [7] in September 2017. The analyses shown in Figure 3D-E and 4G-H were from the larger provisional TCGA dataset. The analysis of copy number variation and mutation shown in Figure 4A was performed on the 2011 dataset [8] as this represents a larger cohort of patients with complete mutation and CNV datasets. For Figure 4G-H, a cut-off for high *CCNE1* mRNA (>0.7 z-score) was determined from natural divisions in the multimodal distribution of the binned dataset (Supplementary Figure S3D).

For CIN25 analysis (Figure 5), normalised gene and protein expression data from the provisional TCGA ovarian study was downloaded from cBioPortal [7] as of September 2017. Normalised individual CIN25 gene expression data was extracted from TCGA datasets based on median of all three available expression platforms, and cyclin E1 protein z-scores from the reverse phase protein array (RPPA) dataset. A cut-off for high cyclin E1 protein (>0.0 z-score) was determined from natural divisions in the multimodal distribution of the binned dataset (Supplementary Figure S3D). The CIN25 score was determined for each sample by the sum of the 25 gene expression values [9].

Supplementary table 1: Distribution of raw scores for INSR (< and ≥ 3) and 19q12 (<4, 4-6 and ≥ 6) by amplification status.

	All (n=262)		**Amplified (n=53)**		**Non-amplified (n=209)**	
	INSR<3 (n=239)	**INSR≥3 (n=23)**	**INSR<3 (n=48)**	**INSR≥3 (n=5)**	**INSR<3 (n=191)**	**INSR≥3 (n=18)**
19q12 ≥6	45	5	45	5	0	0
19q12 4-6	29	8	3	0	26	8
19q12 <4	165	10	0	0	165	10

Supplementary References

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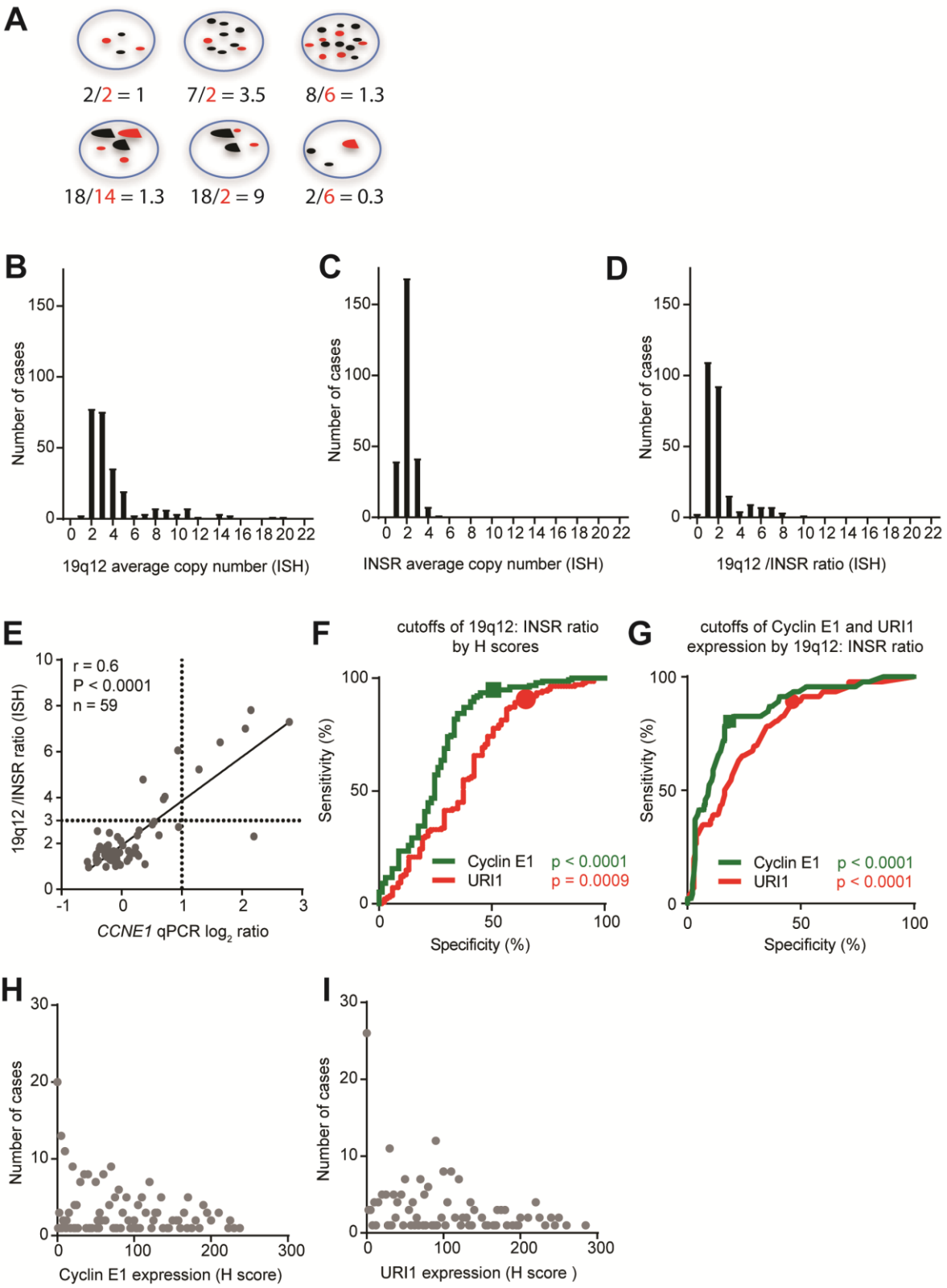
SUPPLEMENTARY FIGURE S1: Assessment of 19q12 status and determination of assay cut points.



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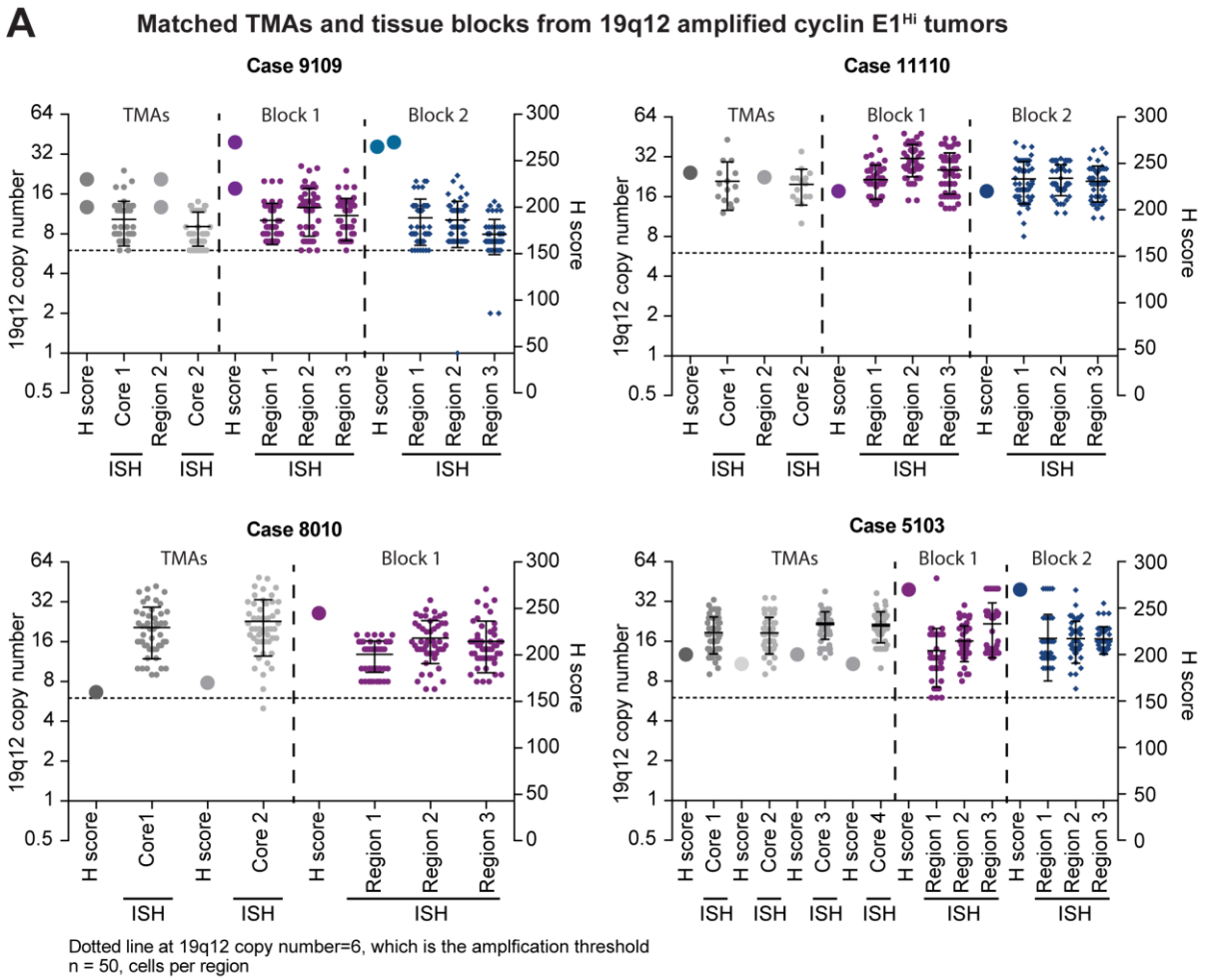
SUPPLEMENTARY FIGURE S2: Homogenous 19q12 amplification and cyclin E1 expression in different sections of HGSOC tumors.



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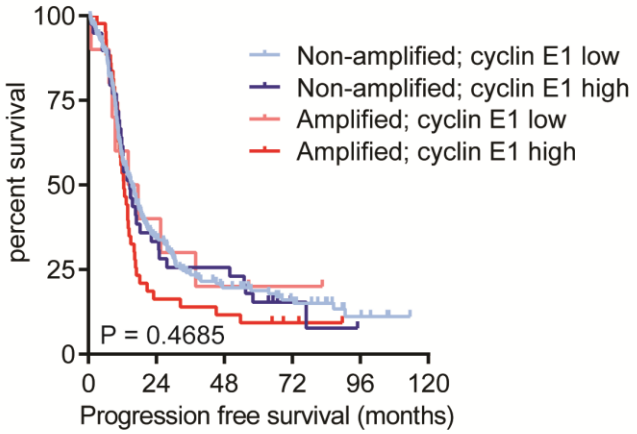
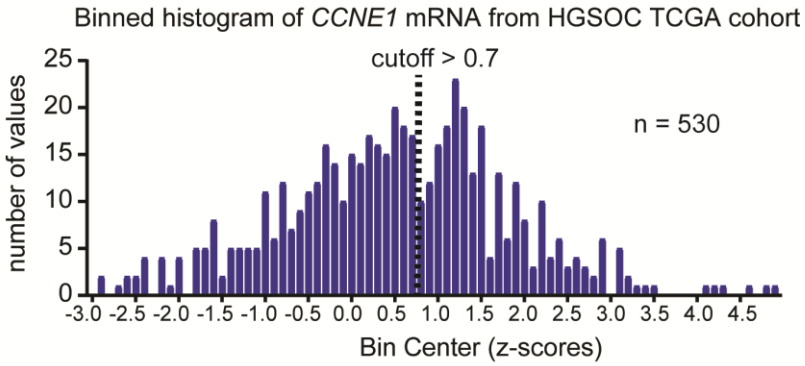
SUPPLEMENTARY FIGURE S3: Effect of 19q12 copy number, cyclin E1 and *CCNE1* expression on progression free survival and overall survival in HGSOC.

A



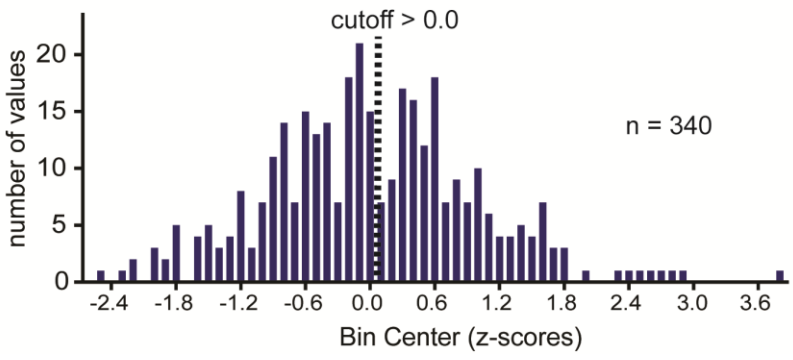
B



C

Comparison	P-value	Significance
non-amplified/*CCNE1*^{lo} versus non-amplified/*CCNE1*^{hi}	P = 0.8321	NS
non-amplified/*CCNE1*^{lo} versus amplified/*CCNE1*^{lo}	P = 0.6966	NS
non-amplified/C*CNE1*^{lo} versus amplified/C*CNE1*^{hi}	P = 0.0125	*
non-amplified/*CCNE1*^{hi} versus amplified/*CCNE1*^{hi}	P = 0.0240	*
amplified/*CCNE1*^{lo} versus amplified/*CCNE1*^{hi}	P = 0.3609	NS

D



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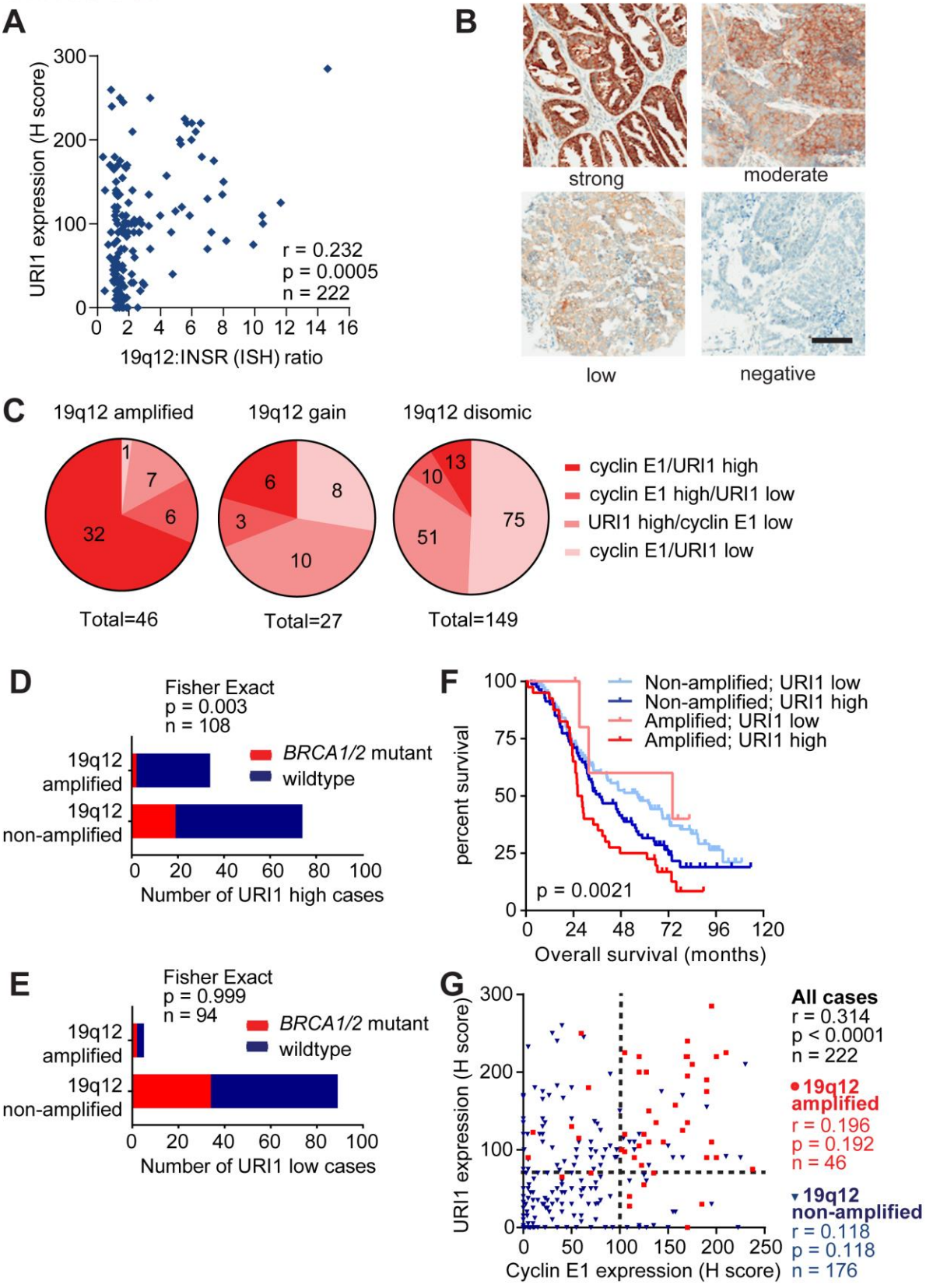
SUPPLEMENTARY FIGURE S4: URI1 amplification and expression in HGSOC.



Chapter 4: The correlation of cyclin E1 deregulation with *BRCA1* inactivation in breast cancer

Preface:

The reported mutual exclusivity of *CCNE1* amplification and *BRCA1* inactivation in HGSOC and the proposed similarity of HGSOC and BLBC prompted us to explore the correlation of cyclin E1 deregulation with *BRCA1* inactivation in breast cancer.

This chapter is drafted in a format to be published in **Clinical Cancer Research Journal**, as per the following:

Aziz, D., Portman, N., Fernandez. K., Lee, C., Alexandrou, S., Yong, A., Sergio, C., Musgrove, E., Ferraro, D., Etemadmoghadam, D., Bowtell, D., kConFab Investigators, Waring, P., Lim, E., Caldon, C. "Cyclin E1 protein is stabilised in *BRCA1* mutated breast cancers resulting in synergy between CDK2 and PARP inhibitors"**(In progress)**

The drafted manuscript and associated supplementary information are presented in this chapter.

Cyclin E1 protein is stabilised in *BRCA1* mutated breast cancers resulting in synergy between CDK2 and PARP inhibitors

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RUNNING TITLE: BRCA1 regulates cyclin E1 in breast cancer

KEYWORDS: BRCA1, cyclin E1, breast cancer, PARP inhibitor

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ABSTRACT

BRCA1 mutant breast cancers occur primarily as basal like breast cancers, which are a highly aggressive subset of breast cancers that respond poorly to available targeted therapies and chemotherapies. In order to provide better therapeutic options for *BRCA1* mutant breast cancers, we explored the co-occurrence of cyclin E1 overexpression and *BRCA1* inactivation.

Using a patient cohort enriched for familial cancer mutations, we showed that patients with *BRCA1* loss and high cyclin E1 had significantly worse overall survival. Moreover, *BRCA1* loss correlated with decreased phosphorylation of cyclin E1, on Threonine 62, and high expression of cyclin E1 deubiquitinase USP28. *BRCA1* loss in cell lines led to cell cycle specific stabilisation of cyclin E1 by reducing cyclin E1 T62 phosphorylation while *BRCA1* overexpression increased cyclin E1 T62 phosphorylation. Overexpression of cyclin E1 with an inactivated cyclin E1 T62 site, to mimic loss of phosphorylation, increased cyclin E1 stability and resistance to Paclitaxel. These findings indicate that cyclin E1 stability is regulated by *BRCA1* in breast cancer cells via cyclin E1 T62 phosphorylation.

Since replication stress caused by cyclin E1 dysregulation is associated with DNA damage, we hypothesised that CDK2 inhibition would enhance sensitivity of *BRCA1* deficient cells to PARP inhibition. CDK2 inhibition induced DNA damage and synergised with the PARP inhibitor Rucaparib in *BRCA1* mutated cell lines. Preliminary data of combination treatment of xenograft models led to tumor regression. Our results suggest a new therapeutic strategy for *BRCA1*-mutant breast cancer by combining CDK2 and PARP inhibitors to enhance synthetic lethality.

INTRODUCTION

Breast cancer with *BRCA1* mutation most often manifests as basal like breast cancer (BLBC) (1), which presents difficulties for treatment as these cancers generally present at an earlier age, at a high grade and with greater tumor burden. Problematically, they are mostly negative for the estrogen receptor and HER2 (2), which means that they are unsuitable for either hormone therapy or targeted HER2 therapy.

BRCA1 is a central component of the homologous repair pathway, and its loss results in compromised DNA damage repair (3). Alterations to *BRCA1* are important founder mutations for breast cancer (4), and notably, more than 70% of *BRCA1* mutation carriers develop early-onset BLBC based on gene expression profiling (5). *BRCA1* mutation directly drives the basal phenotype, as mice with *p53* and *BRCA1* deletion develop mammary tumors with basal-like characteristics (6), and intact *BRCA1* represses the transcription of basal cytokeratins (7).

A previous report identifies that BLBCs from patients with germline *BRCA1* mutation can have high cyclin E1 protein (8). Cyclin E1 is a cell cycle regulatory protein whose gain can promote both increased proliferation and genomic instability in cancer cells, and is frequently elevated in BLBC (9). Perplexingly, in high grade serous ovarian cancer (HGSOC), it is believed that *BRCA1/2* mutation and cyclin E1 gain do not co-occur as they are both drivers of genomic instability, and together they would precipitate lethal genomic damage (10).

We recently described separate subsets of HGSOC, one where cyclin E1 gene amplification and *BRCA1* mutation were mutually exclusive, and another where cyclin E1 protein overexpression was due to post – transcriptional dysregulation rather than gene amplification, and *BRCA1* mutation was common (11). Accordingly, we examined whether *BRCA1* loss and cyclin E1 gain occurred concurrently or independently in breast cancer. We also explored the mechanisms underpinning high cyclin E1 expression in *BRCA1* mutated breast cancer including gene amplification and protein stability. Cyclin E1 protein stability is regulated by a multi-step process of specific phosphorylation and ubiquitination, leading to its cyclic expression and turnover (12). Key regulators in the turnover of cyclin E1, such as the ubiquitin ligase component FBXW7 and the deubiquitinase USP28, are frequently dysregulated in cancer (12-14) leading to altered stability of the cyclin E1 protein.

We here use a cohort of familial breast cancers, enriched for *BRCA1* mutant tumors, to perform a detailed analysis of the relationship between *BRCA1* and cyclin E1 in breast cancer. We identify that *BRCA1* loss/disruption and high cyclin E1 protein are highly correlated in breast cancer, and high cyclin E1 in *BRCA1* mutated patients is associated with reduced overall

survival. Using cell line models we find the mechanistic basis for this relationship: *BRCA1* loss increases the stability of the cyclin E1 protein via a decrease in phosphorylation of the cyclin E1 T62 site. We show that this site contributes to increased cyclin E1 stability, increased cell survival and resistance to paclitaxel. We then tested the hypothesis that disruption of cyclin E1/CDK2 function would sensitise *BRCA1*-mutant cells to PARP inhibition by enhancing synthetic lethality.

METHODS

Patient demographics and tumor samples

The cohort studied includes samples of 308 breast cancers that were provided by the Kathleen Cuningham Foundation Consortium for research into Familial Breast cancer (kConFab) (http://www.kconfab.org, (15)). Ethics board approval was obtained for patients' recruitment, sample collection and research studies. Written informed consent was obtained from all participants for participation in research studies.

Four tissue microarrays (TMA) represented the 308 cases, of which 222 cases were assessable in terms of sufficient tumor tissue and staining quality for immunohistochemistry (IHC), and *in situ* hybridization (ISH). Patient demographics are shown in Table 1. The World Health Organization criteria were used to histologically classify breast cancers (Table 1). Using patient data from kConFab (15), tumors were classified based on estrogen receptor, progesterone receptor, HER2 status and mutation status of *BRCA1*, *BRCA2*, *CHEK1* and *PALB2* (Table 1). Tumors were further classified as basal-like breast cancer (BLBC) if the tumor tissue was CK5/14 and /or EGFR positive (N=75, 31.6%) and non-basal like breast cancer (NBLBC) where the tumor tissue was both CK5/14 and EGFR negative.

Dual-colour *In Situ* Hybridization (ISH) assay for detection of the 19q12 locus amplification

A 19q12 DNP ISH probe that covers the coding sequences of the *CCNE1* and adjacent *URI1* genes and an insulin receptor (INSR) DIG ISH probe, a surrogate reference located on chromosome 19p13.2, were provided by Ventana Medical Systems (Tucson, AZ). The Ventana dual colour 19q12 DNP/INSR DIG ISH assay was optimised for use on the Ventana ULTRA™ platform as previously described (11). Essentially, 4μm sections from formalin-fixed, paraffin-embedded TMAs were deparaffinized, treated with Cell Conditioning 2 for four cycles of 12 minutes each, followed by ISH protease 3 for 24 min. After co-denaturation, 19q12 DNP and INSR DIG probes were hybridized at 47 °C for 6 hours and washed for 3 cycles at 68 °C for 8 minutes. 19q12 DNP and INSR DIG signals were detected using VENTANA ultraView SISH DNP and VENTANA ultraView RED ISH DIG detection kits, respectively, and counterstained with hematoxylin II and bluing reagent. For image display, the brightness and contrast of each image was adjusted using Photoshop (CS6). Amplification status was calculated as

19q12/INSR ratio ≥ 3 and/or 19q12 average number ≥6. The distribution of 19q12/INSR ratio and 19q12 average number are provided in supplementary methods, supplementary figure S1.

Cyclin E1, FBXW7, USP28 and Phospho cyclin E1 (T62) immunohistochemistry

Previously optimised cyclin E1 mouse monoclonal (HE12) (Santa Cruz Biotechnology, CA), FBXW7 rabbit monoclonal (SP-237) (Spring Bioscience, CA) and the USP28 rabbit polyclonal (HPA006778) (Sigma Aldrich) (11) antibody staining were performed using the Ventana Bench Mark ULTRA™ automated staining platform and the Optiview™ Detection kit. The phospho cyclin E1 (T62) polyclonal antibody (Cell Signaling) was the only IHC grade phospho-cyclin E1 antibody available. Since the cyclin E1 T62 phospho-epitope is potentially labile, we confirmed it was not degraded in formalin fixed paraffin embedded tissue under non-ideal fixation conditions (supplementary methods and supplementary figure S2). In addition, we confirmed cyclin E1 T62 stability and specificity by cyclin E1 knockdown by siRNA level (supplementary methods, supplementary figure S2). All were assessed based on nuclear staining using a 0 to 3+ intensity score. For all proteins, heterogeneous expression was captured using the semi-quantitative H score (16), by adding 3 x % of strongly staining (3+) nuclei to 2 x % of moderately staining (2+) nuclei and 1 x % of weakly staining (1+) nuclei, giving a range of 0 to 300. The distribution of H scores for cyclin E1, phospho cyclin E1 T62, FBXW7 and USP28 is shown in supplementary figure s3, and determination of the assay cut points for each marker is detailed in supplementary methods.

Survival Analysis

Kaplan Meier curves were used to plot the overall survival (OS). Assessment of progression free survival was not possible as progression coincided with death in many cases, therefore a single assessment of overall survival was performed.

Cell lines and drug treatment

Cell lines were obtained from ATCC and cultured in RPMI1640, 5–10% fetal calf serum (FCS) and insulin (10 μg/ml). All cell lines were authenticated by STR profiling (CellBank Australia) and cultured for less than 6 months after authentication. Cyclin E1 was mutated by site-directed mutagenesis as described (17). MDA-MB-468 cells expressing the ecotrophic receptor (18) were infected with pMSCV-IRES-GFP retrovirus expressing cyclin E1 wildtype and mutants as described (19). Subpopulations with graded expression of GFP and cyclin proteins were separated by sterile FACS and matched populations selected based on GFP expression. Cells were treated with the following drugs resuspended in DSMO: Rucaparib (Selleck

Biopharmaceuticals), Paclitaxel (Selleck Biopharmaceuticals) or CYC-065 (Cyclacel Pharmaceuticals).

Cell proliferation and survival analysis

Survival assays were performed on MDA-MB-468 cells set up at 15,000 per 6 cm dishes in 50% conditioned medium. Paclitaxel (0, 2.6, 2.8 nM) was added and refreshed every 6-7 days for 3 weeks. Colonies were fixed with trichloroacetic acid (TCA 16%), and stained with 10% Diff Quik Stain 2 (Lab Aids Pty Ltd). Quantification was done with ImageJ and the ColonyArea plugin (20).

Metabolic rate was assessed by Alamar Blue (Invitrogen) to determine IC50 doses. Synergy assays were performed on indicated cell lines in 96 well plates. The concentration of each drug was increased linearly along each axis of the plate, creating a drug matrix of the different drug concentrations. The highest concentration of each drug was IC80, followed by dilutions of 1/2, 1/4, 1/8, 1/16, and no drug. Cell viability was measured after five days using Alamar Blue. Drug synergy was analysed with Combenefit using the BLISS algorithm (21).

siRNA transfection

Gene-specific siRNAs to *BRCA1* (On-Target Plus siRNAs J-003461-11-0005 and J-003461-12-0005); *CDK2* (J-003236-11, J-003236-12, J-003236-13, J-003236-14); CDK9 (J-003243-9-0002, J-003243-10-0002, J-003243-11-0002, J-003243-12-0002); FBXW7 (J-004264–07-10) and controls [On-Target Plus siCONTROLs (D-001810-10, D-001810-1-4)]; siGENOME Nontargeting siRNA #2 (D-001210-02) were purchased from Dharmacon and transfections carried out as described previously (22).

Western blot analysis

Primary antibodies were BRCA1 (#9010, Cell Signalling Technology), USP28 (EPR4249(2), Abcam), CDK2 (M2, Santa Cruz), cyclin E1 (HE12, Santa Cruz), cyclin E2 (EP454Y, Abcam), β-actin (AC-15; Sigma) and GAPDH (4300; Ambion).

Gene expression analysis

Quantitative reverse transcriptase PCR (qRT-PCR) used inventoried TaqMan probes BRCA1 (Hs01556191_m1), cyclin E1 (Hs00180319_m1) and human RPLPO (4326314E; Applied Biosystems). Analysis was performed as previously described using the ΔΔCT method (23).

Flow cytometry

S-phase percentages were measured by flow cytometric analysis of propidium iodide stained, ethanol fixed cells. Cell cycle specific expression of endogenous cyclin E1 and V5-tagged cyclin E1 constructs were assessed by flow cytometry as described (24), with further details provided in the Supplementary Data.

Comet Assay

The alkaline comet assay was performed using the Trevigen Kit (Maryland, USA) according to the manufacturer's guidelines. HCC1937 cells were seeded in a 6 well plate and treated with CYC-065 at the calculated IC5, IC20 or IC50 dose for 5 days, or treated with 10, 20 or 50nM Cdk2 siRNA or Cdk9 siRNA, or 50nM non targeting siRNA for 72 hours. Slides were imaged with a fluorescence microscope (Leica DM5500) and analysed with ImageJ OpenComet software (v1.3.1, (25)).

TCGA datasets

Breast cancer datasets were downloaded via cBioPortal (8) and the BLBC subset identified from PAM50 definitions from TCGA 2015 (27).

Statistical Analysis

Statistical analysis was performed using Prism Software™ version 7 as indicated for each dataset. Data presented as box and whisker plots includes error bars of minimum to maximum, with mean values indicated. Data are presented as column graphs with mean +/- SEM. All experiments were performed in triplicate, except as indicated.

RESULTS

BRCA1 inactivation associates with high cyclin E1 expression in breast cancer

High cyclin E1 protein expression has been reported in the cancers of a cohort of Ashkenasi Jewish women with familial *BRCA1* mutation (8). Curiously, in HGSOC, *BRCA1* mutation and cyclin E1 amplification are mutually exclusive, presumably because both aberrations drive genomic instability and together they are synthetically lethal (10,11,28). Consequently we examined the KConFab cohort, which is enriched for familial cancer mutations, for co-occurrence of germline *BRCA1* mutation and high cyclin E1 expression.

Cyclin E1 expression was examined by IHC (Figure 1A). High cyclin E1 expression was defined as an H score cut-off of ≥45 based on the overall distribution of cyclin E1 expression (Supplementary Figure S3A), the previously reported frequency of high cyclin E1 expression in (9,29) as well as the best association with outcome (minimal p value, Supplementary Table 1). Overall, germline *BRCA1* mutated cancers had significantly higher cyclin E1 protein than the *BRCA1* non-mutant cases, which included not only *BRCA1* wildtype tumors but also tumors with other germline (*BRCA2*, *PALB2*, or *CHK2)* mutations associated with breast cancer (Figure 1B). Moreover, a significantly larger proportion of germline *BRCA1* mutant cases (82.2%) had detectable cyclin E1 protein (83/101) compared to only 38% of non - *BRCA1* - mutant tumors (46/121) ($P<0.0001$, Fisher Exact test).

Notably, 8 of the germline *BRCA1* wildtype samples had high cyclin E1, and we speculated that these may be *BRCA1* methylated since our cohort is selected for familial breast cancers where *BRCA1* methylation is not infrequent (30). Consequently, we examined the relationship between *BRCA1* methylation and cyclin E1 protein by interrogating the breast cancer dataset of the TCGA. 241 cases had available data for *BRCA1* methylation and cyclin E1 protein expression. Using a cut-off of 0.2 for methylation (31), we found that *BRCA1* methylation had a significant positive correlation with cyclin E1 protein expression ($r=0.647$, $P=0.0082$) (Figure 1C).

Since high cyclin E1 co-occurs with *BRCA1* inactivation, we examined the association between cyclin E1 expression and overall survival in germline *BRCA1* mutated breast cancers in our cohort. High cyclin E1 was associated with a significantly reduced overall survival of patients with *BRCA1* mutation (233.4 vs 426.3 months, $P=0.0419$, HR 0.34, CI 0.130-0.895) (Figure 1D).

CCNE1 amplification is not the primary driver of high expression of cyclin E1 in *BRCA1* mutated cancers

The *CCNE1* gene, located on chromosome 19q12, is a frequent site of amplification in cancer. We assessed *CCNE1* amplification by ISH analysis of tissue sections with 19q12 and INSR probes to determine the 19q12/INSR ratio. A ratio of ≥3 19q12/INSR and/or a 19q12 average number ≥6 was classified as amplified (11), and examples of 19q12 non-amplified and 19q12 amplified are shown in Figure 1E. The frequency of 19q12 (*CCNE1)* amplification in the entire cohort was 30/222 or 13.5%. There was not however a strong correlation between high cyclin E1 protein and *CCNE1* gene amplification (r=0.198, P=0.003, Figure 1F).

We then specifically assessed whether *CCNE1* amplification and *BRCA1* mutation co-occurred. In contrast to HGSOC where these do not co-occur (11), 21/101 (20.8%) of *BRCA1* mutant cases had concurrent 19q12 (*CCNE1*) amplification. This was higher than *BRCA1* wildtype cases, where only 8/121 (6.6%) had 19q12 (*CCNE1*) amplification. The 19q12/INSR ratio was also significantly higher in *BRCA1* mutated than wildtype cancers (Figure 1G). Since 19q12 (*CCNE1*) amplification is associated with poor survival in other cancer types we examined the relationship of amplification with overall survival in *BRCA1* mutated breast cancer. Unlike high expression of cyclin E1, which is predictive of poor survival, 19q12 (*CCNE1)* amplification had no prognostic value for overall survival in *BRCA1* mutated breast cancer (Figure 1H).

The cyclin E1 degradation machinery is disrupted in *BRCA1* mutated breast cancers

Since 19q12 status was only poorly predictive of high cyclin E1 expression, we thus investigated other mechanisms that lead to high cyclin E1 expression. One possibility was disruption of the proteasome mediated degradation of cyclin E1, which occurs frequently in cancer (32). Normal cyclin E1 turnover depends upon phosphorylation within two phospho-degrons on the cyclin E1 protein, of which T62 and T395 are crucial phosphorylation sites. The phosphorylated protein is recognised by the FBXW7 module of the SCF^{FBXW7} complex, and ubiquitinated for degradation. The deubiquitinase USP28 can remove ubiquitin from cyclin E1 and antagonise FBWX7-mediated degradation (Figure 2A). Disruption of this process, ie loss of cyclin E1 phosphorylation, loss of FBXW7 or gain in USP28, would be expected to lead to cyclin E1 stabilisation and accumulation.

We used IHC to assess the cyclin E1 degradation machinery in our familial breast cancer cohort, however, tissue exhaustion prevented analysis of the full cohort. We first assessed

cyclin E1 T62 phosphorylation in 195 cases, with examples shown in Figure 2B. We observed only a moderate positive correlation between cyclin E1 T62 phosphorylation and cyclin E1 expression (r=0.212, P=0.003, Spearman), indicating that a proportion of cancers had less than expected cyclin E1 T62 phosphorylation (Figure 2C). Consequently, we assessed the ratio of cyclin E1 phosphorylation to its absolute expression to determine if phosphorylation was specifically dysregulated in certain subsets of patients. We found that the *BRCA1* and *BRCA2* mutant subsets exhibited a significantly lower T62/cyclin E1 ratio (Figure 2D), indicative of a loss of cyclin E1 phosphorylation in the absence of functional *BRCA1* or *BRCA2*.

When we examined FBWX7 expression (Figure 2E) in 218 cases, we found no correlation between cyclin E1 and FBXW7 across any part of the cohort (Figure 2F). This included no difference between *BRCA1* mutated and wild type subset however slightly higher expression compared to the *BRCA2* mutant subset (Figure 2G). By contrast, USP28 expression (Figure 2H) was moderately correlated with cyclin E1 expression (r=0.420, P<0.0001, Spearman) (Figure 2I). USP28 protein expression was significantly higher in the *BRCA1* mutated breast cancers subsets (Figure 2J).

Overall, these data implicate increased cyclin E1 protein stability, rather than gene amplification, as the cause for high cyclin E1 levels observed in *BRCA1* mutated breast cancer. The *BRCA1* mutated subset had a particular loss of relative T62 phosphorylation, suggesting that this could be driving high cyclin E1. Like cyclin E1 overexpressing HGSOC without gene amplification (11), USP28 was also elevated in *BRCA1* mutated cancers.

***BRCA1* loss leads to cell cycle stabilisation of cyclin E1**

Since the cyclin E1 degradation machinery was deregulated in *BRCA1* mutant cancers across our cohort, we investigated whether cyclin E1 turnover is dysregulated in cell lines with mutant *BRCA1* or *BRCA1* loss. The BLBC cell line HCC1937 has a homozygous *BRCA1* 5382C* mutation and the triple negative breast cancer (TNBC) cell line MDA-MB-436 has a *BRCA1* homozygous deletion. We compared these to 4 cell lines with wildtype *BRCA1*: BT-20 and MDA-MB-468 (BLBC cell lines), MDA-MB-231 (TNBC), and SkBr3 (HER2+). Cells were analysed for the expression of cyclin E1 during the cell cycle using flow cytometry. BT-20, MDA-MB-468, SkBr3 and MDA-MB-231 cells showed a typical downregulation of cyclin E1 during S phase (Figure 3A), which we quantitated by comparing the expression of cyclin E1 during the second half of S phase versus the first half of S phase (Figure 3B). The *BRCA1* defective cell lines showed significantly diminished down -regulation of cyclin E1 during S phase: in HCC1937 cells the cyclin E1 levels do not decrease in S phase, but instead marginally

increased, and MDA-MB-436 had only a small decrease in cyclin E1 absolute expression during S phase (Figure 3A/B).

We then investigated whether knockdown of *BRCA1* was able to recapitulate the cell cycle stabilisation of cyclin E1. First, we treated *BRCA1* wildtype T-47D cells, with two different *BRCA1* siRNAs, siRNA#11 and siRNA#12, and a pool of the two siRNAs. *BRCA1* siRNA led to a reproducible increase of cyclin E1 protein (Figure 3C). Notably, this was specific to cyclin E1, and did not lead to any increase in cyclin E2 protein (Figure 3C). We then specifically assessed whether *BRCA1* knockdown led to changes in the cell cycle expression of cyclin E1, and compared this to the knockdown of the known regulator of cyclin E1, FBXW7. T-47D breast cancer cells were transfected with *BRCA1* Pool siRNA followed by flow cytometry analysis of cells immunoprobed for cyclin E1 and co-stained with propidium iodide. *BRCA1* siRNA treatment led to a significant increase in cyclin E1 protein during late S phase and G_2/M of the cell cycle (Figure 3D), and *FBXW7* siRNA treatment led to cyclin E1 stabilisation at the same points of the cell cycle (Figure 3D). We also examined the expression of *CCNE1* by qRT-PCR following *BRCA1* siRNA treatment and found it was not increased (Figure 3E), confirming that the increase in cyclin E1 expression occurs post-transcriptionally.

BRCA1 dysregulation specifically alters T62 phosphorylation of cyclin E1, but not USP28 expression

Since cyclin E1 cell cycle expression is dysregulated with *BRCA1* disruption and we had observed both loss of T62 phosphorylation and gain of USP28 in our cohort, we sought to confirm *in vitro* that *BRCA1* loss alters either T62 phosphorylation or USP28 expression to lead to stabilisation of cyclin E1.

We first tested whether *BRCA1* knockdown would lead to cyclin E1 stabilisation via upregulation of USP28. siRNA mediated knockdown of USP28 protein in MDA-MB-231 cells led to decreased expression of cyclin E1 (Supplementary Fig S4A). However, *BRCA1* siRNA led only to downregulation of BRCA1 and did not lead to any change in USP28 levels (Supplementary Figure S4B). Thus while USP28 is elevated in *BRCA1* mutant cancers, we could not detect its regulation directly downstream of *BRCA1*.

Subsequently we examined whether there was a direct relationship between changes to *BRCA1* expression and cyclin E1 phosphorylation. We initially treated T-47D cells with *BRCA1* siRNA to increase cyclin E1 expression (Figure 4A). We then immunoprecipitated the lysates with the phospho-cyclin E1 T62 and phospho-cyclin E1 S395 antibodies to examine the relative abundance of phosphorylation of cyclin E1 at these sites after *BRCA1* siRNA treatment. We

identified that cyclin E1 T62 is depleted following *BRCA1* siRNA, whereas cyclin E1 S395 expression is sustained (Figure 4A). Thus the increased expression of cyclin E1 following depletion of Brca1 protein is linked directly to cyclin E1 T62 dephosphorylation.

We performed the converse analysis by comparing UWB1.289 ovarian cancer cells which are *BRCA1* mutant (germline *BRCA1* mutation within exon 11 along with deletion of the wild-type allele) to the UWB1.289/*BRCA1* cell line, which stably re-express *BRCA1* (33). The *BRCA1* restored cell line had higher cyclin E1 T62 expression (Figure 4B).

Since T62 dephosphorylation can increase cyclin E1 protein stability, we subsequently analysed the effect of disrupting the phospho-degrons on cyclin E1 by mutating phospho-sites to alanine to mimic the non-phosphorylated state. We performed site-directed mutagenesis within the two phospho-degrons of cyclin E1 (Figure 4C). We created an N-terminal mutant (T62A, designated N-term), a C-terminal mutant (T383A/S387A, designated C-term) and a combined mutant (T62A/T383A/S387A, designated Dual). We stably overexpressed these cyclin E1 mutants as V5 fusion proteins using pMIG retrovirus, and sorted fractions using co-expressed GFP. We expanded cell populations expressing a similar intensity of GFP signal from each cell line, and confirmed expression using western blotting (Figure 4D).

We examined the effect of each mutant on the stability of the cyclin E1 protein by performing flow cytometry for the V5 tag protein during the cell cycle. We measured the fold change in each of the V5 tagged proteins between early and late S phase (Supplementary Figure S5). All three mutants were significantly more stable than the wildtype "high" cyclin E1 protein (Figure 4E). The T62A site in the N-terminus stabilises the cyclin E1 protein during early S phase, and particularly in combination with the C-terminal phospho-sites of cyclin E1.

We then examined the effect of each mutant on cell proliferation. Overexpression of cyclin E1 wildtype and each of the cyclin E1 mutants led to a significant increase in BrdU incorporation compared to the vector control (Figure 4F).

Following this we examined whether these mutants were able to alter the survival of cells when treated with Paclitaxel, a standard chemotherapy for BLBC (34). We treated vector, cyclin E1, and cyclin E1 mutant cells with cytotoxic doses of Paclitaxel, and monitored survival by colony forming assay after 3-4 weeks. Only the Dual mutant, significantly increased survival compared to wildtype cyclin E1 overexpression (Figure 4G/4H).

Overall, *BRCA1* loss led to decreased cyclin E1 T62 phosphorylation, which in turn can increase cyclin E1 protein stability and increase the percentage of cells in S phase. Cyclin E1

T62 was also critical in combination with other cyclin E1 phosphorylation sites to increase cell survival in Paclitaxel.

Synergistic targeting of cyclin E1 and *BRCA1*

Our data showing that *BRCA1* loss has a direct role in sustaining elevated cyclin E1 protein levels during S phase, suggested the utility of co-targeting these proteins. *BRCA1* deficiency leads to susceptibility to PARP inhibition, whereas cyclin E1 activates the therapeutically targetable kinase CDK2. CDK2 also has important roles in DNA repair (35), leading to increased sensitivity of *BRCA1/2* mutant cancers to CDK2 inhibitors (36). We thus hypothesised that treating *BRCA1* mutant cancers with a combination of CDK2 inhibitors and PARP inhibitors would be synergistic due to the simultaneous blockade of cyclin E1 dependent proliferation and exacerbated synthetic lethality from PARP inhibitors due to the additional DNA damage resulting from CDK2 inhibition.

Initially we tested whether CDK2 inhibition induces DNA damage, by treating *BRCA1* mutant HCC1937 cells with a CDK inhibitor, CYC065. CYC065 targets CDK2, CDK5 and CDK9, but with the highest specificity to CDK2 (Cyclacel Pharmaceuticals). After establishing a dose response curve for CYC065 (Supplementary Figure S6A), we identified a dose dependent induction of DNA damage using the alkaline Comet assay, which detects both double strand and single strand DNA breaks (Figure 5A). We subsequently confirmed that DNA damage was occurring via CDK2 action by performing comet assays after CDK2 and CDK9 siRNA treatment. CDK2 siRNA treatment led to an increase in tail moment detection after 72h treatment at all doses (Figure 5B), whereas no effect was seen on DNA damage with CDK9 siRNA treatment (Figure 5C).

We then examined the effect of combining CDK2 inhibition with PARP inhibition. We treated a *BRCA1* wildtype cell line (BT20) and two *BRCA1* mutant cell lines (MDA-MB-468 and HCC1937) with CDK2 inhibitor CYC-065 and Rucaparib (a PARP inhibitor). After first establishing dose response curves (Supplementary data S6B), we treated the cells alone or in combination with IC50 doses of CYC-065 and Rucaparib. We found that the combination treatment was significantly more effective than either drug used as a single agent in the *BRCA1* mutant cell lines (Figure 5D&E), but not in the *BRCA1* wildtype cell line (Figure 5F). We then treated HCC-1937 cells with intersecting dose curves of CYC065 and Rucaparib, and identified a significant synergy between the two drugs using BLISS analysis (Figure 5G).

DISCUSSION

We here identify that cyclin E1 is stabilised in *BRCA1* mutated breast tumors via reduced phosphorylation on cyclin E1 Threonine 62, and this is associated with decreased overall survival of patients. The stabilisation of cyclin E1 leads to increased S phase and survival, consistent with the known role of cyclin E1 in the activation of CDK2 to regulate G_1 phase progression and entry into S phase (39).

Cyclin E1 T62 phosphorylation was originally believed to be of lesser importance in the turnover of cyclin E1, but our work here, and that of others (42), shows that it can have potentially strong effects in tumorigenesis. We find that T62A mutation contributes to cell survival in combination with mutation of the other major phospho-sites of the protein, as well as individually increasing protein stability and BrdU incorporation. Mutation of cyclin E1 T74A and cyclin E1 T393A (equivalent to human cyclin E1 T62 and T395) in a mouse model led to much higher cyclin E1 levels in hematopoietic and epithelial cells compared to T393A mutation alone, as well as haematopoietic neoplasia (42). Delayed mammary gland involution after pregnancy was also observed exclusively in the presence of the T74A mutation (42), highlighting its potential importance in breast tumorigenesis.

The kinase responsible for T62 phosphorylation has not been identified, though it is strongly suspected to be a CDK2 auto-phosphorylation site based on a loose consensus sequence for CDK2 around the T62 site, and the timing of T62 phosphorylation early in G_1 phase soon after partnering with CDK2 (40). Consequently, increased T62 auto-phosphorylation may be the result of a direct physical interaction between BRCA1 and cyclin E1/CDK2 (41) or through downstream effectors of BRCA1 action. We observed that *BRCA1* mutation-mediated stabilisation only occurred for the cyclin E1 protein, but not the closely related ortholog cyclin E2. This is despite the phospho-T62 site being conserved between the two proteins (39). However, downstream of T62 in cyclin E1 there lies a GSK3-B consensus site at S58 which is hypothesised to require T62 phosphorylation for priming, and this site is absent from cyclin E2 (24).

Our finding that *BRCA1* regulates the turnover of cyclin E1 thereby increasing proliferation and survival, provides a new therapeutic opportunity to enhance the synthetic lethality of PARP inhibitors by co-targeting the cyclin E1/CDK axis. The PARP inhibitors olaparib and talazoparib were recently approved by the FDA for use as monotherapies in patients with

metastatic germline *BRCA1/2*-mutated breast cancer based on significant improvement in progression free survival compared to chemotherapy (37). Nevertheless, the efficacy of PARP inhibitors is limited as there was no improvement in overall survival, partial and complete responses are infrequent, and combinations with chemotherapeutic agents are limited by myelosuppression (38). Consequently, there is a compelling unmet clinical need to identify targeted therapies with a different side effect profile that enhance the synthetic lethality of PARP inhibitors.

Pan-CDK inhibitors have been used in pre-clinical TNBC models to create HR deficiency thereby inducing synthetic lethality in combination with PARP inhibitors (26). Notably, the pan-CDK inhibitor Dinaciclib is being trialled in combination with the PARP inhibitor Veliparib in a cohort that includes *BRCA1* associated TNBC (Clinical Trial ID NCT01434316). While Dinaciclib inhibits cancer cells through CDK12, it may show additional benefit from synthetic lethality between CDK2 inhibition and PARP inhibition.

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Figure 1: **Cyclin E1 is elevated in *BRCA1* deficient cancers, and predicts poor prognosis.**

A: Microscope images of high and low expression of cyclin E1 (IHC). Scale bar is 50µM. **B:** Cyclin E1 protein expression (H score) in wildtype, *BRCA1*, *BRCA2, and PALB2*/*CHEK2* mutated cancers, analysis by one-way ANOVA. **C:** Scatter plot of TCGA breast cancer cohort cyclin E1 protein expression (RPPA) versus *BRCA1* methylation by HM27 array. Dashed line indicates cutoff between methylated and non methylated. Correlation analysis of cyclin E1 protein and *BRCA1* methylation performed across the methylated subset, r=Spearman coefficient. **D:** Kaplan Meier curves of overall survival in the KConfab cohort comparing *BRCA1* mutated cyclin E1 high cases versus *BRCA1* mutated cyclin E1 low cases. **E:** Microscope images of 19q12 non-amplified and 19q12 amplified breast cancer cases (ISH); inset shows representative example of each. Scale bar is 20µM. **F:** Scatter plot of cyclin E1 protein expression versus *CCNE1* (19q12/INSR ratio) amplification status in the KConfab cohort. r=Spearman coefficient. **G:** 19q12/INSR ratio (ISH) cases compared to each of wild type, *BRCA1*, *BRCA2, PALB2*/*CHK2* mutated cancers in the KConfab Cohort, analysed by one way ANOVA. **H:** Kaplan Meier curves of overall survival of *BRCA1* mutated breast cancer comparing 19q12 amplified and non-amplified subsets.

Figure 2: **Gain of USP28 and loss of cyclin E1 T62 phosphorylation are associated with *BRCA1* mutation.**

A: Schematic of cyclin E1 turnover. **B:** Microscope images of IHC staining of breast cancer sections with high and low phospho-cyclin E1 T62. Scale bar is 50µM. **C:** Scatter plot of cyclin E1 expression versus phospho-cyclin E1 T62 expression, r=Spearman coefficient. **D:** Phospho cyclin E1 T62/cyclin E1 ratio of expression in wildtype, *BRCA1* mutated, *BRCA2* mutated, *PALB2*/*CHK2* mutated subsets of the KConfab Cohort, analysed by one way ANOVA. **E:** Microscope images of IHC staining of BC sections with high and low FBXW7. Scale bar is 50µM. **F:** Scatter plot of cyclin E1 expression versus FBXW7 expression, r=Spearman

coefficient. **G:** FBXW7 expression in wildtype, *BRCA1* mutated, *BRCA2* mutated, *PALB2*/*CHK2* mutated subsets of the KConfab Cohort, analysed by one way ANOVA. **H:** Microscopic images of IHC staining of BC sections with high and low USP28 expression. Scale bar is 50μM. **I:** Scatter plot of cyclin E1 expression versus USP28 expression, r=Spearman coefficient. **J:** USP28 expression in wildtype, *BRCA1* mutated, *BRCA2* mutated, *PALB2*/*CHK2* mutated subsets of the KConfab Cohort.

Figure 3: **Cyclin E1 protein is stabilized in the absence of functional BRCA1.**

A: Breast cancer cell lines (SkBr3, MDA-MB231, BT20 and MDA-MB-468, MDA-MB-436 and HCC1937) were analysed by flow cytometry for intracellular cyclin E1 and DNA content (propidium iodide). **B:** Quantitation of fold change in expression of cyclin E1 from early S phase to late S phase as measured by flow cytometry in (A.). Data is mean +/- SEM, analysed by t-test. **C:** T-47D cells were treated with siRNAs for 48h (siRNAs: NTP (non-targeting pool) Brca1 Pool, #11 and #12), and lysates western blotted for BRCA1, cyclin E1, cyclin E2 and GAPDH. **D:** T-47D cells treated for 48h with BRCA1 pool siRNA, FBXW7 pool siRNA and non-targeting pool siRNA were analysed by flow cytometry for cyclin E1 expression and DNA content (propidium iodide).The geometric mean expression of cyclin E1 at G_1, early S, mid S, late S and G_2M phase was quantitated for each treatment, and data is mean +/- SEM. **E:** T-47D cells treated for 48h with BRCA1 pool siRNA, FBXW7 pool siRNA and non-targeting pool siRNA were analysed by qRT-PCR for *CCNE1* expression, and data is mean +/- range of duplicate experiments assayed in triplicate.

Figure 4: ***BRCA1* loss leads to decreased cyclin E1 T62 phosphorylation, which alters protein stability, and contributes to proliferation and cell survival.**

A: T-47D cells were treated with siRNAs for 48h (siRNAs: NTP (non-targeting pool) Brca1 Pool, #11), and lysates western blotted for Brca1, cyclin E1, and GAPDH. Lysates were also immunoprecipitated with phospho T62-cyclin E1, phospho-T395 cyclin E1 and a rabbit IgG

control, and immunoprecipitates western blotted for cyclin E1. **B:** UWB1.289 and UWB1.289 BRCA1 cells were western blotted for BRCA1, phospho-T62 cyclin E1, cyclin E1 and GAPDH. **C:** Schematic of site-directed mutagenesis of phospho-sites of cyclin E1. **D:** MDA-MB-468 cells were retrovirally infected with V5-tagged cyclin E1 constructs (N-term, C-term, Dual, wildtype), sorted by flow cytometry for populations with matched GFP expression, and lysates western blotted for cyclin E1 and GAPDH. **E:** MDA-MB-468 cells expressing cyclin E1 constructs (N-term, C-term, Dual, wildtype) were analysed by flow cytometry for V5-cyclin E1 expression and DNA content (propidium iodide).The geometric mean expression of cyclin E1 at early S and late S phase was quantitated for each treatment, and the fold change from early to late S phase is shown as the mean +/- SEM of triplicate experiments. Data analysed by one-way ANOVA. **F:** MDA-MB-468 cells expressing cyclin E1 constructs (N-term, C-term, Dual, wildtype) were analysed by flow cytometry for BrdU incorporation. Data is the mean +/- SEM of triplicate experiments, analysed by one-way ANOVA. **G:** MDA-MB-468 cells expressing cyclin E1 constructs (N-term, C-term, Dual, wildtype) were treated with Paclitaxel (0nM, 2.6nM, 2.8nM) for 3 weeks, and colony formation detected with Diff Quick Stain 2. **H:** Colony formation was quantitated using the ColonyArea ImageJ plugin from triplicate assays. Data is the mean +/- SEM analysed by two-way ANOVA.

Figure 5: **CDK2 inhibition induces DNA damage to synergise with PARP inhibition in Brca1 defective breast cancer cells.**

A: HCC1937 cells treated with CYC-065 or vehicle for 5 days were analysed by alkaline Comet assay, 90-300 tails quantitated/treatment in duplicate experiments. Data analysed by one-way ANOVA. Representative images shown. **B:** HCC1937 cells treated with CDK2 siRNA or Non-targeting Pool siRNA for 72h were analysed by alkaline Comet assay, 190-250 tails quantitated/treatment. Data analysed by one-way ANOVA. Representative images shown. **C:** HCC1937 cells treated with CDK9 siRNA or Non-targeting Pool siRNA for 72h were

analysed by alkaline Comet assay, 190-250 tails quantitated/treatment. Data analysed by one-way ANOVA. Representative images shown,. **D:** HCC1937 cells were treated with IC50 doses of CYC-065, Rucaparib, or CYC-065 + Rucaparib for 5 days, as well as vehicle control, and cell viability measured by Alamar Blue. Data analysed by one way ANOVA. **E:** MDA-MB-436 cells were treated with IC50 doses of CYC-065, Rucaparib, or CYC-065 + Rucaparib for 5 days, as well as vehicle control, and cell viability measured by Alamar Blue. Data analysed by one way ANOVA. **F:** BT20 cells were treated with IC50 doses of CYC-065, Rucaparib, or CYC-065 + Rucaparib for 5 days, as well as vehicle control, and cell viability measured by Alamar Blue. Data analysed by one way ANOVA. **G:** HCC1937 cells were treated with doses of CYC-065 and Rucaparib for 5 days, and viability measured by Alamar Blue. Synergy analysis was performed by BLISS, where blue indicates synergy, and red indicates antagonism. Data is pooled from 5 replicates.

Table 1: **Patient Characteristics**

Parameter			
Age			
Mean		43.7	
Range		19 - 74	
Histological Subtype		**N**	**%**
Infiltrating duct carcinoma, NOS		179	80.6
Lobular carcinoma, NOS		9	7.2
Carcinoma (NOS)		16	4.1
Infiltrating duct and lobular carcinoma		8	3.6
Medullary carcinoma, NOS		5	2.3
Others		5	2.3
Grade			
1		19	8.6
2		61	27.5
3		119	53.6
No Grade		23	10.4
Nodal status			
N0		95	42.8
N1 (1-3)		38	17.1
N2 (4-9)		14	6.3
N3 (10 or >)		6	2.7
Not known		69	31.1
Germline *BRCA1/2* Status			
BRCA1		101	45.5
BRCA2		45	20.3
CHEK2		2	0.9
PALB2		4	1.8
Wildtype		70	31.5
BLBC (Ck5/14 and /or EGFR) positive (75)			
ER/PR Positive, HER2 Negative		6	8
ER/PR Positive,HER2 Positive		3	4
ER/PR Negative, HER2 Positive		13	17.3
Triple Negative		51	68
Not tested		2	2.7
NBLBC (Ck5/14 and /or EGFR) negative (143)			
ER/PR Positive, HER2 Negative		80	55.9
ER/PR Positive,HER2 Positive		30	21
ER/PR Negative, HER2 Positive		5	3.5
Triple Negative		24	16.8

Not tested		4	4
Progression-free survival (all cases)			
Events	60		
Median months	160.8		
Progression-free survival (*BRCA1* mutant)			
Events	28		
Median months	157.5		
Overall survival (all cases)			
Events	57		
Median months	161.2		
Overall survival (*BRCA1* mutant)			
Events	26		
Median months	161.4		

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FIGURE 1: Cyclin E1 protein is elevated in *BRCA1* deficient cancers, and predicts poor prognosis

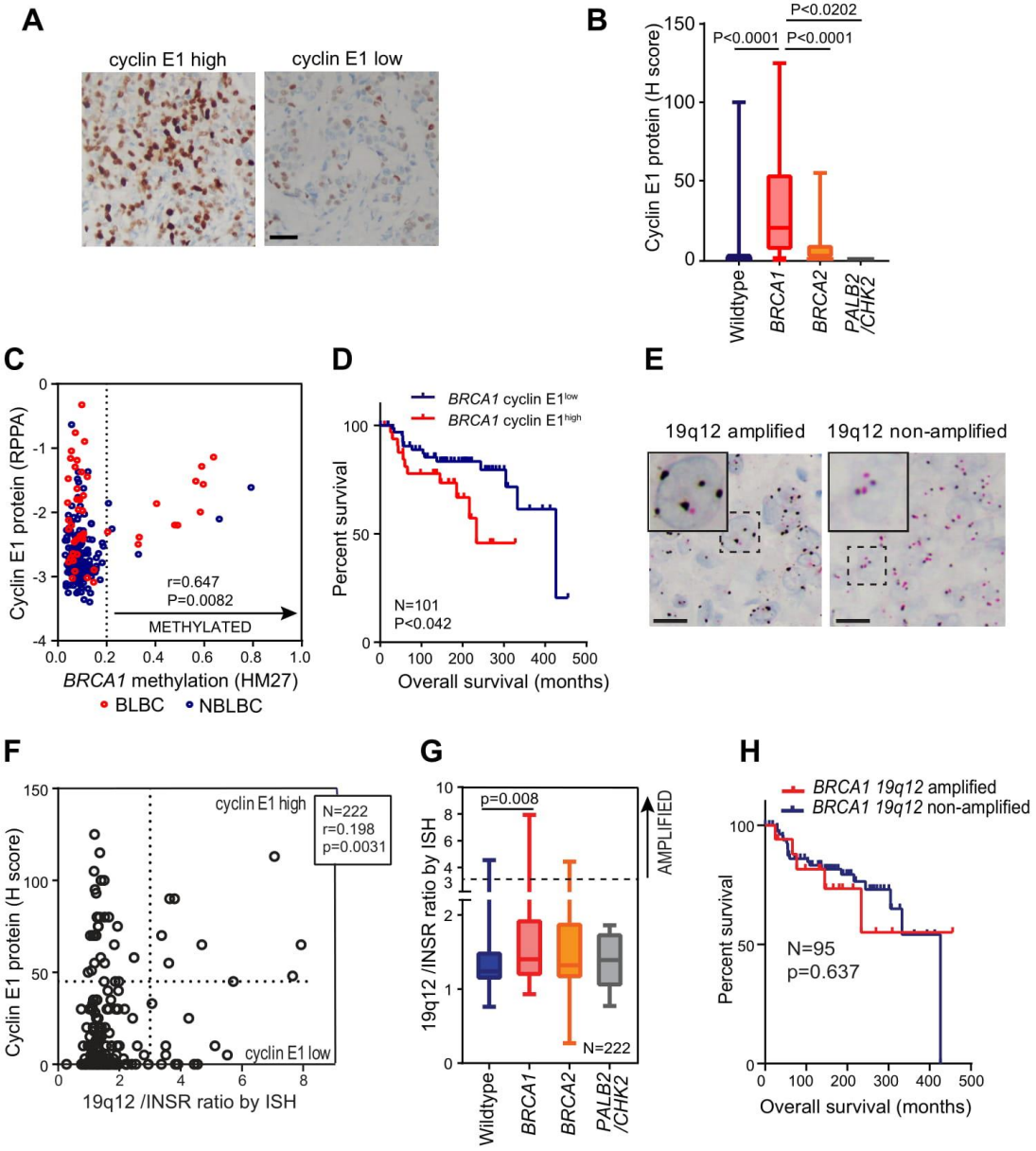

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FIGURE 2: Loss of cyclin E1 T62 phosphorylation and gain of USP28 expression are associated with *BRCA1* mutation.

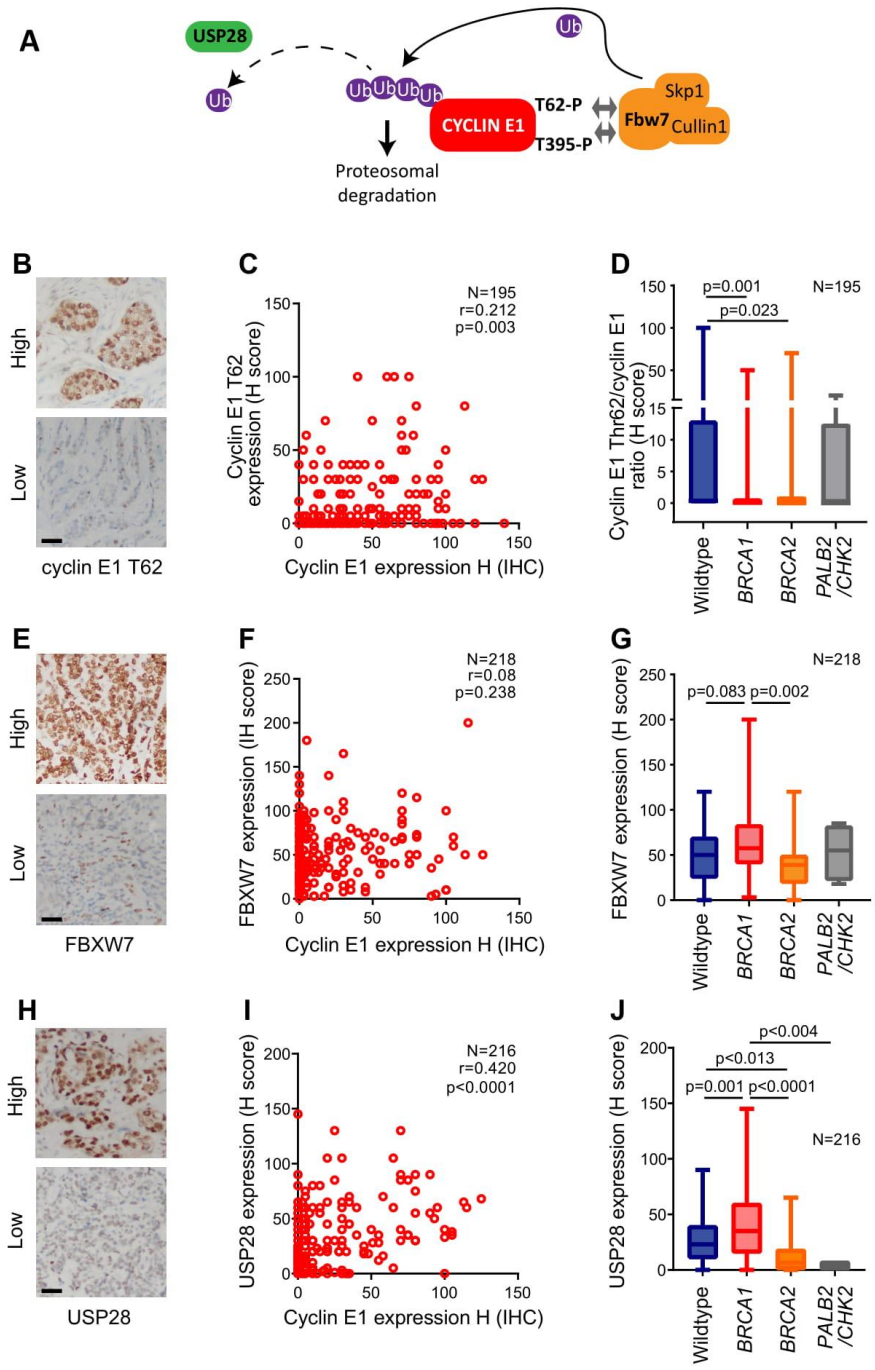

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FIGURE 3: Cyclin E1 protein is stabilised in the absence of functional BRCA1.

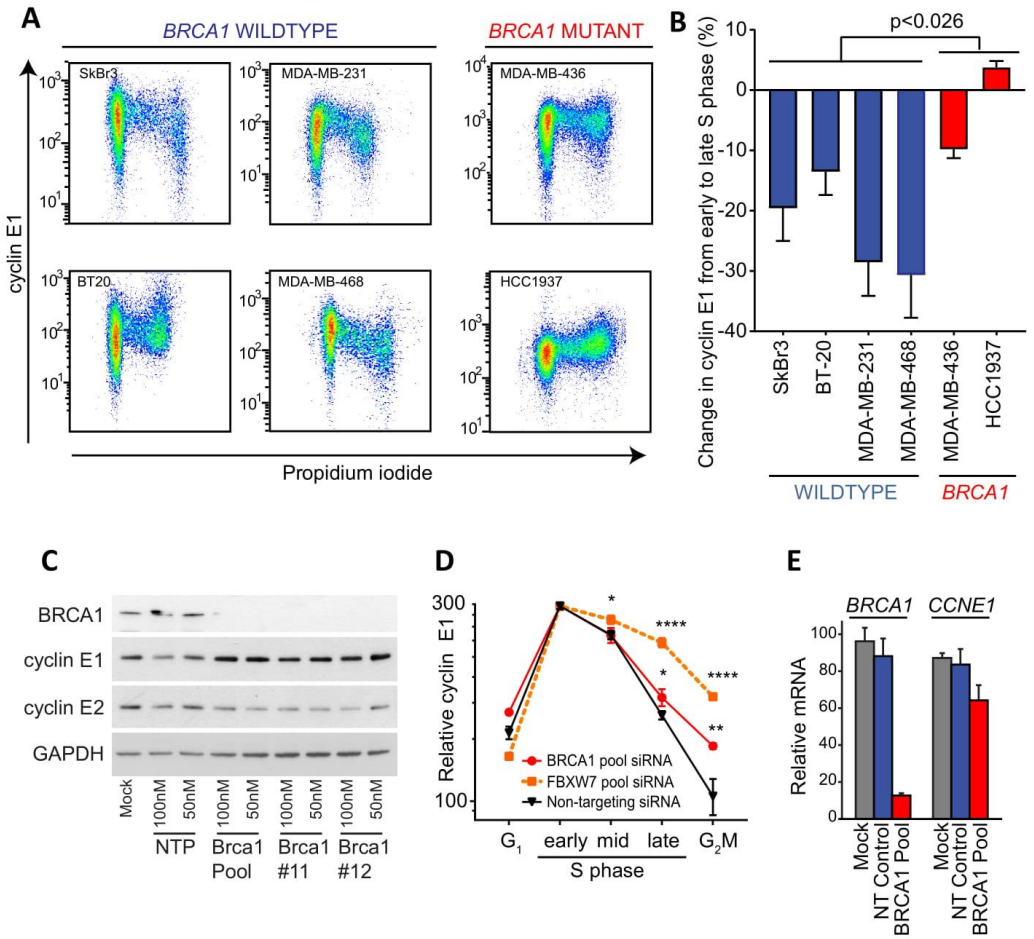

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FIGURE 4: *BRCA1* loss leads to decreased cyclin E1 T62 phosphorylation, which alters protein stability, and contributes to proliferation and cell survival.

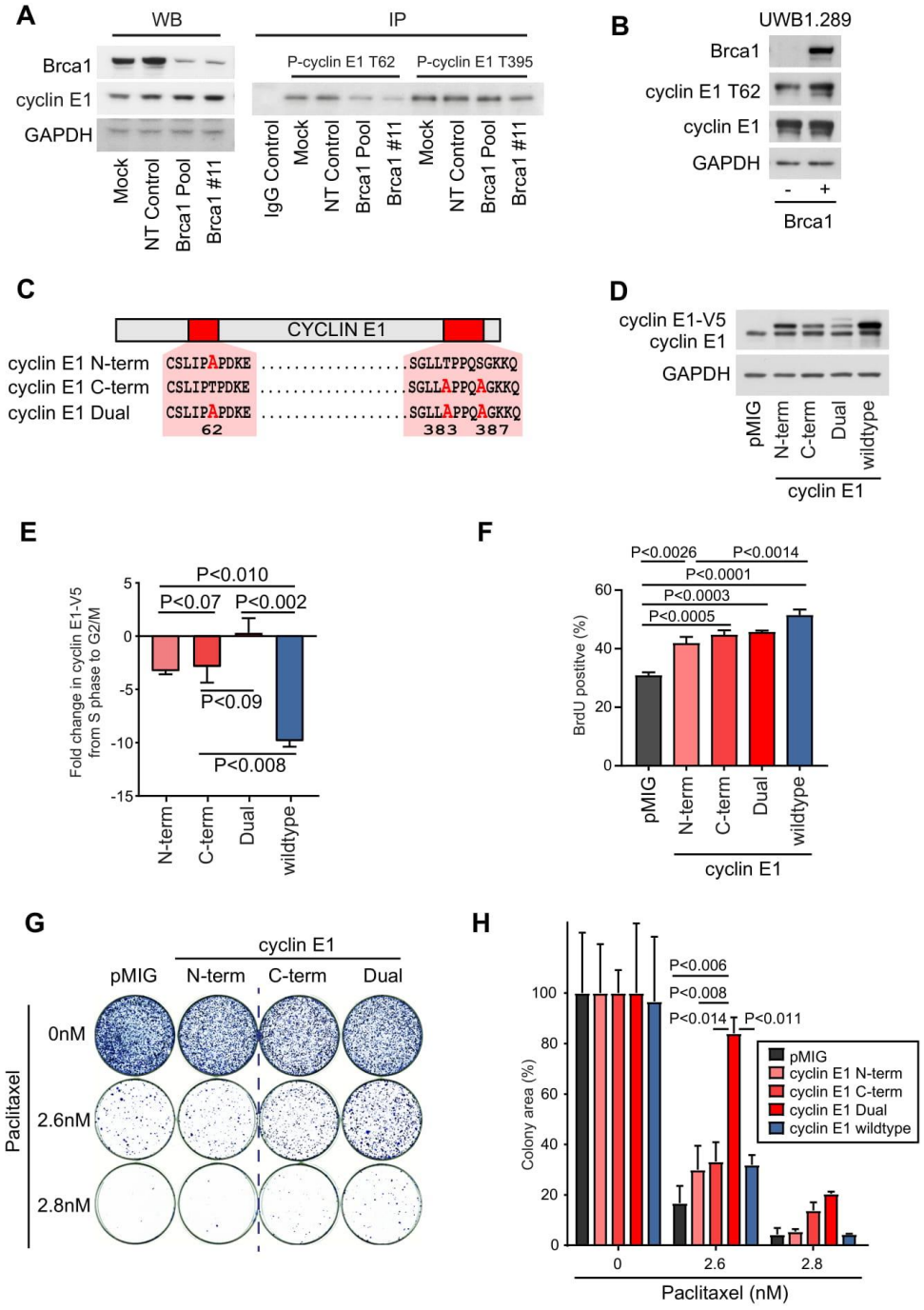

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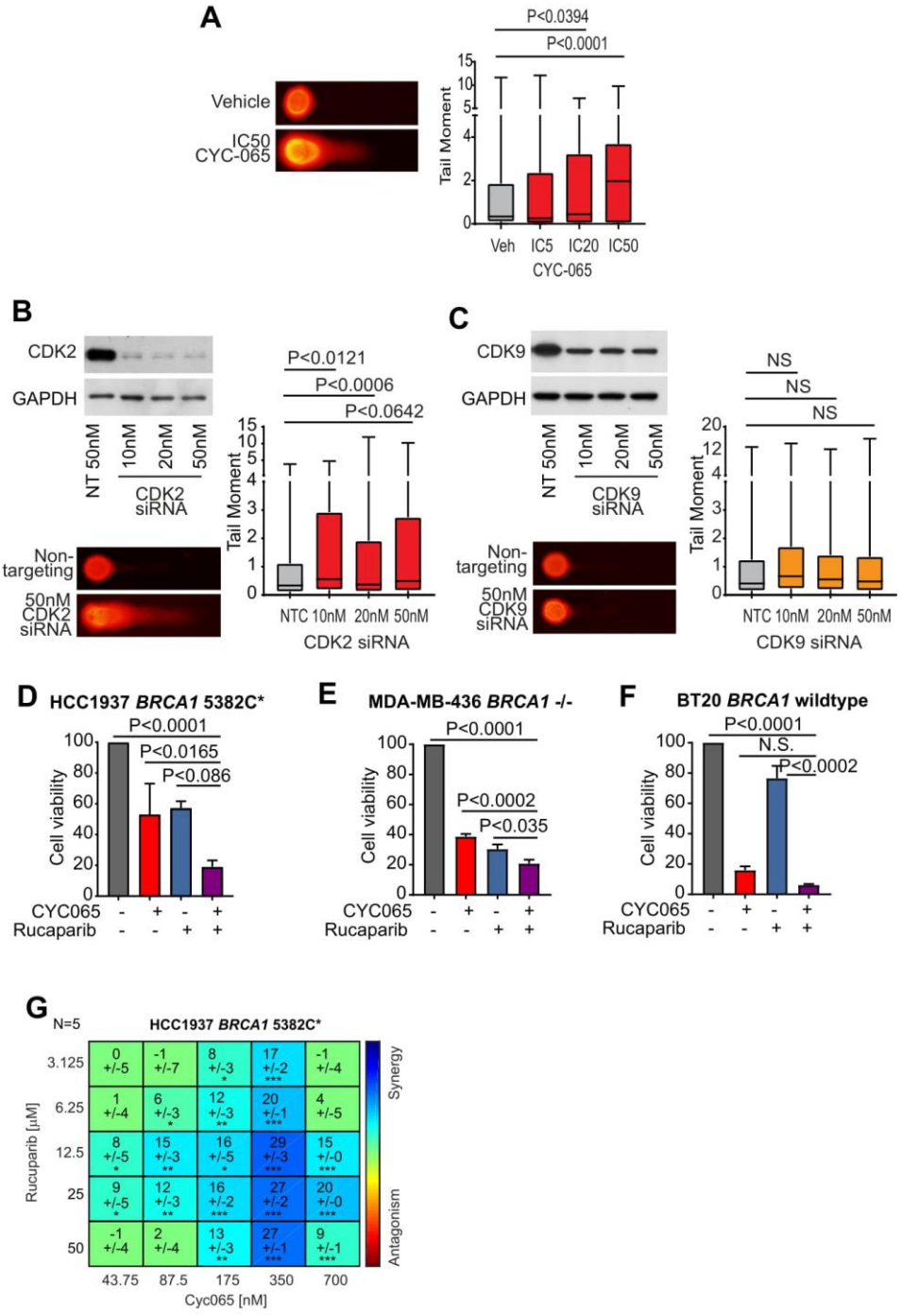
FIGURE 5: CDK2 inhibition induces DNA damage to synergise with PARP inhibition in *BRCA1* defective breast cancer cells



Supplementary information

Cut point determination for *in situ* hybridization

Cut-off thresholds for 19q12 amplification were guided by our previously optimised 19q12 ISH assay (1). Amplification status was calculated as 19q12/INSR ratio ≥ 3 and/or 19q12 average number ≥6. The distributions of 19q12/INSR ratio and 19q12 average number are provided in supplementary Figure S1A and S1B respectively.

T62 antibody validation

In order to confirm the robustness and the stability of T62 antibody detection of protein under different fixation conditions we used paraffin embedded cell blocks of HCT116 colorectal cancer cell lines that were **v**ariably fixed in formalin at variable conditions. Different fixation conditions included both variable time to fixation, between pellet stage and 4% paraformaldehyde (PFA) addition; and variable time of fixation, the duration for which the pellet was fixed in 4% PFA. Sections from all variably formalin fixed paraffin embedded cell blocks were stained using the optimized protocol and revealed acceptable robustness of detection (Supplementary Figure S2A).

To further assess the reliability of the p T62 antibody, we tested specificity of the T62 antibody detection by examining cyclin E1 siRNA treated MDA-MB-436 cells. Western blot revealed that cyclin E1 and cyclin E1 T62 expression is lower in cyclin E1 siRNA knocked down cells, which should lead to reduced cyclin E1 T62 expression. Sections from formalin fixed paraffin embedded blocks of each of the cyclin E1 siRNA and control treated cells were stained with the optimized protocol and revealed aligned protein expression to those seen in western blot (Supplementary Figure S2B).

As those experiments revealed the stability, reliability and specificity of T62 antibody, the antibody was next used to stain sections from TMAs of samples of patients enrolled in the KConfab cohort.

H score and cut point determination for immunohistochemistry

The overall distribution of H score of cyclin E1 expression in all cases (N=222), ranged between 0 and 125 with a median of 5 (Supplementary Figure S3A). The cutoff between high and low cyclin E1 H score was determined by two factors: (1) the previously reported frequency of high cyclin E1 expression in breast cancer and (2) the best association with outcome (minimal p value, Supplementary Table 1), leading to the selection of an H score cut-off of 45. The distribution of mean H scores for phospho-cyclin E1 T62, FBXW7 and USP28 are shown in Supplementary Figure S3B, S3C and S3D respectively. The median H scores were 5 (range: 0 - 100) for cyclin E1 T62 (N=195), 50 (range: 0 - 200) for FBXW7 (N=218) and 20 (range: 0 - 145) for USP28 (N=216). Exhaustion of tissue cores led to unequal numbers of assessable cases for each antibody.

Flow cytometry for cell cycle specific expression of cyclin E1 and V5

Cells were incubated overnight at 4°C with antibodies to the E-cyclins (E1: EP435E (Epitomics) or V5 (Invitrogen)) followed by 1 h incubation at room temperature with secondary antibodies (allophycocyanin conjugated goat anti-mouse, fluorescein 5-isothiocyanate conjugated goat anti-rabbit, Jackson Immunoresearch), co-stained with 10μg/mL PI (Sigma) for 2–5h, and incubated with 50μg/mL RNase A (Sigma). Flow cytometry was performed on a FACSCanto (BD Biosciences). Data were analyzed using FlowJo (2). Cells were separated into early, mid and late S phase by identifying the G_1 and G_2/M peaks and then partitioning the intervening S phase. Each S phase partition was analysed for expression of cyclin E1 or the V5 tag, where signal intensity was calculated by obtaining the geometric mean signal per cell in gated regions (3,4). Cyclin E1 turnover was calculated as the ratio of expression of late S phase/early S phase. V5-tagged protein turnover was calculated as the ratio of expression of late S phase/early S phase normalized to V5 expression in the pMIG control cell line.

Supplementary references

1. Aziz D, Etemadmoghadam D, Caldon CE, Au-Yeung G, Deng N, Hutchinson R, et al. 19q12 amplified and non-amplified subsets of high grade serous ovarian cancer with overexpression of cyclin E1 differ in their molecular drivers and clinical outcomes. Gynecologic oncology 2018;151(2):327-36 doi 10.1016/j.ygyno.2018.08.039.
2. FlowJo. 7.2.5. Ashland, OR, USA: Tree Star Inc.; 2008.
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4. Flow Cytometry: Principles and Applications. Macy MG, editor. Totowa, New Jersey: Humana Press; 2007. 290 p.

Supplementary figure legends

Supplementary Figure S1: **Distribution of 19q12 ISH scores in breast cancer cases**

A: The distribution of 19q12/INSR ratio by ISH. **B:** The distribution of 19q12 average number.

Supplementary Figure S2: **Phospho cyclin E1 T62 antibody optimisation**

A : Phospho cyclin E1 T62 expression in HCT116 cell lines at different times to fixations (TTF) and time of fixation (TOF). **B:** Phospho cyclin E1 T62 expression in MDA-MB-436 cells treated with non-targeting control siRNA and cyclin E1 siRNA.

Supplementary Figure S3: **Distribution of IHC H scores in breast cancer cases**

A: The distribution of mean IHC H scores for cyclin E1 expression in all breast cancer cases. **B:** The distribution of mean H scores for cyclin E1 T62 expression. **C:** The distribution of mean H scores for FBXW7 expression. **D:** The distribution of mean H scores for USP28 expression.

Supplementary Figure S4: *BRCA1* siRNA does not alter expression of USP28

A: USP28 siRNA was transfected into MDA-MB-468 cells, and lysates collected after 48h and western blotted for USP28, cyclin E1 and GAPDH. **B:** *BRCA1* siRNA was transfected into MDA-MB-468 cells, and lysates collected after 48h and western blotted for Brca1, USP28 and GAPDH.

Supplementary Figure S5: Stability of cyclin E1 constructs in S phase

A: Cells expressing each of the cyclin E1 constructs (pMIG, N-term, C-term, Dual) were analysed by flow cytometry for DNA content (propidium iodide) and V5 (using anti-V5 antibody). Cells were partitioned into early, middle and late S-phase using propidium iodide expression, and V5 expression is shown (x-axis).

Supplementary Figure S6: Dose-response curves for cell viability assessment in the BLBC cell lines MDA-MB-468, BT20 and HCC1937.

A: Cells were treated with a range of doses of CYC-065 for 5 days and relative Alamar Blue staining measured. **B:** Cells were treated with a range of doses of Rucaparib for 5 days and relative Alamar Blue staining measured. Experiments performed in triplicate.

Supplementary Table 1: Determination of cyclin E1 cut-off based on frequency and minimum p-value

H score cut-off	% cyclin E1 high	OS P value	OS HR	CI of HR
40	42.7	0.067	0.866	0.365-2.054
45	40	0.040	0.866	0.365-2.054
50	36	0.063	0.548	0.233-1.289
55	33.3	0.055	0.548	0.233-1.289
60/65	29.3	0.198	0.866	0.365-2.054
70	26.7	0.101	0.866	0.365-2.054

Supplementary Table 2: Distribution of *BRCA1* mutated and *BRCA1* non-mutated breast cancer cases versus 19q12 amplification and cyclin E1 expression status

All cases (222)	**19q12 amplified (30)**		**19q12 non amplified (192)**	
	Cyclin E1≥45	**Cyclin E1<45**	**Cyclin E1≥45**	**Cyclin E1<45**
***BRCA1* mutated (101)**	9 (8.9%)	13 (12.9%)	24 (23.8%)	55 (54.4%)
***BRCA1* non-mutated (121)**	2 (1.8%)	6 (5.4%)	6 (5.4%)	107 (96.4%)
Total	11	19	30	162

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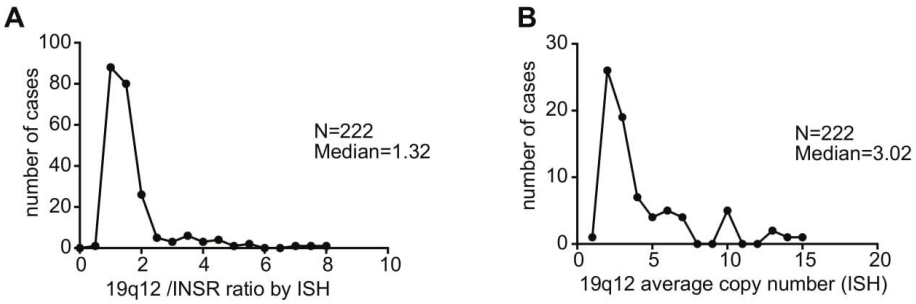
Supplementary Figure S1: Distribution of 19q12 ISH scores in breast cancer cases



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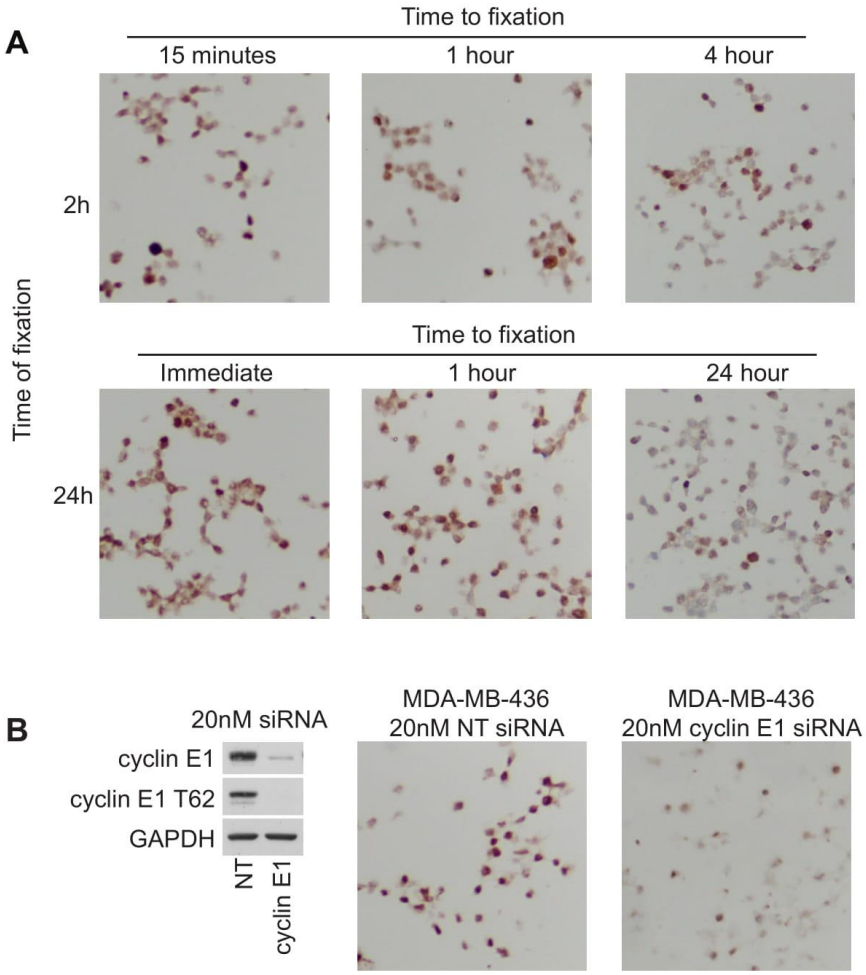
Supplementary Figure S2: Phospho cyclin E1 T62 antibody optimisation



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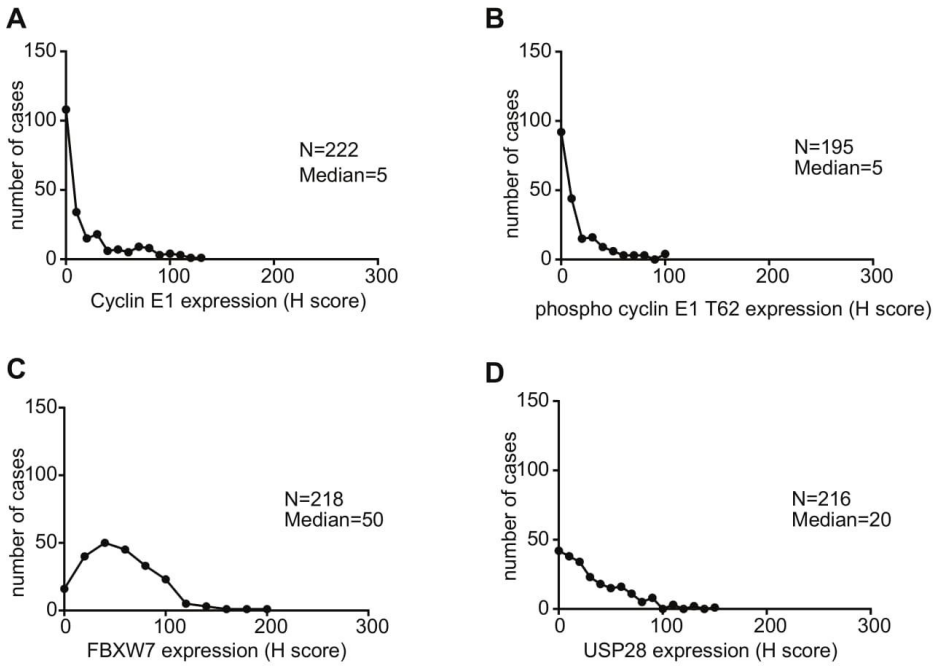
Supplementary Figure S3: Distribution of H scores in breast cancer cases



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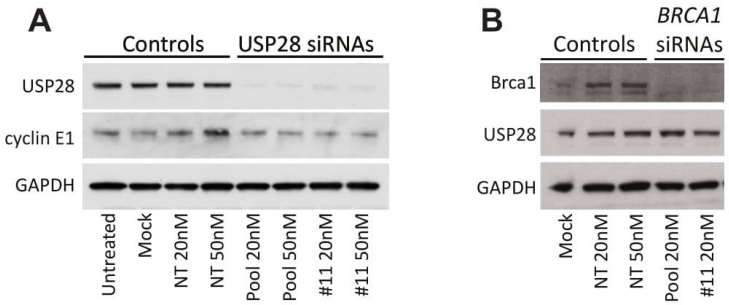
Supplementary Figure S4: *BRCA1* siRNA does not alter expression of USP28



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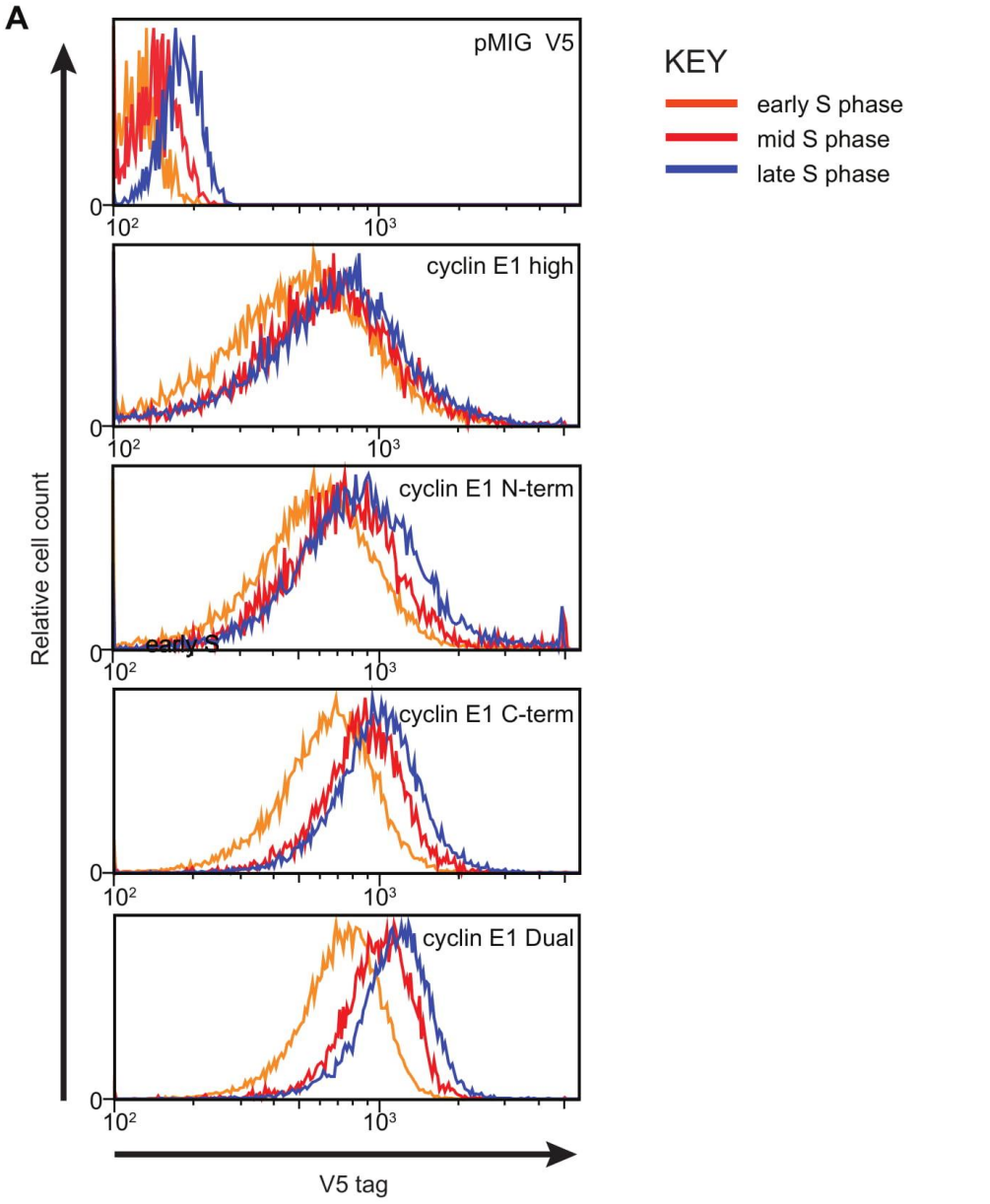
Supplementary Figure S5: Stability of cyclin E1 constructs in S phase



B

$$\text{Relative protein turnover of } \chi = \frac{\text{geometric mean (late S phase) } \chi}{\text{geometric mean (late S phase) pMIG-V5}} \div \frac{\text{geometric mean (early S phase) } \chi}{\text{geometric mean (early S phase) pMIG-V5}}$$

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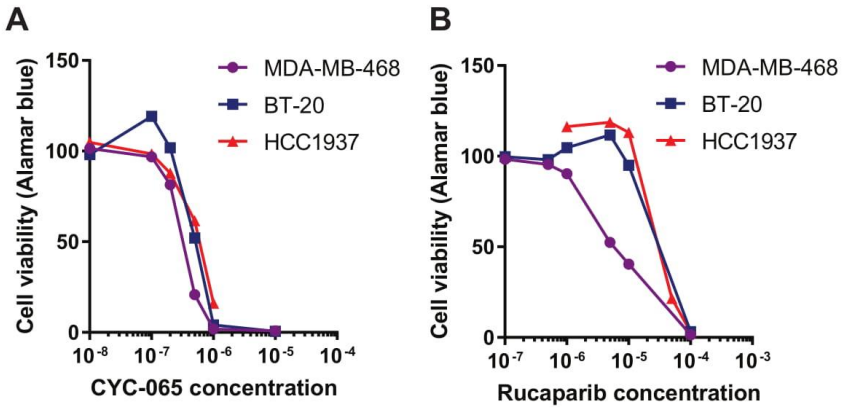
Supplementary Figure S6: Dose-response curves for cell viability assessment in the BLBC cell lines MDA-MB-468, BT20 and HCC1937



Chapter 5: Cyclin E1 as a shared biomarker of HGSCO and BLBC

Preface

This chapter compares systematically cyclin E1 deregulation in the supposedly similar cancers of HGSOC and BLBC

This chapter is drafted in a format to be published as short communication format for: **International Journal of Cancer**, as per the following:

Aziz D, Lee,C., Chin,V., Fernandez,K., kConFab Investigators, AOCS, Etemadmoghadam, D., Bowtell,D. Waring,P. Caldon, C. "High cyclin E1 protein is a shared biomarker for specific subsets of basal-like breast cancer and high grade serous ovarian cancer"**(under review).**

The drafted manuscript and associated supplementary information are presented in this chapter.

High cyclin E1 protein is a shared biomarker for specific subsets of basal-like breast cancer and high grade serous ovarian cancer

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KEYWORDS

Cyclin E1; BRCA1; CCNE1 amplification; 19q12; Basal like breast cancer; High grade serous ovarian cancer

Short communication format for:

International Journal of Cancer

Abstract

Basal-like breast cancer (BLBC) and high grade serous ovarian cancer (HGSOC) have a shared aetiology and similar molecular signatures. With the increased drive towards biomarker driven treatments, it is important to determine which subsets of these disease have the greatest similarity in terms of biomarkers of response. Cyclin E1 gene (*CCNE1*) amplification and protein overexpression are common features of both BLBC and HGSOC. Using a BLBC patient cohort, we find cyclin E1 protein overexpression but not *CCNE1* amplification to be prognostic for overall survival. By comparison, both cyclin E1 protein expression and *CCNE1* gene amplification are prognostic for HGSOC, which we establish with a meta-analysis of existing studies. We examined the nature of *CCNE1* amplification to find that HGSOC has an enhanced degree of amplification of *CCNE1* with 5.6x copies compared to 3.9x *CCNE1* copies in BLBC, and this was associated with vastly different expression of cyclin E1 protein. We find that BLBC cancers, with or without *CCNE1* amplification, have similarities in dysregulation of regulators of cyclin E1 protein stability. Since cyclin E1 is only expressed at high levels in HGSOC and not BLBC, we examined the consequence of very high cyclin E1 expression in a BLBC cell line, MDA-MB-468. Moderate overexpression of cyclin E1 showed increased survival upon treatment with Paclitaxel, however very high expression of cyclin E1 gave less survival benefit. We conclude that high cyclin E1 expression associated with *CCNE1* amplification gives rise to a unique phenotype in HGSOC, but otherwise the high expression of cyclin E1 protein may be a similar prognostic marker for BLBC and HGSOC.

Background

Both basal-like breast cancer (BLBC) and high grade serous ovarian cancer (HGSOC) are aggressive diseases with high frequency of *Tp53* mutation, *BRCA1* mutation and/or *CCNE1* gene amplification [1]. Overall, the genomic and transcriptomic signatures of BLBC are more similar to HGSOC than to other breast cancer subtypes [1]. The significant co-occurrence of both tumour types in patients suggests that they could have a common aetiology [2]. Multiple clinical trials are now grouping these patients to access larger cohorts when testing new drugs or drug combinations, especially PARP inhibitors in the context of *BRCA1*/*BRCA2* mutation (eg. Clinical Trial NCT01623349, NCT02203513, NCT00679783, EMBRACE).

Recently, we described separate subsets of HGSOC, one where cyclin E1 amplification and *BRCA1/BRCA2* mutation were mutually exclusive (the *CCNE1*^{Amp} subset), and another with *BRCA1/BRCA2* mutation co-occurred with high cyclin E1 protein overexpression (the cyclin E1^{Hi} subset) [3]. We subsequently described co-occurrence between *BRCA1* mutation and cyclin E1 protein in breast cancer, in a cohort that is highly enriched for BLBC. In order to guide biomarker driven treatment we aimed to determine the similarities and differences between these disease subsets. Accordingly, we assessed the overlap between BLBC and HGSOC subsets in terms of the relative expression of cyclin E1, *CCNE1* gene amplification, *BRCA1/2* status and impact on outcome.

Materials and methods

Patient cohorts

We reinterrogated previously collected data from cohorts from the Australian Ovarian Cancer Study (AOCS) and Kathleen Cunningham Foundation Consortium for research into Familial Breast cancer (KConFab).

Meta-analysis

The prognostic power of high cyclin E1 protein expression and *CCNE1* amplification in overall survival was assessed by meta-analysis using Revman 5.0 software. Publications were identified through searches of the PubMed database, Google Scholar and SCOPUS, and included in the meta-analysis (Supplementary Figures 2A-D). To be included, studies needed to report the primary outcome measure of overall survival rate at 50 months, and these data were extracted by D.A. and C.E.C.. The cohorts also needed to have >40 patients, and report the HGSOC and BLBC subgroups separately.

BLBC was defined according to cytokeratin 5/6/14 staining, or PAM50 status. Bias within each meta-analysis was assessed with FUNNEL plots.

Public Datasets

BLBC public datasets were downloaded via cBioPortal [4] and identified from PAM50 defined subsets of TCGA 2015 [5] and METABRIC [6]. Estimates of *CCNE1* amplification in cell lines was downloaded via cBioPortal [4] from the Cancer Cell Line Encyclopaedia [7]. Gene expression and ASCAT estimates of ploidy from HGSOC and BLBC cohorts were accessed through COSMIC (December 2018).

Cell lines and treatment

Cell lines were obtained from ATCC and cultured in RPMI 1640, 5–10% fetal calf serum and insulin (10 μg/ml). All cell lines were authenticated by STR profiling (CellBank Australia) and cultured for less than 6 months after authentication. True HGSOC and BLBC cell lines were classified as described [8, 9].

MDA-MB-468 cells expressing the ecotrophic receptor [10] were infected with pMSCV-IRES-GFP retrovirus expressing cyclin E1 as described [11]. Subpopulations with graded expression of GFP and cyclin proteins were separated by sterile FACS. Cells were treated with Paclitaxel (Selleck Biopharmaceuticals). S-phase percentages and cellular ploidy were measured by flow cytometric analysis (BD FACS Canto II) of propidium iodide–stained, ethanol-fixed cells. Survival assays were performed on MDA-MB-468 cells set up at 15,000 per 6 cm dishes in 50% conditioned medium. Paclitaxel (0, 2.6, 2.8, 3.2, 3.6 nM) was added and refreshed every 6-7 days for 3 weeks. Colonies were fixed with trichloroacetic acid (TCA 16%), and stained with 10% Diff Quik Stain 2 (Lab Aids Pty Ltd). Quantification was done with ImageJ and the ColonyArea plugin [12].

Results and discussion

High cyclin E1 protein expression and *CCNE1* amplification occur in BLBC, but only high protein expression is prognostic for poor overall survival

High expression of cyclin E1 has been observed in BLBC [13] and is associated with poor survival in young breast cancer patients [14]. To confirm these reports, we first explored the expression of cyclin E1 in PAM50-defined basal-like breast cancer using the TCGA reverse phase protein array (RPPA) dataset [15] which showed that cyclin E1 protein is indeed significantly increased in BLBC compared to other PAM50 – defined breast cancer subtypes (Figure 1A). BLBC also had the highest rate of *CCNE1* amplification of breast cancer subsets, as determined by analysis of the METABRIC cohort (Figure

1B). Since PAM50 molecular typing does not completely overlap with immunohistochemical (IHC) definitions for BLBC, we further quantified expression, by H score, of cyclin E1 in IHC - defined BLBC using the KConFab familial breast cancer cohort (unpublished, Aziz et al 2019). As expected, cyclin E1 expression was significantly higher in BLBC compared to non-BLBC (NBLBC) cases (P<0.0001) (Figure 1C).

We then examined the relationship between cyclin E1 expression and outcome in BLBC. High cyclin E1 expression was defined as an H score cut-off of ≥45 (unpublished, Aziz et al 2019). BLBC cases with cyclin E1 expression ≥45 had significantly shorter overall survival compared to the cyclin E1 low subset (<45) (368.9 vs 426.3 months, P=0.040, HR 0.39, CI 0.156-0.958) (Figure 1D). By contrast, high expression of cyclin E1 was not predictive of poor overall survival in NBLBC (Supplementary Figure 1).

Amplification of the *CCNE1* gene is a major contributor to high cyclin E1 expression in HGSOC [3, 16], so we assessed whether *CCNE1* amplification is associated with high cyclin E1 expression in BLBC. 19q12 (*CCNE1*) was amplified in 19/75 (25.3%) and 11/147 (7.5%) of tumors within the BLBC and NBLBC subsets respectively (P=0.001) (Figure 1E). Since 19q12 (*CCNE1*) amplification is associated with poor survival in other cancer types we examined the relationship of amplification with overall survival in BLBC. Unlike high expression of cyclin E1, which is predictive of poor survival, 19q12 (*CCNE1)* amplification had no prognostic value for overall survival in BLBC (Figure 1F).

Meta-analysis identifies that only cyclin E1 protein expression is prognostic for BLBC, but both *CCNE1* amplification and cyclin E1 protein are prognostic for HGSOC

The inconsistency between the reported significance of *CCNE1*^{Amp} on overall survival across HGSOC and BLBC [17-19] prompted us to combine our data with published reports in a meta-analysis (Figure 2A). We identified 6 HGSOC cyclin E1^{Hi}, 6 HGSOC *CCNE1*^{Amp}, 4 BLBC cyclin E1^{Hi} and 3 BLBC *CCNE1*^{Amp} studies that were suitable for inclusion in the meta-analysis (PRISMA diagram, Supplementary Figure 2A-D). Studies were weighted to patient number and analysed using a random effects model. Cyclin E1^{Hi} and *CCNE1*^{Amp} were both associated with greater risk of cancer death in HGSOC (Figure 2B/2C). The odds ratio for cyclin E1^{Hi} was 1.63 ([1.04, 2.54], P<0.03) and the odds ratio for *CCNE1*^{Amp} was 1.90 ([1.11, 3.26], P=0.02). By contrast only cyclin E1^{Hi}, and not *CCNE1*^{Amp}, was associated with decreased overall survival in

BLBC (Figure 2D/E). FUNNEL plots did not overall show significant bias (Supplementary Figure 3A-D). An exception was the study of Sapoznik *et al* (2017) on cyclin $E1^{Hi}$ HGSOC. This was the smallest scale study (n=40) included in any meta-analysis which may have contributed to its variance from other published studies.

The *CCNE1*^{Amp} subset of HGSOC expresses higher cyclin E1 protein than *CCNE1*^{Amp} BLBC

The meta-analysis showed that *CCNE1*^{Amp} is prognostic of poor overall survival for HGSOC, but not for BLBC. Across the studies in the meta-analysis, the rate of amplification of BLBC (16.0%) was similar to HGSOC (21.7%) (Supplementary Figure 4). We examined the level of protein expression related to *CCNE1*^{Amp} in BLBC from the KConFab cohorts and HGSOC from the AOCS cohort [3](unpublished , Aziz et al 2019). These cohorts were stained by the same protocol and used the same positive control, allowing their direct comparison. Each study was also within the confidence interval of the meta-analysis. Protein expression was ~2.4 fold higher in *CCNE1*^{Amp} HGSOC than in *CCNE1*^{Amp} BLBC (P<0.0001, Figure 3A). Since HGSOC *CCNE1*^{Amp} had significantly more protein than BLBC *CCNE1*^{Amp}, we assessed the degree of 19q12 amplification in each subset. We found that the average *CCNE1* 19q12/INSR ratio in *CCNE1*^{Amp} BLBC was 3.9, which was significantly lower than the average ratio of 5.6 in HGSOC (P<0.0047, Figure 3B). In fact there was no correlation between cyclin E1 protein expression and the degree of *CCNE1*^{Amp} across the cohort (Figure 3C).

We further examined the protein expression of BLBC and HGSOC in cell lines with different reported levels of amplification. Most BLBC cell lines have no amplification or low level amplification and we do not see a significant difference in protein expression in these models (Figure 3D). Cyclin E1 expression in these models was less or comparable to expression in the OvCa3 HGSOC model which lacks *CCNE1*^{Amp}. One BLBC cell line with high level *CCNE1*^{Amp} (HCC1806) shows comparable cyclin E1 protein expression to the HGSOC *CCNE1*^{Amp} cell line, CaOv3.

From these data we conclude that while cyclin E1 expression is high in BLBC, *CCNE1* does not undergo the same degree of amplification in BLBC, and it does not lead to the same extremes of cyclin E1 expression as in HGSOC.

***CCNE1*$^{Non\text{-}Amp}$ HGSOC and BLBC show similar changes in drivers of cyclin E1 stability**

Our previous studies have identified that the proteins USP28 and FBXW7, which are involved in regulating cyclin E1 stability, are dysregulated in HGSOC [3]. We evaluated

whether these two proteins could be common drivers of high cyclin E1 protein expression in the absence of the very high *CCNE1*^{Amp} observed in HGSOC.

First, we assessed the prevalence of high USP28 in *CCNE1*^{Amp} and *CCNE1*$^{Non\text{-}Amp}$ subsets of BLBC and HGSOC, defined by equal to or greater than median USP28 expression for each cohort. *CCNE1*$^{Non\text{-}Amp}$ HGSOC, *CCNE1*^{Amp} BLBC and *CCNE1*$^{Non\text{-}Amp}$ BLBC all showed increased USP28 expression associated with high cyclin E1 protein. By contrast, the *CCNE1*^{Amp} HGSOC subset had significantly fewer USP28^{Hi} cancers compared to all other subsets (p=0.001, Fischer Exact) (Figure 3E). We then performed a similar analysis of FBXW7 expression. The *CCNE1*^{Amp} HGSOC subset was significantly enhanced for FBXW7^{Lo} cases compared to *CCNE1*$^{Non\text{-}Amp}$ HGSOC, *CCNE1*^{Amp} BLBC and *CCNE1*$^{Non\text{-}Amp}$ BLBC (Figure 3E).

Overall, high USP28 in association with increased cyclin E1 protein is a common feature of *CCNE1*$^{Non\text{-}Amp}$ HGSOC and all BLBC, whereas low FBXW7 associates with *CCNE1*^{Amp} HGSOC.

Moderate expression of cyclin E1 provides the greatest survival advantage in BLBC

Since *CCNE1*^{Amp} BLBC did not express cyclin E1 at the same level as in *CCNE1*^{Amp} HGSOC, this led us to speculate that very high expression of cyclin E1 does not lead to a growth or survival advantage for BLBC cells. We tested this hypothesis by overexpressing cyclin E1 at different levels in a BLBC cell line. We selected MDA-MB-468 which has modest endogenous expression of cyclin E1 (Figure 3D). Using retroviral infection, we overexpressed cyclin E1.V5-fusion protein using the pMSCV vector, which co-expresses GFP using an IRES sequence. Populations expressing different levels of cyclin E1.V5 were selected by flow cytometry and validated by western blot (Figure 4A). Moderate overexpression of cyclin E1 was commensurate with non-genomic overexpression seen in ovarian cancer CaOv3 cells, whereas high overexpression corresponded to the levels seen with *CCNE1*^{Amp} in the HGSOC cell line, OvCa3. The third population, expressing "extreme" levels of cyclin E1, was >2-fold higher than observed with amplification in OvCa3 cells.

We determined the effect that these expression levels had on the cell cycle by performing propidium iodide staining for DNA content. There was a dose dependent effect, where the S phase fraction of the cells increased with increased expression of cyclin E1 (Figure 4B). We examined the colony forming ability of these cell lines following Paclitaxel treatment to determine the effect of cyclin E1 expression on survival. Paclitaxel reduces colony forming ability, but cells overexpressing any level

of cyclin E1 form more colonies at 2.6nM Paclitaxel (Figure 4C/D). Moderate level cyclin E1 overexpression led to a marked increase in colony number at higher doses of Paclitaxel, but "high" and "extreme" cyclin E1 were not effective at increasing survival.

We then examined how expression of cyclin E1 related to overall survival in our patient cohort. The patient cohort was split three ways based on the cyclin E1 H score, low: 0-40, moderate: 40-80 and high: >80. Compared to low expression, moderate expression associated with poor overall survival (P<0.046, Logrank, Mantel-Cox) whereas high expression showed no significant different in overall survival (P<0.326, Logrank, Mantel-Cox) (Figure 4E).

In HGSOC the amplification of *CCNE1* is specifically associated with an increase in cancer cell ploidy[23]. We thus examined our cyclin E1 overexpressing cell lines for differences in ploidy by measuring >4N content by propidium iodide staining. We observed that moderate overexpression of cyclin E1 did not lead to a significant increase in polyploid cells, but both "high" and "extreme" overexpression had higher levels of polyploid cells, which was significantly increased in the case of the "extreme" overexpression (Figure 4F). We assessed the association between cyclin E1 expression and polyploidy in BLBC and HGSOC. In the METABRIC dataset, using ASCAT estimations of ploidy derived from COSMIC, 56% of BLBC and 63% of HGSOC are high ploidy cancers (Supplementary Figure 5A-B). When the expression of CCNE1 mRNA is compared between diploid and polyploid cancers for both cancer types, CCNE1 mRNA is similar in both diploid and polyploid BLBC, but is significantly higher in the HGSOC polyploid compared to diploid cancers (Figure 4G).

Overall these data demonstrate that moderate level protein overexpression of cyclin E1 increases proliferation in BLBC, but does not show an association with increased ploidy as observed in HGSOC.

Conclusions

Here we describe that moderate expression of cyclin E1 is associated with poor overall survival in BLBC, but very high expression by *CCNE1*^{Amp} does not frequently occur. We speculate that BLBC may not show a high degree of *CCNE1*^{Amp} because of the prevalence of *BRCA1* mutation and the related "Brcaness" phenotype in BLBC. "Brcaness" describes tumours with *BRCA1/BRCA2*–like properties which most likely derive from similar homologous recombination defects. *CCNE1*^{Amp} is mutually exclusive with *BRCA1/2* mutation[16] and "Brcaness" in ovarian cancer[24], which is thought to account for ~60% of HGSOC[24]. By contrast, almost all of BLBC shows a homologous repair deficiency signature[25], which could preclude the high amplification

of *CCNE1* through synthetic lethality [26]. We note that the MDA-MB-468 cell line used in this study, despite not having a *BRCA1* mutation[27], presents with a "Brcaness" profile[28] and low homologous repair function[29], which could explain its intolerance to high cyclin E1.

There are several studies describing *CCNE1*^{Amp} in other breast cancer subtypes. *CCNE1*^{Amp} was associated with poor progression-free survival in a small cohort of Trastuzamab treated HER2+ disease (n=34) [20], but not in a similar study of 185 patients [21]. In a study of chemotherapy treated triple negative breast cancer (TNBC) disease (n=59), which included some BLBC cases, *CCNE1*^{Amp} was associated with poor overall survival [22]. We note that including this study in our meta-analysis does not alter the conclusion that *CCNE1*^{Amp} is not predictive of overall survival in BLBC/TNBC (Supplementary Figure 6A-B), and speculate the association of *CCNE1*^{Amp} with poor survival may be due to the other TNBC subtypes in this cohort. Further larger scale studies are required to conclusively define the relationship between *CCNE1*^{Amp} and survival across other subtypes of breast cancer.

We conclude that *CCNE1*^{Amp} has different associations with protein expression and patient outcome in BLBC and HGSOC. Therapies and biomarkers developed for *CCNE1*^{Amp} HGSOC, an essentially *BRCA1*/BRCA2 wildtype population, are thus unlikely to be transferable to BLBC. However, cyclin E1 overexpressing BLBC and *CCNE1*$^{Non\text{-}Amp}$ HGSOC show great similarity in their drivers and prognostic value. Common features are *BRCA1* mutation and possible "Brcaness" phenotypes, as well as high expression of the ubiquitin serine protease USP28. Consequently these cancers may have similar therapeutic targets, and it may be valuable to group these patients in the future.

Figure legends

Figure 1: High cyclin E1 protein expression, but not *CCNE1* amplification, is prognostic for outcome in BLBC

A: Cyclin E1 protein expression by RPPA (Reverse Phase Protein Array) in 273 TCGA breast cancer cases categorized into PAM50 defined subtypes Luminal A, Basal like, HER2 enriched and Luminal B. B. Relative amplification across the molecular subtypes of breast cancer, including BLBC, based on amplification (GISTIC=2) in the METABRIC dataset (n=1974). C: Cyclin E1 protein expression in BLBC compared to non-BLBC (NBLBC) in the KConFab cohort, analysed by T test. D: Kaplan Meier curves of overall survival in the KConFab cohort comparing BLBC cyclin E1 high cases to BLBC cyclin E1 low cases. D: 19q12/INSR ratio (ISH) in BLBC compared to non-BLBC in the KConFab Cohort, analysis by T test. F: Kaplan Meier curves of overall survival of BLBC comparing 19q12 amplified and non-amplified subsets.

Figure 2: Meta-analysis identifies that only cyclin E1 protein expression is prognostic for BLBC, but both *CCNE1* amplification and cyclin E1 protein expression are prognostic for HGSOC

A. Schematic of meta-analysis of cyclin E1 protein expression and *CCNE1* amplification in BLBC and HGSOC. B-E. Meta-analyses performed on studies using random-forest analysis of a dichotomous value of overall survival at 50 months. B. Meta-analysis of association of cyclin $E1^{Hi}$ protein with overall survival in HGSOC. C. Meta-analysis of association of $CCNE1^{Amp}$ with overall survival in HGSOC. D. Meta-analysis of association of cyclin $E1^{Hi}$ protein with overall survival in BLBC. E. Meta-analysis of association of $CCNE1^{Amp}$ with overall survival in BLBC.

Figure 3: *CCNE1* undergoes low range amplification in BLBC, leading to moderate cyclin E1 protein expression

A. Cyclin E1 protein expression related to amplification status in kConFab and AOCS. Analysed by non-parametric Mann-Whitney test. B. The relative 19q12/INSR ration of the amplified subsets of HGSOC and BLBC. Compared using a non-parametric Mann-Whitney test. C: Scatter plot of cyclin E1 protein expression versus *CCNE1* (19q12/INSR ratio) amplification status in the KConFab cohort. Correlation analysis performed across all breast cancers (BC) and the BLBC and NBLBC subsets, r=Spearman coefficient. D. Western blots of HGSOC and BLBC cell lines with different degrees of *CCNE1* gene amplification. Amplification levels derived from [7]. *** = High amplification (GISTIC=2), * = low level amplification (GISTIC=1), - =

unamplified or allelic loss. (GISTIC=-1 or 0). E. Contingency analysis of the *CCNE1*^{Amp} HGSOC, *CCNE1*$^{Non\text{-}Amp}$ HGSOC, *CCNE1*^{Amp} BLBC and *CCNE1*$^{Non\text{-}Amp}$ BLBC subsets, and how high cyclin E1 relates to either high USP28 or Low Fbxw7 in these subsets. Fisher Exact tests were used for contingency. All pairs without p-values were non-significant.

Figure 4: Moderate cyclin E1 expression in the BLBC cycE1^{Hi} subset is associated with increased proliferation, but not genomic instability

A. Western blot of cyclin E1 expression in HGSOC cell lines (CaOv3 and OvCa3) and in MDA-MB-468 BLBC cell line, with cyclin E1-V5 overexpression from the pMIG vector at Moderate, High and Extreme levels. GAPDH used as loading control. B. Cell cycle phase of cyclin E1 overexpressing MDA-MB-468 cells determined by propidium iodide staining and flow cytometry. C. MDA-MB-468 cells expressing different levels of cyclin E1 were treated with Paclitaxel for 3 weeks, and analysed by colony forming assay. D. Quantitation of colony forming assay performed in triplicate and analysed by two-way ANOVA. E. Kaplan-Meier overall survival curve of basal-like breast cancer patients with low, medium or high expression of cyclin E1 protein. F. Percentage of polyploid cells in cell lines overexpressing cyclin E2. Polyploidy measured by propidium iodide staining and identification of >4N populations on flow cytometry. Performed in triplicate and analysed by one-way ANOVA. G. Expression of *CCNE1* mRNA in non-genome doubled (NGD) and genome doubled (GD) cancers identified as BLBC or HGSOC from the METABRIC dataset.

List of abbreviations

BLBC	basal like breast cancer
HGSOC	high grade serous ovarian cancer
METABRIC	Molecular Taxonomy of Breast Cancer International Consortium
mRNA	messenger RNA
OS	overall survival
TNBC	Triple negative breast cancer

Supplementary figure legends

Supplementary Figure S1: High expression of cyclin E1 is not predictive of poor overall survival in NBLBC

Supplementary Figure 2: PRISMA analysis to identify studies to include in meta-analyses

Supplementary Figure 3: FUNNEL plots of meta-analyses

Supplementary Figure 4: *CCNE1* amplification rates across the meta-analyses of *CCNE1* relationship to overall survival in BLBC and HGSOC

Supplementary Figure 5: Diploid and genome doubled cancers in Metabric/TCGA

Supplementary Figure 6: Meta-analysis of *CCNE1* amplification in combined BLBC/ER negative breast cancer

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Figure 1: High cyclin E1 protein expression, but not *CCNE1* amplification, is prognostic for outcome in BLBC

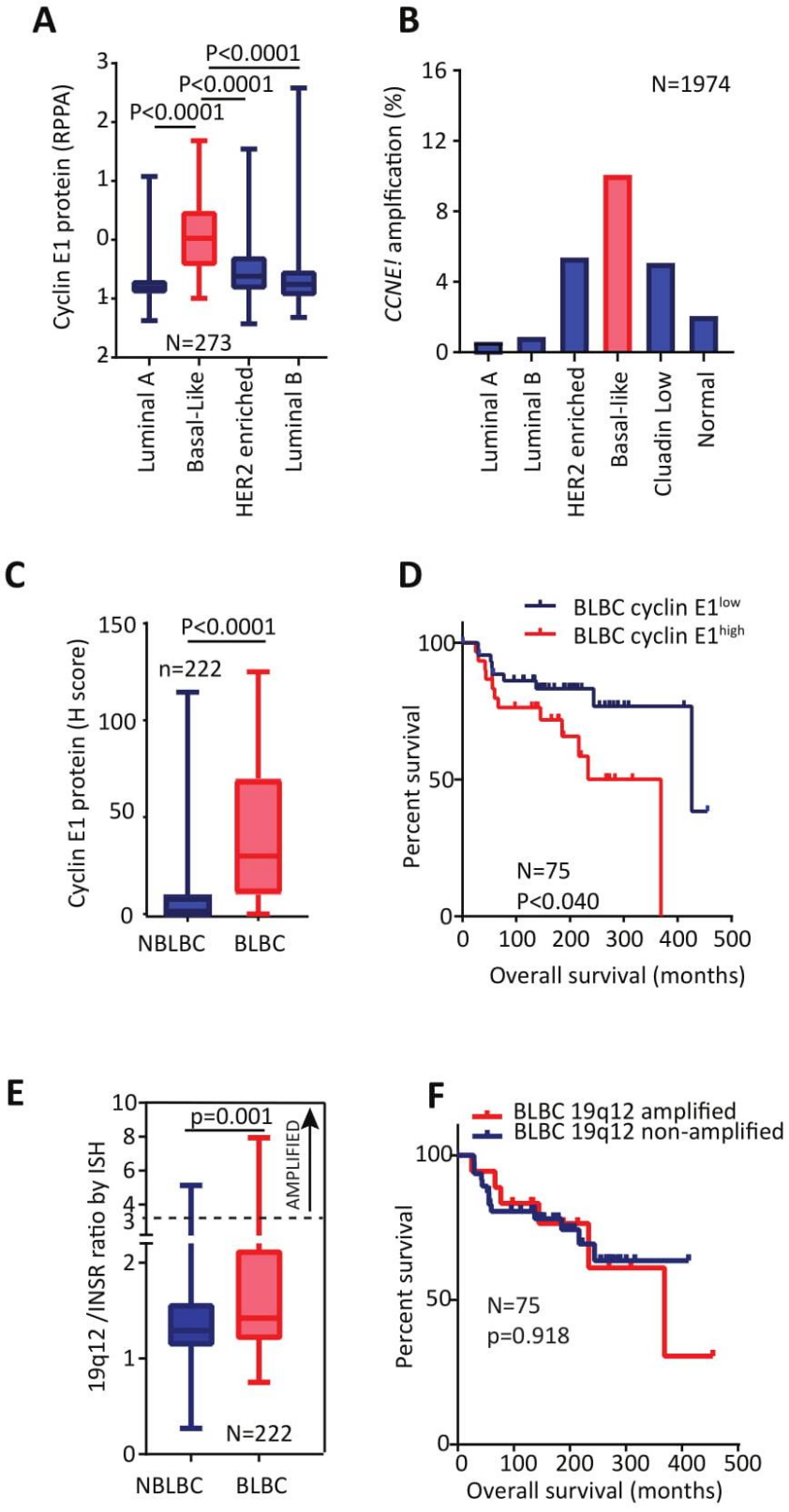


Figure 2: Meta-analysis identifies that only cyclin E1 protein expression is prognostic for BLBC, but both CCNE1 amplification and cyclin E1 protein are prognostic for HGSOC

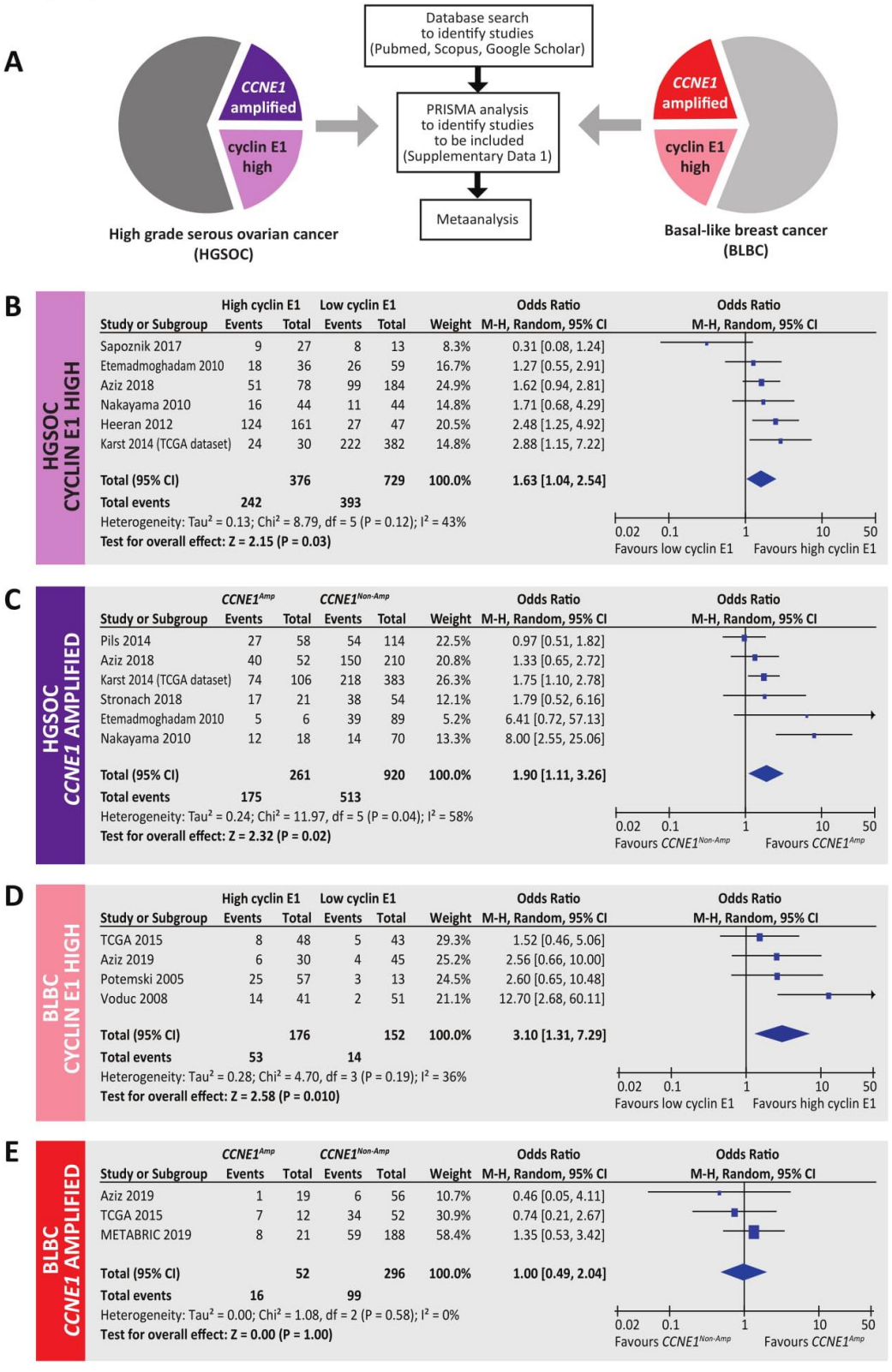


Figure 3: *CCNE1* undergoes low range amplification in BLBC, leading to moderate cyclin E1 protein expression

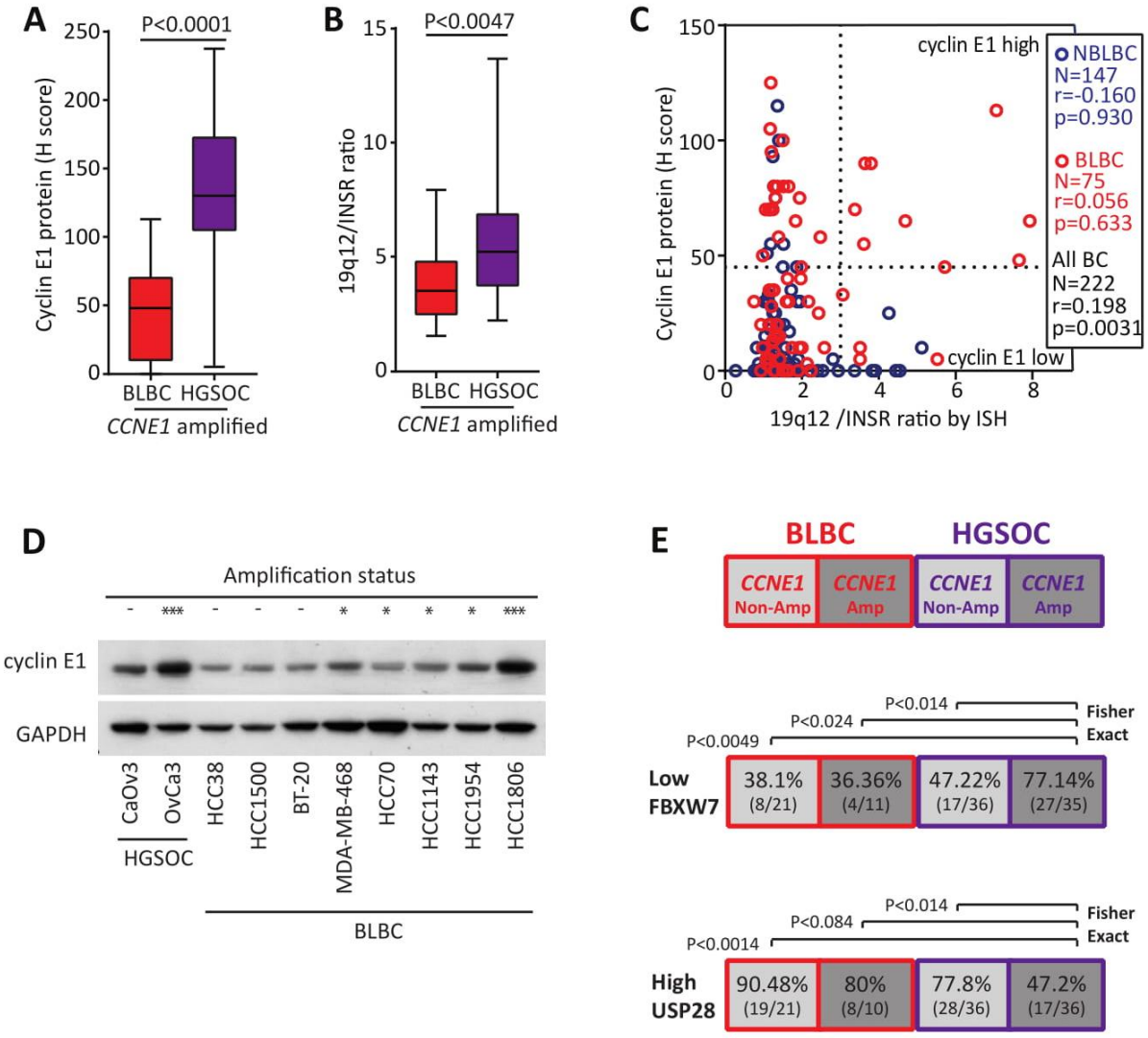
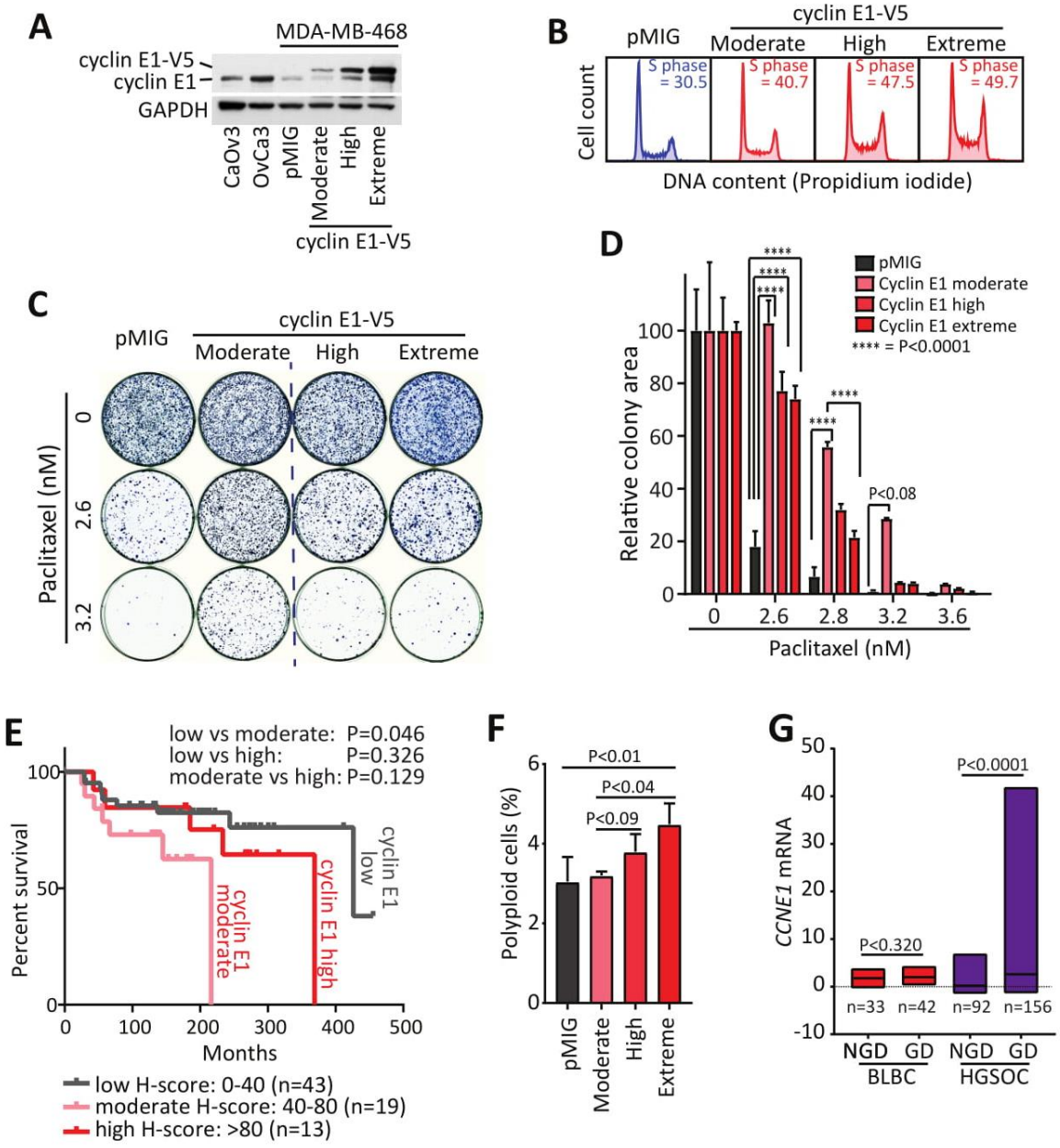
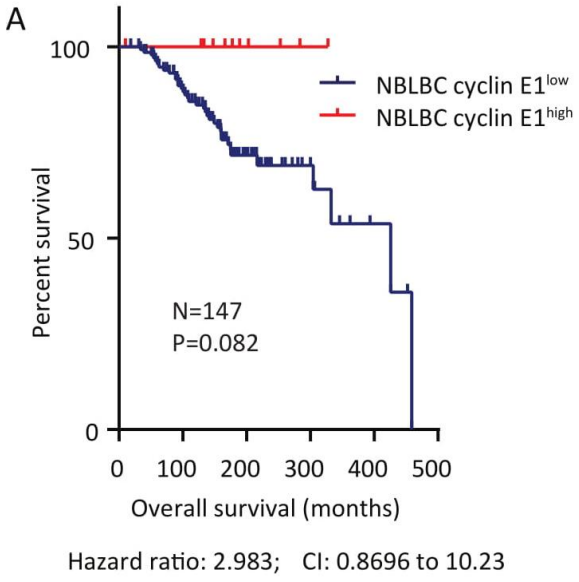


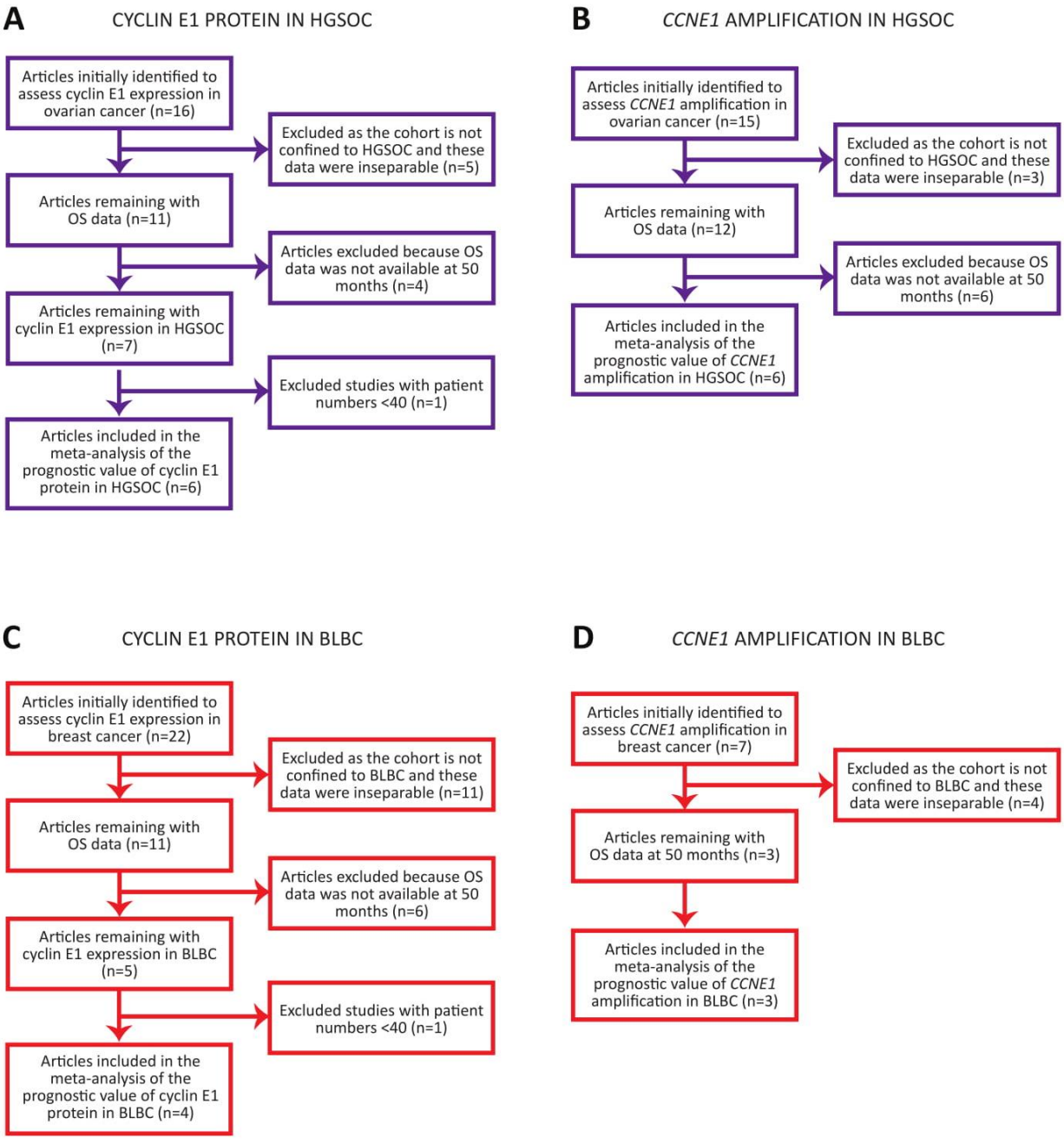
Figure 4: Moderate cyclin E1 expression in the BLBC cycE1^{Hi} subset is associated with increased proliferation, but not genomic instability



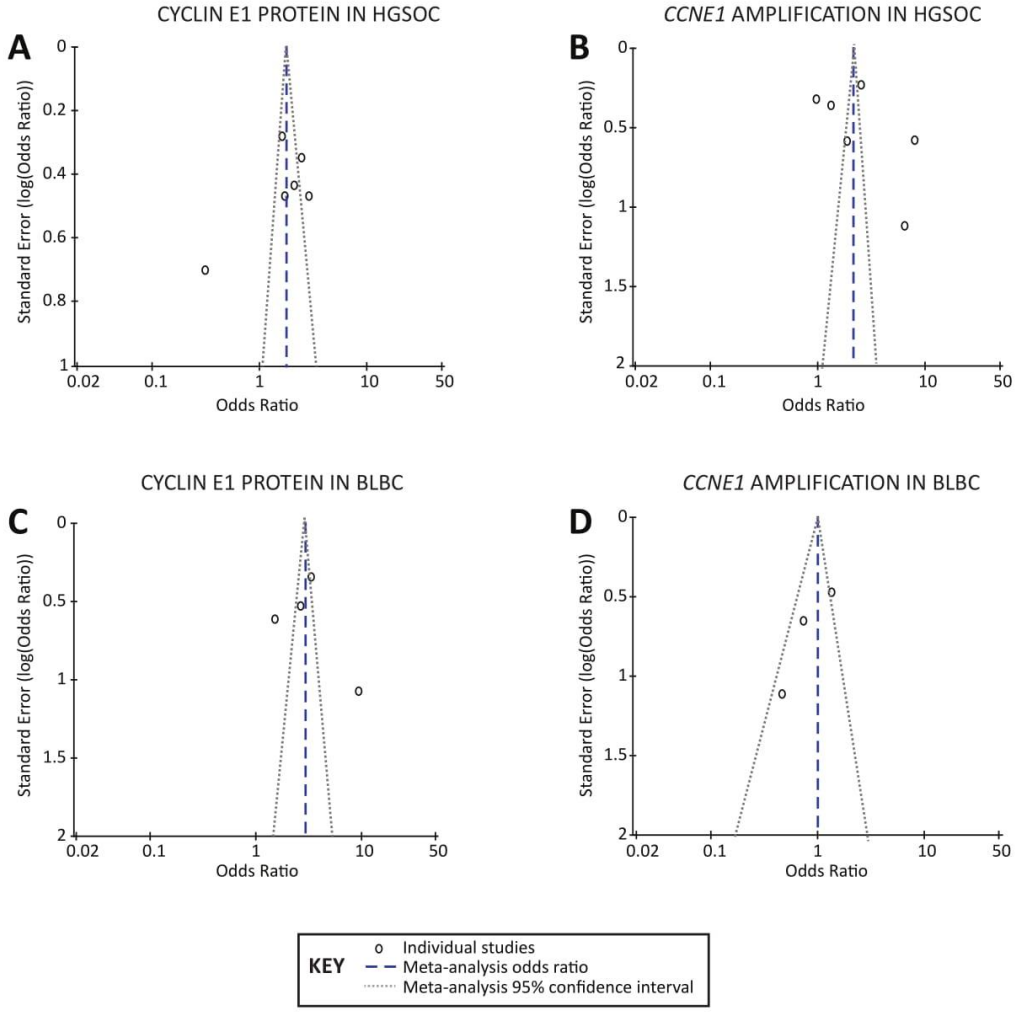
Supplementary Figure S1: High expression of cyclin E1 is not predictive of poor overall survival in NBLBC



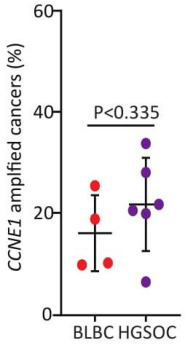
Supplementary Figure 2: PRISMA analysis to identify studies to include in meta-analyses



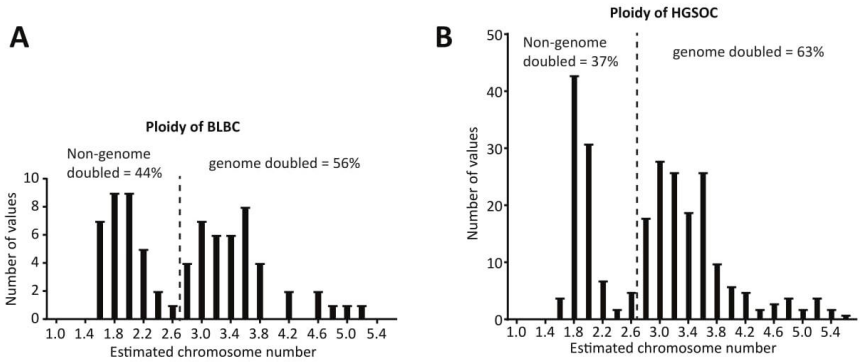
Supplementary Figure 3: FUNNEL plots of meta-analyses



Supplementary Figure 4: *CCNE1* amplification rates across the meta-analyses of *CCNE1* relationship to overall survival in BLBC and HGSOC

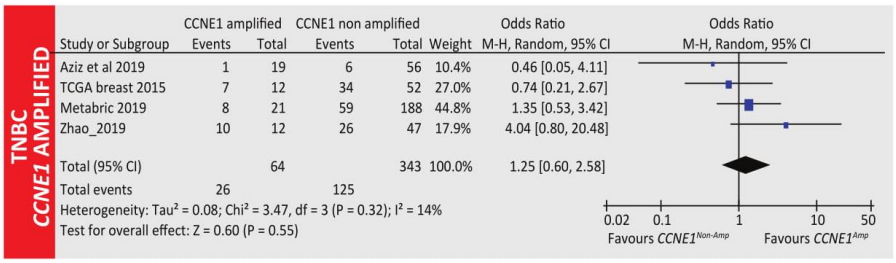


Supplementary Figure 5: Diploid and genome doubled cancers in Metabric/TCGA



Supplementary Figure 6: Meta-analysis of *CCNE1* amplification in combined BLBC/ER negative breast cancer

A



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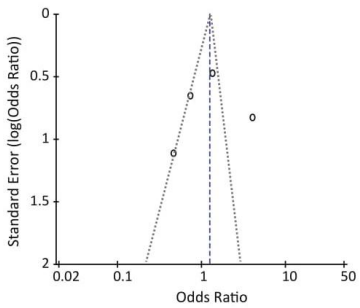

Chapter 6: Discussion

6.1 Summary of the results

HGSOC and BLBC are aggressive cancers that share numerous molecular features and significantly co-occur in patients and are therefore suggested to have a common etiology (Begg, C. B. *et al.* 2017). Among the shared molecular characteristics is the high frequency of *TP53* mutation, *BRCA1/2* mutation and cyclin E1 gene (encoded by *CCNE1*) amplification (Cancer Genome Atlas Research Network 2012). In one report, 4 out of 14 (28.5 %) amplified DNA regions in *BRCA1* mutant BLBC were found to parallel characteristic regions of HGSOC carcinomas (Prat, A. *et al.* 2014).

Given the shared features and the suggested common etiology of these cancers, many clinical trials are now grouping patients with HGSOC and BLBC into cohorts when testing new drugs or drug combinations, especially PARP inhibitors in the context of *BRCA1*/*BRCA2* mutation (e.g. Clinical Trial NCT01623349, NCT02203513, NCT00679783, and EMBRACE).

We sought to test whether deregulated cyclin E1 is another shared biomarker of subsets of both HGSOC and BLBC and can therefore be used to group patients with both diseases for therapeutic purposes. Cyclin E1, is a cell cycle regulatory protein whose gain can promote both increased proliferation and genomic instability in cancer cells. Increased cyclin E1 expression is reported to drive poor outcome in various cancer types including HGSOC and BLBC (Potemski, P. *et al.* 2005, Voduc, D. *et al.* 2008, Nakayama, N. *et al.* 2010, Karst, A. M. *et al.* 2014).

We first assessed the significance of deregulated cyclin E1 in a well characterised cohort of HGSOC from patients enrolled in the AOCS and a breast cancer cohort enriched for BLBC cancers and familial cancer mutations from patients enrolled in the KConFab study. For each disease, we tested the prevalence, possible drivers, and clinical impact of deregulated cyclin E1 and systematically compared our findings for both groups with the overall aim of guiding future selection of targeted therapies for similar subsets.

The findings of our study are summarised below:

6.1.1 Cyclin E1^{hi}/*CCNE1* amplified HGSOC subset differs from cyclin E1^{hi}/*CCNE1*non-amplified group

The results of our study of the AOCS cohort are described in Chapter 3 and published in Gynaecological Oncology (Aziz, D. *et al.* 2018). In brief, using pre-validated automated tissue based assays, we reported high expression of cyclin E1 in 31.3% (82/262) of HGSOC cases and we could divide these cyclin E1 high cases into two separate subsets. The *CCNE1* amplified subset accounted for 16.4% (43/262) and the *CCNE1* non-amplified subset accounted for 14.9% (39/262) of patients.

The majority of amplified cases co-expressed both URI1 and cyclin E1. *URI1* is a gene neighboring *CCNE1* that is spanned by our dual ISH probe and is reported to be amplified in HGSOC (Theurillat, J. P. *et al.* 2011). However, the full impact of URI1 on HGSOC is hard to assess due to its close correlation with *CCNE1*and their frequent co-amplification that, in a way, suggest a cooperation between both proteins in driving HGSOC growth and survival.

The *CCNE1* amplified-cyclin E1 high subset of HGSOC typically had low FBXW7 expression, lower incidence of *BRCA1/BRCA2* mutation, and worse outcome. We speculate that the high prevalence of low FBXW7 expression in the *CCNE1* amplified cohort reflects disrupted or slower degradation of cyclin E1. This is in an agreement with reports that *CCNE1* amplified are addicted to, and reliant on, cyclin E1 expression for their survival (Etemadmoghadam, D. *et al.* 2010). However, the mechanism of low FBXW7 expression is not identified, and mechanisms such as epigenetic inactivation are implicated (Akhoondi, S. *et al.* 2007, Yokobori, T. *et al.* 2009, Kitade, S. *et al.* 2016).

The *CCNE1* non-amplified subset could be distinguished by significantly high USP28 expression and higher incidence of *BRCA1/BRCA2* mutation. The overall survival in this subset was longer than those in the amplified group, however, the difference was not statistically significant.

Next, we assessed the extent of genomic instability in HGSOC cancers with high cyclin E1, with and without *CCNE1* amplification using TCGA data and CIN25 gene signature. CIN25 is the sum expression of 25 genes that correlate most highly with functional aneuploidy in tumors (Spruck, C. H. *et al.* 1999). We observed a stronger correlation of CIN25 with *CCNE1* amplification and with amplified cyclin E1 high

compared to the non-amplified cohort and non-amplified cyclin E1 high group respectively.

These findings collectively suggest a different impact of high cyclin E1 in the absence and presence of *CCNE1* amplification and consequently define two different subsets of cyclin E1 high HGSOC that might require different therapeutic approaches.

Next we assessed cyclin E1 deregulation in breast cancer and the findings are summarized below:

6.1.2 In BLBC, cyclin E1 overexpression is commonly associated with increased cyclin E1 stability, and only occasionally with *CCNE1* amplification

Based on our reports of the two different cyclin E1 ^{hi} HGSOC, we sought to test whether similar subsets can be identified in the BLBC cohort. Since the majority of *CCNE1* amplified HGSOC subset lacked *BRCA* inactivation, we asked whether the same is true for BLBC. In literature, the majority of *BRCA1* mutated breast cancers are BLBC (Kobayashi, S. 2008, Rakha, E. A. *et al.* 2008). However, BLBC are commonly cyclin E1 high expressers (Foulkes, W. D. *et al.* 2004). Therefore we asked whether in breast cancer, as in the HGSOC scenario, the *BRCA* inactivation is mutually exclusive from *CCNE1* gene amplification due to the reported synthetic lethality (Etemadmoghadam ^{A,} D. *et al.* 2013). Subsequently, we aimed to assess the *BRCA1*/cyclin E1 association in our *BRCA* enriched cohort of breast cancer with the results shown in Chapter 4.

In summary, cyclin E1 expression occurred in 18.5% (41/222) of all BC cases and the majority of high expressers, 73.2% (30/41), did not have associated *CCNE1* amplification. Unlike in HGSOC, the expression of cyclin E1 was significantly higher in *BRCA1* related breast cancer. Similarly, *BRCA1* mutant breast cancer was associated with high USP28 expression and decreased phosphorylation of cyclin E1, on Threonine 62 compared to the non *BRCA1* mutant cases. Fittingly, we showed by *in vitro* study that cyclin E1 stability is regulated via *BRCA1* through regulating its phosphorylation at T62. We concluded that the reported high cyclin E1 in the *BRCA1* mutant cases was mainly due to disrupted degradation and only occasionally associated with gene amplification.

Within the *BRCA1* mutant and the BLBC subsets, cases with cyclin E1 high cancers had significantly worse overall survival compared to cases with cyclin E1 low cancers while the gene amplification did not seem to impact patients' survival in both groups.

Consequently, we searched for potential therapeutic combinations to target the cyclin E1 high *BRCA1* mutated (and BLBC) cancers and cancer cell lines. Since CDK inhibitors are reported to induce DNA damage, we hypothesised that the use of CDK inhibitors, in addition to targeting cyclin E1 activity, will enhance the synthetic lethality of PARP inhibitors. Using comet assay, we confirmed that treating cell lines with the CDK inhibitor, CYC065, was associated with increased DNA damage and this was significantly higher with higher dose. Using the combination therapy of CDK and PARP inhibitors, we reported for the first time, a synergistic action between PARP inhibitor Rucaparib and CDK2 inhibition that led to decreased cancer cell survival *in vitro*. Our in vivo data are underway but the preliminary data are supportive of our hypothesis. Based on our findings we propose a new approach for targeting cyclin E1 high *BRCA1* mutant BLBC.

Next we asked whether the cyclin E1 high BLBC shares feature with cyclin E1 high HGSOC subsets as this might pave the way for grouping similar subsets for future targeted therapies. Therefore, we systematically compared cyclin E1 high HGSOC and cyclin E1 high BLBC. These data are described in Chapter 5 and are summarized below:

6.1.3 Cyclin E1^{hi} BLBC group shares features with cyclin E1^{hi}-*CCNE1* non-amplified HGSOC

In order to assess the clinical impact of cyclin E1 high and *CCNE1* amplification in BLBC compared to the impact observed in HGSOC, we did a meta-analysis that included our cohorts as well as those in literature. The meta-analysis finding was well-matched to our observation and revealed that in HGSOC patients, both high expression of cyclin E1 and *CCNE1* amplification had an adverse impact on OS while only high protein expression was prognostic for BLBC.

Next, we compared the four cyclin E1 high subsets of HGSOC and BLBC, amplified or not for variable cyclin E1 related parameters. The comparison included prevalence, level of cyclin E1 expression and *CCNE1* gene amplification, the prevalence of low FBXW7, high USP28 and *BRCA1/2* mutations as well as the impact on outcome. The findings

are summarized in table 6-1. Overall, amplified and non-amplified subsets of BLBC were almost indistinguishable. Moreover, both cyclin E1 high subsets of BLBC shared phenotypic features with the *CCNE1* non-amplified cyclin E1 high HGSOC subset such as USP28 dysregulation and the *BRCA1/2* association.

Notably, in *BRCA1* mutant breast cancer and/or BLBC, we did not observe the mutual exclusivity reported between 19q12 amplification and *BRCA1* loss in HGSOC. In fact, within the KConFab cohort, we reported higher prevalence of 19q12 amplification and cyclin E1 high expression in *BRCA1* mutant compared to *BRCA1* intact breast cancers. However, 19q12 amplification only occasionally associated with high cyclin E1 expression and the level of amplification was lower than those observed in HGSOC. We suggest that the apparently paradoxical co-occurrence of 19q12 amplification with *BRCA1* inactivation, might indicate that amplification in those cases is due to drivers other than *CCNE1*, *URI1* for instance. Consistently, a functional characterisation of 19q12 amplicon in ER negative breast cancer, a subset that overlaps greatly with both *BRCA1* mutant breast cancer and BLBC, revealed that *CCNE1* is only occasionally amplified in 19q12 amplicon and reported other potential drivers as *POP4*, *PLEKHF1*,*TZH3* (Natrajan, R. *et al.* 2012). Interestingly, 19q12 amplification level and cyclin E1 expression were significantly higher in BLBC compared to NBLBC cases but lower than those reported in HGSOC suggesting lower tolerability to higher expression and/or amplification. This notion is reinforced by our reports of the overall lower range of USP28 and higher range FBXW7 expression compared to HGSOC coupled with low cyclin E1 T62 (Table 6-1, chapter 5-figure 3E) , all suggesting the inclination of BLBC to have lower expression of cyclin E1 than those reported in HGSOC. In agreement with this suggestion, are our observations that moderate expression was associated with the greatest survival advantage of BLBC cell lines to paclitaxel treatment and their association with worst patients' outcome compared to low and high expression.

Table 6-1: Comparison of cyclin E1 related parameters in cyclin E1 high subsets of HGSOC and BLB, amplified and non-amplified.

Category	**Amplified/cyclin E1 high HGSOC**	**Non-amplified/cyclin E1 high HGSOC**	**Amplified/cyclin E1 high BLBC**	**Non-amplified/cyclin E1 high BLBC**
Prevalence	(43/262) 16.4%	(39/262) 14.9%	(11/75) 14.7	(19/75) 25.3%
Cyclin E1 expression (H score)	102-238 (median 145)	100-230 (median 135)	45-113 (median 65)	40-125 (median 75)
***CCNE1* copy number**	2.20-14.62 (median 10.9)	0.88-2.73 (median 3.02)	5.36-14.53 (median 7)	1.77-5.85 (median 3.17)
Low FBXW7 expression	(27/35) 77.1%	(17/37) 44.2%	(4/11) 36.4%	(7/19) 36.8%
USP28 expression	(45/72) 47.2%	(28/36) 77.8%	(8/10) 80%	(17/19) 89.5%
BRCA1/2	(2/34) 5.9%	(9/37) 24.3%	(9/11) 81.8%	(16/19) 84.2%
BRCAness	?	?	?	?
Impact on outcome (HR)	HR 0.63 (95%CI, 0.43 to 0.91)	HR 0.66 (95%CI, 0.41 to 1.07)	HR 0.92 (95%CI, 0.29 to 2.87)	HR 1.10 (95%CI, 0.3488 to 3.413)
Potential targetability	CDK2+AKT_Chk1/WEE1 inhibitors	CDK2+/-PARP inhibitors	CDK2inhibitors+PARP inhibitors?	CDK2 inhibitors +PARP inhibitors

1. Note that the cohort is from familial breast cancers and includes a large number of patients with Brca1 mutation. High cyclin E1 expression is enriched due to the prevalence of Brca1 mutation.
2. Green marks similarities, red marks differences, light green tendency to be similar

All these findings together suggest tendency for overall lower expression level of cyclin E1 in BLBC compared to those observed in HGSOC, possibly because these cases are unable to tolerate higher expression of cyclin E1 and replication stress due to their *BRCA1* loss related genomic instability.

Our findings collectively suggest that cyclin E1 protein is likely to be a shared biomarker for HGSOC and BLBC, however for HGSOC, the impact and correlations varies with the 19q12 associated amplification status. Our data has shown that cyclin E1 high subset of BLBC shares features with the non-amplified cyclin E1 high HGSOC subset and suggest that these 2 groups can possibly be grouped for therapeutic purposes.

6.2 Final discussion and future direction

Our work provides new insights for characterizing cyclin E1 high HGSOC and BLBC subsets of ovarian and breast cancer. It also provides guidance for future selection of targeted therapies to allocate each subset into their selective therapeutic options.

Overall, we suggest that *CCNE1* amplification is the oncogenic driver that is responsible for the carcinogenesis of a subset HGSOC that is *CCNE1* amplified, possibly in cooperation with other factors. Conversely, in *CCNE1* non amplified cases with high cyclin E1 protein expression the driver is unlikely to be cyclin E1 and the effect of high cyclin E1 is mainly the increased cellular proliferation.

6.2.1 *CCNE1* amplification as a driver oncogene in HGSOC

CCNE1 amplification has previously been described as the driver of *CCNE1* amplified HGSOC subset (Nakayama, N. et al. 2010) that is responsible for their evolution from their alleged precursor, STIC lesions. Fittingly, both cyclin E1 expression (Karst, A. M. et al. 2014) and *CCNE1* amplification (Snijders, A. M. *et al*. 2003, Nowee, M. *et al*. 2007) have been reported in early STIC lesions of HGSOC. Moreover, we, and others (Zhong, Q. *et al.* 2016) have shown that amplification of *CCNE1* in HGSOC is homogenous, a characteristic of an early event in tumorigenesis (McGranahan, N. and Swanton, C. 2017). In addition, amplification in most of the cases was associated with very high expression of cyclin E1. This finding fits nicely with literature description of a driver oncogene as a gene that is when amplified, typically associates with high expression of their encoding protein (Ohshima, K. et al. 2017).

We hypothesise that persistently high cyclin E1 expression during S phase, exacerbated by FBXW7 loss, forces normally quiescent fallopian tube epithelial cells into cell division and enhances cell proliferation. Excess cyclin E1 results in replication stress which is the slowing of the DNA replication fork (Minchom, A. *et al.* 2018). Replication stress initially drives unscheduled replication origin firing, results in depletion of nucleotide pool and impaired ability to repair all the stalled replication forks in the face of continuous origin firing (Teixeira, Leonardo K. *et al.* 2015, Doberstein, K. *et al.* 2018). If replication stress persists, stalled forks are converted into 'collapsed forks' (Lambert, S. and Carr, A. M. 2005, Minchom, A. *et al.* 2018) and eventually to DNA DSBs, causing replication catastrophe and cell death (Toledo, L. I. *et al.* 2013, Minchom, A. *et al.* 2018). In order to avoid the otherwise toxic level of replication stress, cyclin E1 deregulated cancer cells adopt certain coping mechanisms (Doberstein, K. *et al.* 2018). These mechanisms include loss of G1/S checkpoint via p53 inactivation (Minella, A. C. *et al.* 2007, Schoonen, P. M. *et al.* 2019) to allow cells to progress though the cell cycle. Relevantly, the vast majority of HGSOC cases, including the *CCNE1* amplified subset, are *TP53* mutant (Cancer Genome Atlas Research Network 2011). In p53 inactivated cells with a failed G_1/S checkpoint, stressed cells rely on a successful replication stress response to maintain replication competency and would therefore depend upon various intact DNA repair mechanisms. This dependency on an intact DDR is currently been translated into targeted therapy as detailed later in this chapter.

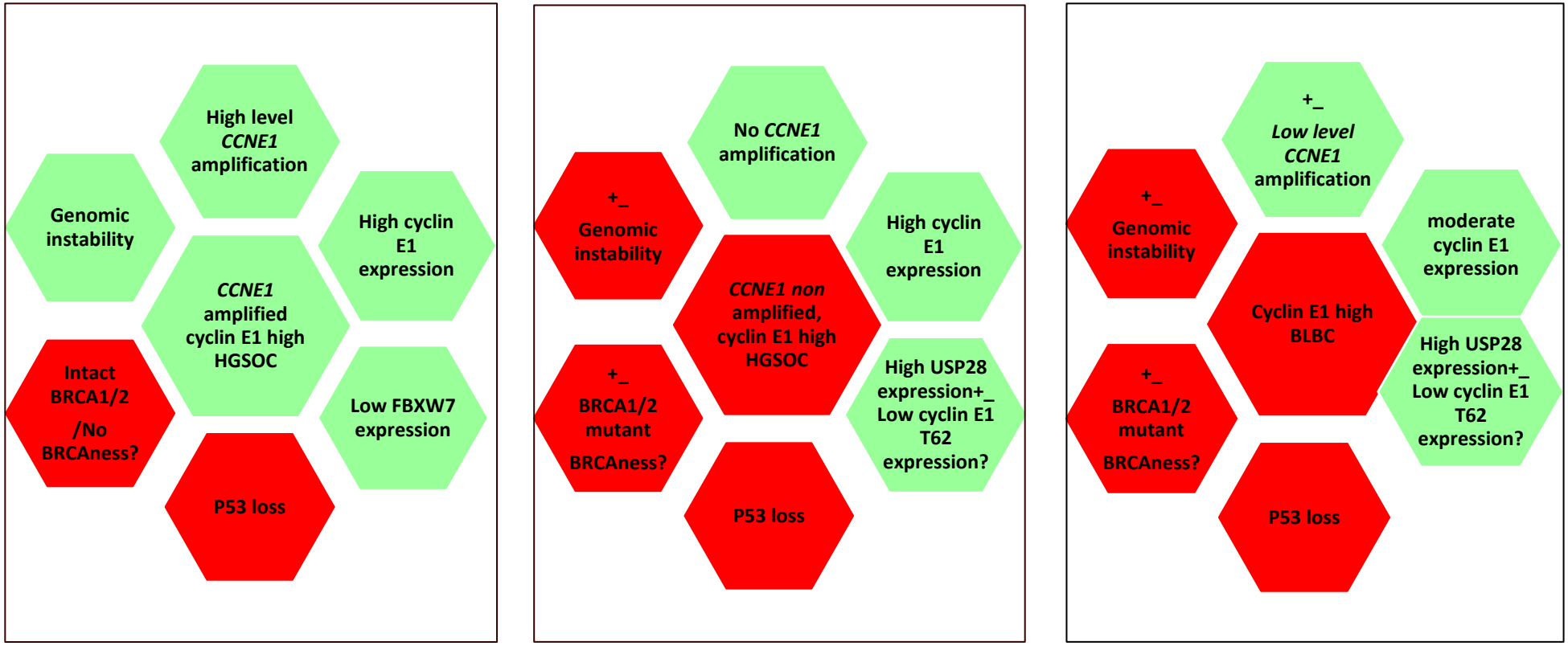

Figure 6-1: Hexagons summarizing the characteristic features of the three cyclin E1 high groups, *CCNE1* amplified HGSCO (left), *CCNE1* non amplified HGSOC (middle) and cyclin E1 high BLBC subset. Color code refers to association between parameters.

6.2.2 *CCNE1* amplification and cyclin E1 disrupted degradation as two different pathways of cyclin E1 deregulation

Based on our assessment of cyclin E1 deregulation in HGSOC and BLBC we suggest that there are at least two different pathways for cyclin E1 deregulation with different effects and associations in these diseases (figure 6-2).

First, the *CCNE1* amplification pathway that is usually of high cyclin E1 expression and associates with persistently high level of cyclin E1 via FBXW7 loss. These harbor higher genomic instability and associate with extensive replication stress and therefore require an intact *BRCA1/2*. This pathway seems to dominate in HGSOC over BLBC, is typically high level amplification that associates with very high cyclin E1 expression in the majority of cases.

The second cyclin E1 deregulation pathway is related to disrupted degradation of cyclin E1 and occurred in both HGSOC and *BRCA1* mutant/BLBC group. Disrupted degradation was in part due to the prevalent USP28 expression, the functional deactivator of FBXW7. The second mechanism of disrupted degradation of cyclin E1 reported in this study is the increased cyclin E1 stability due to *BRCA1* loss mediated decrease in T62 phosphorylation. We observed this in cancer cell lines and also documented a decrease in cyclin E1 T62 expression in *BRCA1* mutant breast cancer of cases included in the KConfab Cohort.

When there was disrupted degradation rather than gene amplification, the expression of cyclin E1 tended to be lower in each of the BLBC cohorts and BLBC cell lines compared to HGSOC. We suppose that the mechanisms that lead to cyclin E1 high expression in this group is less powerful than those occurring in the amplified pathway. For instance, we suggest that the functional inactivation of FBXW7 is less capable of inactivating FBXW7 compared to the complete or nearly complete loss of FBXW7 expression reported in the *CCNE1* amplified HGSOC. Moreover, when lniked to disrupted degradation, moderate rather than high expression of cyclin E1 was associated with worse survival as we have reported in the KConFab cohort and in BLBC cell lines. We suggest that the observed lower expression of cyclin E1 in this group is due to *BRCA1* linked -genomic instability that makes cancer cells intolerant to the added replication stress that would accompany higher cyclin E1 level. This suggestion

is in an agreement with Chen *et al* that have reported that the increased expression of cyclin E1, via CDK2 inhibitors, rendered TNBC cells vulnerable to DDR interruption i.e. increased their sensitivity to the cell cycle checkpoint inhibitors, WEE1 inhibitors (Chen, X. *et al.* 2018). This further support our findings that the level of cyclin E1 in BLBC, is not as high as those reported in HGSOC and is not associated with the same level of genomic instability observed in HGSOC , especially in the amplified group. Details of targeted therapies is provided below.

6.2.3 Targeting cyclin E1 deregulation in HGSOC and BLBC

In the literature, in *vitro* and in *vivo* studies have provided pre-clinical evidence for many therapeutic approaches to target cyclin E1 deregulation in cancers (Etemadmoghadam, D. *et al.* 2010, Etemadmoghadam [A,] D. *et al.* 2013, Etemadmoghadam [B,] D. *et al.* 2013, Yang, L. *et al.* 2015, Au-Yeung, G. *et al.* 2018, Xu, H. *et al.* 2018). One proposed targeted therapy for cyclin E1 deregulation is direct targeting of cyclin E1-CDK signaling, for example via CDK2 inhibitors (Wiedemeyer, W. R. *et al.* 2014). This approach is usually based on the reported reliance of *CCNE1* amplified cells on cyclin E1 for their survival (Etemadmoghadam, D. *et al.* 2010, Natrajan, R. *et al.* 2012). Notably, a number of CDK inhibitors have entered clinical trials for the treatment of cancer over the last few years and the use of CDK4/6-selective inhibitors has led to significant improvement in outcomes in breast cancer treatment. However, the development of CDK2 inhibitors have been slow because of the challenge of the selectivity (Tadesse, S. *et al.* 2019). Remarkably, inhibition of CDKs has been reported to have other therapeutic impact including inducing DNA damage thorough CDK2 inhibition (Wohlbold, L. *et al.* 2012), and enhancing the synthetic lethality of the combination treatments including CDK1 inhibition with PARP inhibitors in TNBC (Johnson, S. F. *et al.* 2016) and CDK2 inhibition with WEE1 inhibitors in TNBC (Chen, X. *et al.* 2018) and CDK4/6 inhibition with platinum therapy in HGSOC (Iyengar, M. *et al.* 2018). Similarly we have reported a synergistic effect via synthetic lethality of a combination therapy of CDK2 inhibitor and PARP inhibitors in cyclin E1 high *BRCA1* mutant BC cell lines.

In fact, creating synthetic lethality via interference with DNA damage repair machinery of cancer cells is attracting huge interest as an exciting therapeutic approach not only

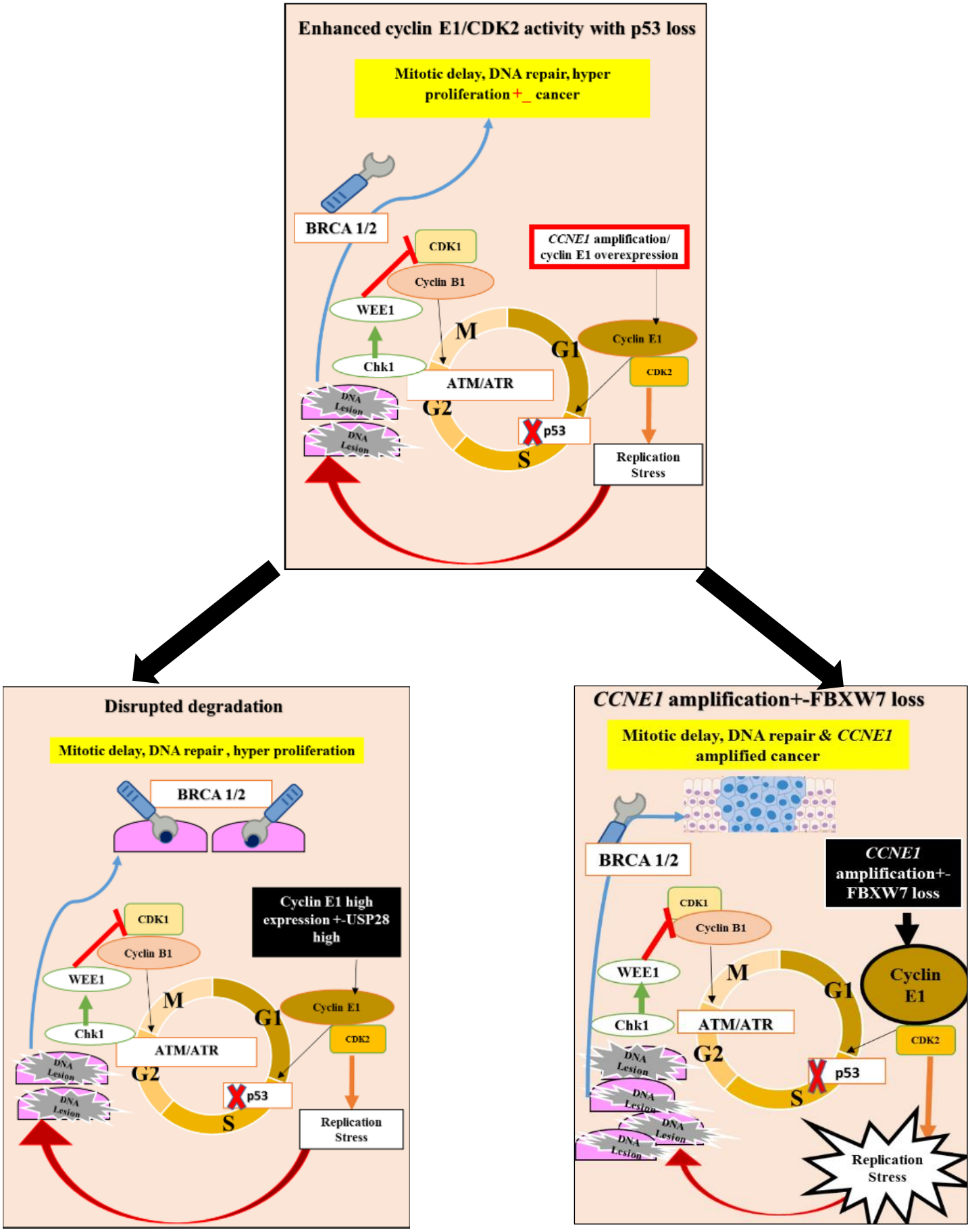

Figure 6-2: suggested two different pathways for cyclin E1 deregulation: (left) disrupted degradation likely to accompany prevalent USP28 expression and lower replication stress and is linked to increased proliferation of cancer cells, (right) *CCEN1* amplification that is likely to be coupled with FBXW7 loss, lead to excess replication stress and evolution of *CCNE1* amplified cancer.

for HGSOC and BLBC but also for other hard to treat cancers (Bartek, J. and Lukas, J. 2003, Chen, T. *et al.* 2012, Visconti, R. *et al.* 2016, Minchom, A. *et al.* 2018). Variable approaches can be adopted to induce synthetic lethality in these diseases (Minchom, A. *et al.* 2018)and these are discussed below in the light of our findings and those in literatures.

6.3 Creating synthetic lethality in HGSOC and BLBC

Both BLBC and HGSOC are genomically unstable cancers that are susceptible to rapid acquisition of genomic gains, losses and chromosomal rearrangements. These ongoing genomic alterations can render even initially responsive cancers to targeted therapy into resistant and result in treatment failure (Patch, A. M. *et al.* 2015, Schoonen, P. M. *et al.* 2019).

The driver of genomic instability in these cancers is broadly believed to be either due to HR defect caused by germline or somatic mutation in *BRCA1*, *BRCA2* or other HR genes (Patch, A. M. *et al.* 2015). Alternatively, genomic instability might be the result of replication stress in these cancers (Halazonetis, T. D. *et al.* 2008, Gaillard, H. *et al.* 2015) and, other than c-Myc and Ras, one of the few oncogenes that has been extensively studied in the context of replication stress is *CCNE1* (Schoonen, P. M. *et al.* 2019). The key concept in targeting these genomically unstable cancers is that these tumors are reliant on an intact DNA repair mechanisms, especially PARP and ATR respectively, for their survival (Schoonen, P. M. *et al.* 2019). Therefore drugs targeting DDR pathways taking advantage of clinical synthetic lethality present a promising therapeutic approach in these diseases. In fact, PARP inhibitor such as Olaparib has already shown clinical benefit in *BRCA*-mutant ovarian and breast cancer (Audeh, M. W. *et al.* 2010, Gelmon, K. A. *et al.* 2011, Ledermann, J. *et al.* 2012, Pujade-Lauraine, E. *et al.* 2017, Minchom, A. *et al.* 2018). Other agents including ATR pathway inhibitors are currently in development (Minchom, A. *et al.* 2018). Typically, PARP inhibitors show greater efficacy in HR deficient ovarian and breast cancer while ATR inhibitors are more likely to be effective in genomically unstable HR proficient cancers, the *CCNE1* amplified group for instance. However, though HR deficient and HR proficient-*CCNE1* amplified cancer do not seem to overlap, there is an increased tendency to group cancers with either aberration in clinical trials, DDR inhibitors trials in particular (Xu, H. *et al.* 2018, U.S. National Library of Medicine 2019).

In our cohorts of HGSOC and BLBC we suggest the genomic instability to be linked to both *CCNE1* amplification and HR defect in HGSOC and mostly to HR defect in BLBC.

Based on the previous reports of reliance of *CCNE1* amplified cancers on intact *BRCA* coupled with the proposed efficacy of cell cycle checkpoint inhibitors, we and others suggest that targeting CHK1 and WEE1 would induce therapeutic synthetic lethality in *CCNE1* amplified cases via aggravating replication stress, genomic instability and inducing mitotic catastrophe (Do, K. *et al.* 2013, Wiedemeyer, W. R. *et al.* 2014, Matheson, C. J. *et al.* 2016, Geenen, J. J. J. and Schellens, J. H. M. 2017, Lin, A. B. *et al.* 2017, Doberstein, K. *et al.* 2018, Xu, H. *et al.* 2018) (Figure 6-3). Alternatively, for cyclin E1 high HR deficient cancers we propose, supported by our findings, that aggravating DNA damage via CDK inhibitors would enhance PARP inhibitors synthetic lethality in *BRCA1* mutant cancers (Figure 6-3). We therefore provide a framework for future directions for the use of DDR inhibitors as targeted therapy in each group as summarised below.

6.3.1 Creating synthetic lethality in the HR deficient *BRCA1* mutant cancer HGSOC and BLBC via PARP inhibitors

The genetic interaction between PARP and *BRCA* can be described as synthetic lethal since individual loss of either gene is compatible with life, but simultaneous loss of both results in cell death (Hartwell, L. H. *et al.* 1997, Helleday, T. 2011, Dziadkowiec, K. N. *et al.* 2016). In fact, the PARP-*BRCA* interaction represents the first successful example of synthetic lethality approach that is currently used in clinic (Helleday, T. 2011). However, PARP inhibitors are unlikely to be effective in HR-proficient cancers (Bouwman, P. and Jonkers, J. 2014) including *CCNE1* amplified cancers. Moreover, a proportion of HR deficient cancers were reported to either acquire resistance that is usually linked to restoration of HR (Bouwman, P. and Jonkers, J. 2014, Johnson, S. F. et al. 2016) or exhibit primary resistance mediated by incomplete loss of HR function (Johnson, S. F. et al. 2016). These challenges have prompted interest in combining PARP inhibitors with agents capable of simultaneous suppression of multiple HR genes in order to resensitise resistant cells to these agents (Johnson, S. F. *et al.* 2016). Inhibition of some of the CDKs has been reported to create HR deficiency (Deans, A. J. et al. 2006) in pre-clinical TNBC models and subsequently enhanced synthetic lethality of PARP inhibitors (Johnson, S. F. *et al.* 2016, Chen, X. *et al.* 2018). Moreover,

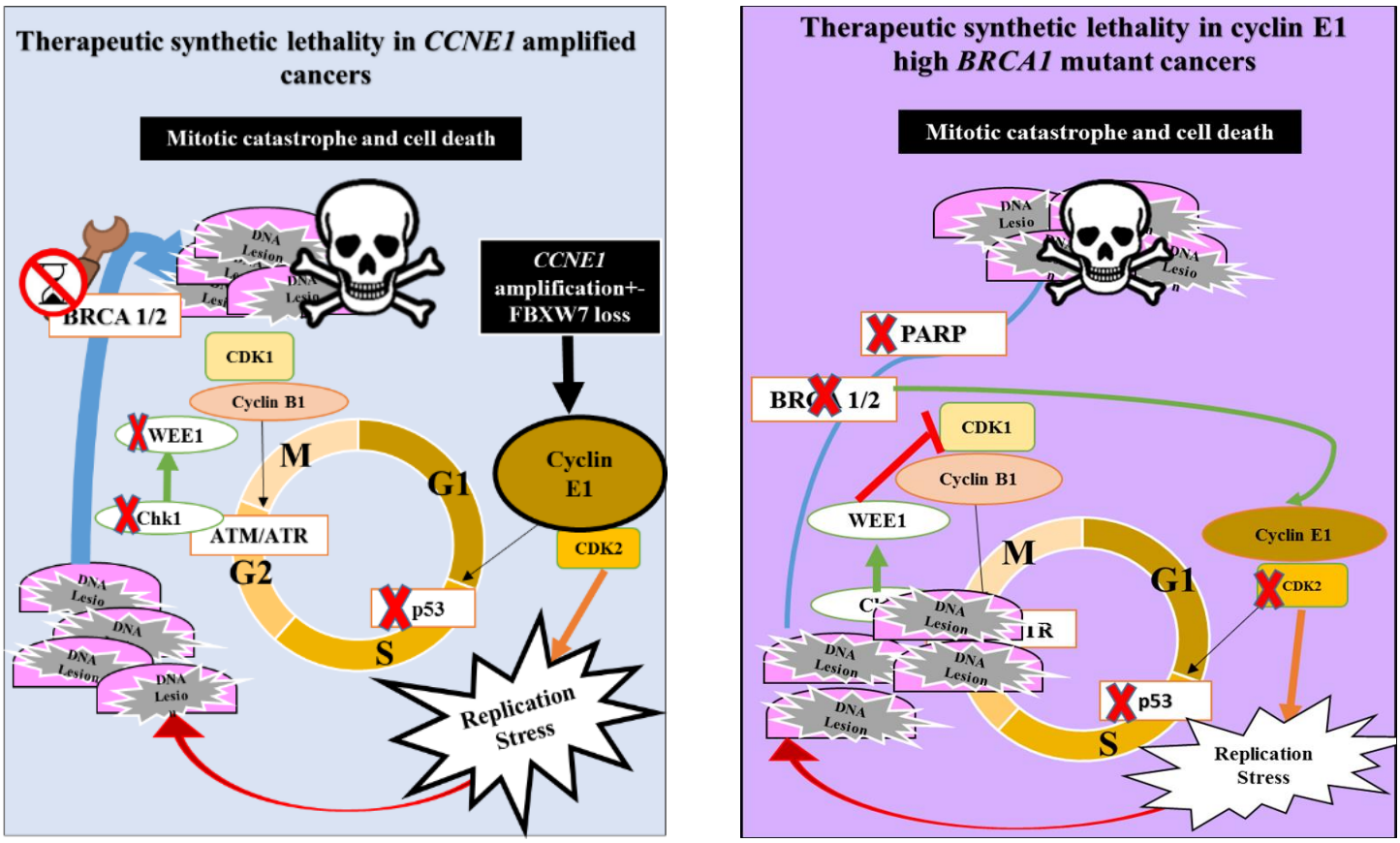

Figure 6-3: Creating synthetic lethality in cyclin E1 deregulated cancers: Left: the use of CHK1 and WEE1 inhibitors in *CCNE1* amplified cancers with excess cyclin E1 and replication stress push the cell with extensive DNA damage into mitotic and allow no time for BRCA to correct errors resulting in mitotic catastrophe. Right: CDK2 inhibitors increases DNA damage and enhances PARP inhibitors synthetic lethality in cyclin E1 high *BRCA1* mutant cancers.

the CDK inhibitor, Dinaciclib and PARP inhibitors combination is currently under trial in a cohort that includes *BRCA1* associated TNBC (Clinical Trial ID NCT01434316). In our work, we reported higher expression of cyclin E1 in the *BRCA1* mutant breast cancers that was associated with worse outcome. We also provided evidence that the stability of cyclin E1 was increased by *BRCA1* loss as *BRCA1* downregulates cyclin E1 phosphorylation at T62. Conversely, in HGSOC *BRCA1* mutant cases did not seem to have higher expression of cyclin E1 compared to the wild type. Probably because wild type comprised the *CCNE1* amplified cancers and cases with possible 'BRCAness'. Future research can therefore focus on testing the stability of cyclin E1 in the *BRCA* mutant HGSOC via testing cyclin E1 T62 expression in those cases as well as assessing the impact of *BRCA* loss on cyclin E1 expression in HGSOC cell lines.

Importantly, we have revealed for the first time an approach that is very likely to be effective in cyclin E1 high- *BRCA1* mutant BLBC as we have reported a synergy between CDK inhibitor and PARP inhibitors in the cyclin E1 high- *BRCA1* mutant BLBC cell lines and xenografts. We suggest that the observed synergy in our study is due to the enhancement of PARP inhibitors synthetic lethality via the CDK inhibitors induced DNA damage (figure 6-3). We suggest testing this combination in a proof of concept future clinical trials that would typically recruit cyclin E1 high *BRCA1* mutant BLBC patients.

Theoretically, the reported synergy between CDK and PARP inhibitions is applicable to the comparable group of HGSOC, however, future research need to test this combination in HGSOC cell lines and xenografts.

6.3.2 Creating synthetic lethality in the HR proficient *CCNE1* amplified HGSOC via ATR pathway inhibition

The reported genomic instability in the *CCNE1* amplified group is mainly due to the extensive replication stress that associates with *CCNE1* amplification and high cyclin E1 expression (Zeman, M., K. and Cimprich, K., A. 2014, Schoonen, P. M. *et al.* 2019). As with other genomically unstable cancer, *CCNE1* amplified HGSOC adopts mechanisms to avoid the otherwise intolerable replication stress. As previously discussed, these mechanisms include loss of p53 (Minella, A. C. *et al.* 2007) and subsequent reliance of an intact G2/M checkpoint (ATR) and HR for their survival (Toledo, L. I. *et al.* 2013, Doberstein, K. *et al.* 2018, Xu, H. *et al.* 2018, Schoonen, P. M. *et al.* 2019). This explains the observed mutual exclusivity of *CCNE1* amplification and *BRCA* inactivation (Etemadmoghadam, D. et al. 2013) as *BRCA* loss significantly impair HR, and if combined with the extensive replication stress that accompanies *CCNE1*amplification mitotic catastrophe would be inevitable (Minchom, A. et al. 2018). Similarly, loss of the G2/M checkpoint would be synthetically lethal with *CCNE1* amplification.

Normally, ATR signaling promotes cell-cycle arrest via activating multiple downstream effectors of which CHK1 and WEE1 are the most well categorized (Schoonen, P. M. *et al.* 2019). This result in cell cycle arrest at M phase and allow for DNA repair through HR (Schoonen, P. M. et al. 2019). Therefore, inhibitors of ATR pathway (Schoonen, P. M. *et al.* 2019) would force cancer cells to bypass G2/M arrest

and continue through mitosis with unrepaired DNA damage and result in mitotic catastrophe (Vitale, I. *et al.* 2011, Chen, T. *et al.* 2012, Visconti, R. *et al.* 2016). Thus, these ATR pathway inhibitors are strongly proposed to be beneficial in *CCNE1* amplified HGSOC as well as in other hard to treat cancers. Currently, many clinical trials that are assessing ATR pathway inhibitors are recruiting patients with *CCNE1* amplified cancers (table 6.2) (U.S. National Library of Medicine 2019). Remarkably, those trials are grouping patients with *CCNE1* amplification and those with HR deficiency together based on their shared genomic instability implying a unified approach in targeting HR deficient and HR proficient cancers. This suggest another approach of grouping HGSOC and BLBC together for therapeutic purposes, in addition to grouping *BRCA* mutant cancers for PARP inhibitors that is currently in practice, and our suggested approach of grouping cyclin E1 high *BRCA1* mutant BLBC with cyclin E1 high *CCNE1* non amplified HGSOC that still needs further investigation especially in HGSOC.

6.4 Conclusion

We conclude that there are two cyclin E1 high HGSOC subsets, with and without gene amplification. The latter shares numerous features with a cyclin E1 high BLBC subset, amplified or not. We strongly suggest that *CCNE1* amplification is the driver of the subset of HGSOC that harbours them, associates with high expression of cyclin E1 that is reinforced by loss of FBXW7, is responsible for their genomic instability and requires an intact DNA repair machinery and associates with worst outcome. Alternatively, in the *CCNE1* non-amplified HGSOC subset, at least for some of the cases, and for most of BLBC cases the driver is the *BRCA* inactivation and the associated high cyclin E1 is a cofactor in driving poor outcome via the increased proliferation of cancer cells.

We have reported a synergy of a combination of CDK2 and PARP inhibitors in cyclin E1 high BLBC. We suggest that this combination is likely to be effective in the comparable HGSOC subset, cyclin E1 high *CCNE1* non amplified group. Further research is need to support our suggestion.

We strongly support the notion that checkpoint inhibitors will soon be the targeted therapy of choice cancers in the group that desperately need them, the*CCNE1* amplified

HGSOC especially when combined with DNA damaging agents to enhance their synthetic lethality.

Table 6-2: List of ATR pathway inhibitors that are currently recruiting *CCNE1* amplified cancers

ClinicalTrials.gov identifier	Title	Phase	Drug used	Drug target
NCT02797964	A Phase 1/2 Trial of SRA737 in Subjects With Advanced Cancer	I/II	SRA737	CHK1 inhibitors
NCT02797977	A Phase 1/2 Trial of SRA737 in Combination With Gemcitabine Plus Cisplatin or Gemcitabine Alone in Subjects With Advanced Cancer	I/II	SRA737	CHK1 inhibitors
NCT02873975	A Study of LY2606368 (Prexasertib) in Patients With Solid Tumors With Replicative Stress or Homologous Repair Deficiency	II	LY2606368 (Prexasertib)	CHK1 inhibitors
NCT03718091	M6620 (VX-970) in Selected Solid Tumors	II	M6620	ATR enzyme inhibitor
NCT03253679	AZD1775 in Treating Patients With Advanced Refractory Solid Tumors With *CCNE1* Amplification	II	Adavosertib	WEE1 inhibitor

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