The role of cyclin E1 expression on sensitivity to DNA damage response modulators in Osteosarcoma

Irene Sechi

St Hilda College, University of Oxford
Thesis submitted for the degree of Master of Science by Research
Trinity Term 2019

CRUK/MRC Oxford Institute for Radiation Oncology
Department of Oncology, University of Oxford

CANCER RESEARCH UK | MRC Medical Research Council

OXFORD INSTITUTE FOR RADIATION ONCOLOGY

The role of cyclin E1 expression on sensitivity to DNA damage response modulators in Osteosarcoma

Irene Sechi
St Hilda College, University of Oxford
Trinity Term 2019

Abstract

Osteosarcoma (OS) is an aggressive malignant bone tumour mostly present in childhood. In particular, the retinoblastoma tumour suppressor gene (*RB1*) has an essential role in the pathogenesis of OS. In response to growth stimuli, an altered Retinoblastoma/E2F pathway mediates an aberrant expression of downstream cell cycle promoters. Specifically, ectopic expression of cyclin E1 accelerates DNA replication timing leading to genomic instability and DNA replication stress. Consequently, DNA replication stress triggers an intra-S phase checkpoint that is dependent on Ataxia Telangiectasia and Rad3 related (ATR) kinase signalling. Oncogene activated DNA replication stress is a common feature of cancer cells. ATR inhibition selectively targets tumour cells exploiting endogenous vulnerabilities and increasing DNA damage to induce cancer cell death.

This research project aims to investigate the oncogenic role of cyclin E1 in modulating sensitivity to DNA damage response inhibitors (DDRi). Here, we use the U2OS osteosarcoma cell line with a stably integrated TET-off inducible genes system to investigate the role of ectopic expression of cyclin E1 in early priming and firing of DNA replication origins, replication stress and genomic instability.

Biomarkers for replication origin firing (pCDC6ser54), DNA replication stress (pRPAser33, pRPAser4/8) and DNA damage response pathways (pCHK1, pKAP1) were explored by western blot analysis. Whether variabilities in cell proliferation upon DDR inhibitors exposure were examined with resazurin-based fluorescence viability assays. Additionally, we extended our findings to PC-9 and A549 human lung carcinoma cell lines to explore DDRi activity regarding differences in cyclin E1 expression.

ATR inhibition (ATRi) using AZD6738 exhibited an unexpected disparity in selectively decreasing viability of cells with ectopic expression of cyclin E1. In particular, ATRi sensitivity was characterised by an increase in biomarkers for replication stress and DNA damage response pathways such as pATM. This result suggests that AZD6738 could selectively target tumour cells, including osteosarcoma, with aberrant expression of cyclin E1, although further study of the relationship between cyclin E1 expression and sensitivity to ATRi is required.

Acknowledgements

When I started this new experience, I hoped to meet a supervisor that could guide me in my research studies. I am deeply grateful to Andy Ryan for being a constant inspiration as person and supervisor. I will never forget his constant support and our ideas-discussion during my time in his lab. I will take all his advice with me in my future research experience.

Sincere thanks to all members of the Ryan lab for the constant help, it was such a nice company during the working hours in the lab. I hope we will keep in touch in the future.

I am extremely grateful to my family for their endless love and for supporting me to pursue this opportunity. They have been an example of how difficulties in life can be overcome with courage, patience and a little bit of irony. Despite the distance, I have always felt them with me.

A big thanks also to my five wonderful best friends for our special friendship that can break every distance. It is absolutely unique. A special thanks to Miranda for being such as an amazing friend. In Oxford, I had the opportunity to meet another little family to share trips, crazy nights and time to laugh. I would like to thank all of the beautiful people have made this experience unforgettable. Especially, I am very thankful to Saif for our scientific discussions and his unlimited encouragement.

I can't forget to thank Prof. Paul Brennan and Saleta Vasquez for giving me the opportunity to join the University of Oxford and make a change in my life. I will work hard for following my aspirations.

Finally, I would like to dedicate my research thesis to the young little heroes that are fighting against a destiny they haven't chosen. I am sure they won't never give up.

- *Beautiful things happen to who believes, even more beautiful things happen to who is patient, but the best things happen to who never gives up, leaves things to happen and follows the waves of his own destiny* -

Word Count: 18801

Table of contents

1. Introduction……12

1.1. Osteosarcoma……...12

1.2. The regulation of G1-S phase transition..... 14

1.3. The impact of cyclin E1 overexpression 18

1.4. The DNA damage response (DDR)..... 20

1.5. The role of ATR in response to DNA damage..... 22

1.6. Targeting the DDR for cancer therapy..... 25

1.7. Development of ATR inhibitors in clinic..... 27

1.8. Aim of this study..... 32

2. Material and Methods 33

2.1. Mammalian Cell Culture 33

2.2. Maintenance of cell lines..... 33

2.3. Cell counting and seeding 34

2.4. Cryostorage and thawing of cells..... 35

2.5. Drug Treatments..... 35

2.6. Cell Proliferation Assay with Resazurin..... 36

2.7. PicoGreen Cell Proliferation Assay 36

2.8. Protein Detection by Western Blotting..... 37

2.8.1. Preparation of cell extracts, gel electrophoresis and transfer 37

2.8.2. Immunoblotting and Protein Detection using LI-COR..... 40

2.9. Cell Synchronisation..... 42

2.10. Flow Cytometry..... 42

2.11. Data Analysis and Statistics 45

3. The U2OS Cyclin E cell line……46

3.1. The TET-off system in U2OS Cyclin E cell line……..……..46

3.2. Cell proliferation assays……..……..……...……..……51

3.3. Cell Cycle Analysis: the impact of Cyclin E1 overexpression..... 52

4. Cyclin E1 overexpression and sensitivity to DDR inhibitors……..……54

4.1. Sensitivity to ATR inhibitors 54

4.2. Sensitivity to other DNA Damage response modulators..... 56

4.3. Signalling Responses: cell synchronisation and mechanistical studies 58

4.3.1. Replication stress analysis…….……58

4.3.2. ATM signalling pathway……60

4.4. Key Findings..... 61

5. Cyclin E1 as a novel biomarker…….…….……...63

5.1. Lung cancer cell lines..... 63

5.2. Signalling response in PC9 and A549 cells..... 66

5.3. ATM signalling pathway..... 67

6. Exploring a possible role for cyclin F…….…...……...70

6.1. The Ubiquitin-proteolysis system……..70

7. Discussion……..……..……72

8. Conclusion……76

9. References……...……...……77

List of figures

Figure 1.1. Schematic showing different cyclin/CDK partners controlling different phases of the cell cycle…….14

Figure 1.2. DNA replication origins during G1 and S phase……..17

Figure 1.3. ATR is activated when replication stress is induced causing long stretches of ssDNA……..23

Figure 1.4. ATR-induced cell cycle arrest is mediated via CHK1 activity……25

Figure 2.1. Cell Synchronisation Analysis with FACS – Flow Cytometry……44

Figure 3.1. Schematic representation of the TET-OFF inducible system in U2OS-Cyclin E osteosarcoma cell line……...……47

Figure 3.2. Characterisation of the system TET-off in U2OS Cyclin E cell line……48

Figure 3.3. Evaluation of cyclin E1 overexpression in Parental U2OS and U2OS Cyclin E cell lines…….…….…….49

Figure 3.4. Time-course experiment to assess the constitutive expression of cyclin E1 in U2OS Cyclin E cell line…….…….…..……50

Figure 3.5. Cyclin E1 overexpression does not affect proliferation of U2OS Cyclin E cells *in vitro*……...……...51

Figure 3.6. Western blot analysis of the cell cycle in normal and overexpressing cyclin E1 cells**.**……..53

Figure 4.1. Sensitivity of U2OS Cyclin E cell line to ATR and CHK1 inhibitors……..…….55

Figure 4.2. Sensitivity of U2OS Cyclin E cell line to different classes of DNA damage response modulators…….……...…….57

Figure 4.3. Replication stress analysis in U2OS Cyclin E cell line. ……..……....59

Figure 4.4. ATM signalling pathway in U2OS Cyclin E cell line. ……..…..61

Figure 5.1. PC9 and A549 cell proliferation……..….63

Figure 5.2. Sensitivity of PC-9 cell line to ATR and CHK1 inhibitors. ……64

Figure 5.3: Sensitivity of A549 cell line to ATR and CHK1 inhibitors…….66

Figure 5.4. Western blot analysis of S phase cyclins (A and E) in PC-9 and A549 cells.…….....67

Figure 5.5. Western blot analysis of markers of DNA replication stress and ATM activity in PC-9 and A549 cells…….....69

Figure 6.1. CDK1 inhibition and cyclin F protein levels…..……...71

List of tables

Table 1.1: DDR inhibitors, their targets and their chemical structure.....31

Table 2.1: Cell lines used in this study.....34

Table 2.2: DNA lysis buffer composition.....37

Table 2.3: Lysis buffer stock components for western blot cell extract collection.....39

Table 2.4: Buffers and solutions used in immunoblotting.....40

Table 2.5: Primary antibodies used for immunoblotting analysis.....41

Table 2.6: Secondary antibodies used for two-color detection with infrared fluorescence.....42

Table 2.7: IC_{50} values for the sensitivity of U2OS Cyclin E cell line to ATR and CHK1 inhibitors……..56

Table 2.8: IC_{50} values for the sensitivity of U2OS Cyclin E cell line to DDR modulators..…..58

Table 2.9: IC_{50} values for the sensitivity of PC9 cell line to ATR and CHK1 inhibitors…….. 65

Table 2.10: IC_{50} values for the sensitivity of A549 cell line to ATR and CHK1 inhibitors……..66

Abbreviations

53BP1	tumour protein p53 binding protein 1
A-T	Ataxia-telangiectasia
ATCC	American Type Culture Collection
ATM	ataxia-telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia-telangiectasia and Rad3-related
ATRi	ATR inhibitor/ inhibition
ATRIP	ATR interacting protein
C	Celsius
CCNA2	cyclin A2
CCNE1	cyclin E1
CDC	cell division cycle
CDK	Cyclin-dependent kinase
CDT1	chromosome licensing and DNA replication factor 1
CHK1	checkpoint kinase 1
CHK2	checkpoint kinase 2
CO2	carbon dioxide
CV	crystal violet
DAPI	4’,6-diamidino-2-phenylindole
DDR	DNA damage response
dH2O	distilled water
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	deoxynucleotide triphosphate
DSB	double-strand break
FBS	foetal bovine serum
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
g	gram(s)
Gln	glutamine

G1	phase gap phase of cell cycle before DNA replication
G2	phase gap phase of cell cycle before mitosis
hr	hour(s)
H2O	water
HCl hydrochloric acid	hydrochloric acid
IC50	half-maximal inhibitory concentration
KAP1	Krüppel-associated box-associated co-repressor 1
kDa	kilo-Dalton
KPA	key pathway analysis
L	litre
M	molarity
m	metre
MCM	minichromosome maintenance
min	minute(s)
M phase	mitosis
mAb	monoclonal antibody
Na_3VO_4	sodium orthovanadate
NaCl	Sodium chloride
NaF	sodium fluoride
NaOH	sodium hydroxide
NER	nucleotide-excision repair
NSCLC	non-small cell lung cancer
OS	Osteosarcoma
p53	(TP53) tumour protein 53
pAb	polyclonal antibody
PARP	poly (ADP ribose)-polymerase
pATM	phospho-ATM
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline with Tween 20
pCHK1	phospho-CHK1
pCHK2	phospho-CHK2
pHH3	phospho-histone H3
PI	propidium iodide
PIKK	phosphatidylinositol 3-kinase-related kinase
pKAP1	phospho-KAP1

PPIB	peptidyl-prolyl cis-trans isomerase B (Cyclophilin B)
pRB	Retinoblastoma protein
RNA	ribonucleic acid
RPA	replication protein A
RT	room temperature
S phase	DNA replication or synthesis phase of the cell cycle
SDS	sodium dodecyl sulphate
sec(s)	second(s)
SEM	standard error of the mean
ssDNA	single-stranded DNA
SKP1	S-phase kinase-associated protein 1
t	time
TBS	tris-buffered saline
TBS-T	tris-buffered saline with Tween 20
UV	ultraviolet
γH2AX	phosphorylated histone H2AX

CHAPTER 1

1. Introduction

1.1. Osteosarcoma

Osteosarcoma is the most common primary malignant bone tumour with the majority of cases arising in children and adolescents younger than 20 years of age. (Longhi et al. 2006; Marina 2004) The overall incidence is 5 cases per million people per year. However, among childhood malignancies, osteosarcoma is the eighth most common behind leukaemias, lymphomas, and neurological malignancies. (Choong et al. 2011) Current treatments for osteosarcoma are based on well-established approaches that combine multi-agent chemotherapy and surgery. Drugs currently included in standard chemotherapy protocols that have been used since 70-80s include: doxorubicin (adriamycin), cisplatin (cis-diamminedichloroplatinum), high-dose methotrexate and/or ifosfamide and/or etoposide. (Ferrari et al. 2005; Hattinger et al. 2015, 2019) Despite the use of high dose of chemotherapeutics and deep resection of the tumour area, 30-40% of patients succumb to the disease mainly due to refractory and/or recurrent disease. (Baumhoer, Amary, and Flanagan 2019; Sajadi et al. 2004) Hence, there is a real need to improve current treatment strategies and to develop novel approaches for treating osteosarcoma.

Different studies investigating the pathogenesis of OS reported genomic instability as a significant feature correlated with cytogenetic abnormalities and wide heterogeneity between cells within the same patient. Karyotype complexity is also related to high expression of tissue invasion-related genes and lower expression of genes involved in cell

cycle regulation and DNA repair. (Roschke et al. 2015) Specifically, mutation or deregulation of retinoblastoma (*RB*1) and p53 genes, fundamental in regulating cell cycle progression, are well-known molecular changes occurring in OS. (Longhi 2001; Masuda et al. 1987; Scholz et al. 1992) In particular, loss of heterozygosity or deletion of the *RB1* gene located on chromosome 13q14.2 has a prominent role in hereditary retinoblastoma tumour and predisposes to development of osteosarcoma. (Friend et al. 1986; Toguchida 2016; Zhang et al. 2015) Mutation or genetic loss of both *RB1* and p53 is found in about 50% of osteosarcoma cases. (Heinsohn et al. 2007; Toguchida 2016) Overall, genetic studies revealed several other mutational drivers implicated in the genesis of OS. For instance, these include overexpression of MDM2 (murine double minute 2) gene that inactivates the p53 protein, amplification of cyclin-dependent kinases 4 (CDK4) that inactivate *RB1* or INK4A deletion. (Rickel, Fang, and Tao 2017) Mutations associated with alteration of integrating factors of either one or both pRB and p53 pathways abrogate regulation of cell cycle transition from G1 to S-phase. (López-Guerrero et al. 2004; Rickel, Fang, and Tao 2017) In particular, an uncontrolled G1-S phase transition in the cell cycle leads to chromosomal abnormalities exposing the genome to DNA damage accumulation. (Benassi et al. 1997; Marina 2004)

Since specific genetic changes leading to an uncontrolled G1-S phase transition are a common feature of osteosarcoma, novel treatment approaches, designed according to the biological characteristics of each individual tumour, may provide more precise therapy and improve current clinical results in OS. Furthermore, there is also a need to identify biomarkers that can be used to classify tumours and patients into those that are likely either to respond or not respond to targeted therapies, as well predicting individual susceptibility to treatment-associated toxicities. (Hattinger et al. 2019)

1.2. The regulation of G1-S phase transition

The cell cycle consists in a series of well-orchestrated events divided in discrete phases (G1, S, G2, M) ending with division of a cell into two daughter cells. (Fig.1.1) Each event is precisely regulated at designated checkpoints and at each phase transition. Each phase of the cell cycle has a unique profile of cyclin-dependent kinases (CDKs) whose activity regulates progression through the cell cycle including the critical transitions between the G1-S and G2-M phases. (Morgan 1992) (Fig1.1) CDKs contain two subunits, a catalytic kinase subunit and a regulatory cyclin subunit that is required for CDK activity. The periodicity of increased cyclins levels is an essential feature of normal cell cycle regulation. (Resnitzky and Reed 1995)

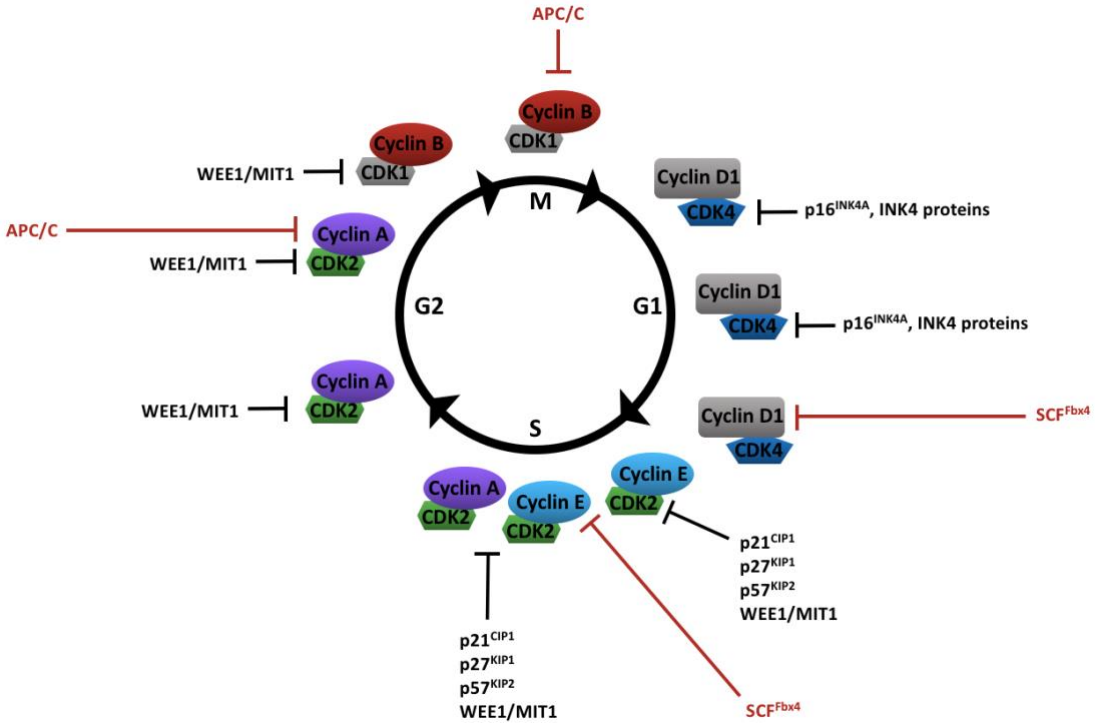


Figure 1.1: Schematic showing different cyclin/CDK partners controlling different phases of the cell cycle. Adapted from Morgan & Reuter, 2006.

Retinoblastoma protein (pRB) acts as a growth-suppressive regulator of cell cycle progression that forms a complex with, and inactivates, E2F (a DNA-binding transcription factor). pRB exists in three phosphorylated forms: un-phosphorylated, mono-phosphorylated

or multi-phosphorylated. When levels of phosphorylation are low, pRB acts as a tumour suppressor by binding E2F, whereas increased levels of pRB phosphorylation lead to release of E2F from the complex and subsequent activation of E2F-dependent transcription. (Fischer and Müller 2017; Weinberg 1995) pRB phosphorylation levels are tightly regulated by cyclin-CDK complexes. The generally accepted order of pRB phosphorylation by cyclin-CDKs through the cell cycle is cyclin D1/CDK4/6 in early G1 phase, cyclin E/CDK2 in late G1, and cyclin A/CDK2 in S phase. The differential responses to phosphorylation on pRB function correspond with modification of distinct sets of phosphorylation sites. (Connell-Crowley, Harper, and Goodrich 1997) In response to growth signals cyclin D1/CDK4/6 complex phosphorylates pRB allowing the release of E2F transcription factor thereby promoting gene transcription of downstream positive regulators of cell cycle progression. (Antonescu et al. 2000; Lundberg and Weinberg 1998; Weinberg 1995) Although the activity of E2F family members E2F-1, E2F-2, and E2F-3 are controlled by pRB, E2F1 in particular plays a major role in controlling cyclin E1 (CCNE1) expression. Cyclin E1 interacts with high specificity with the cyclin kinase 2 (CDK2) and the resulting complex (cyclin E1-CDK2) acts as a master regulator of the G1-S phase cell cycle transition. (Koff et al. 1992; Resnitzky et al. 1994)

There are two forms of cyclin E (cyclin E1 and cyclin E2) which have high sequence similarity and both of which are able to complex with CDK2. (Caldon and Musgrove 2010) However, there are crucial independencies in gene transcriptional and post-transcriptional regulation. Cyclin E1 and cyclin E2 are assumed to be functionally redundant. In particular, cyclin E1 complexed with CDK2 appears to have a predominant role in cell cycle control and it is found to be highly expressed in several types of cancer. (Caldon and Musgrove 2010) Potential oncogenic effects of cyclin E2 have not been reported suggesting that cyclin E1 and cyclin E2 may not have equivalent roles in tumorigenesis. Interestingly, one of the

roles of cyclin E/CDK2 comples is to induce its own expression through a positive feedback loop promoting phosphorylation of pRB and the consequent increase in transcriptionally active E2F1 levels. The expression of E2F gene targets (cyclins, etc) gradually increases during G1 and must reach a critical level in order for cells to overcome the 'restriction point', a point where cells become committed to progression into S phase. (Blagosklonny, Pardee, and Pardee 2016; Resnitzky and Reed 1995) There have been several reports suggesting that expression of cyclin E1 alone is able to induce S phase entry from G1, without the requirement of pRB phosphorylation or the transcription of E2F-activated genes. (Dyson 1998; Leng et al. 1997; Lukas, Hansen, et al. 1997) However, studies have also demonstrated that E2F overexpression can prompt S phase entry without cyclin E/CDK2 activity. (Degregori et al. 1995; Leone et al. 1999) Taken together, this suggests cooperative interaction of E2F and cyclin E/CDK2 for promoting entry into S phase. Moreover, cyclin E1 promotes the assembly of the pre-initiation replication complex onto the DNA. Priming of the origin replication complex depends on recruitment of CDC6 and CDC10-dependent transcript 1 (also known as DNA replication factor CDT1) to the origin replication complex (ORC). ORC and CDC6 cooperates to identify DNA replication origin sequences in an ATP-dependent manner. (Speck et al. 2005) When an ORC is situated in non-origin DNA sequences, CDC6 hydrolyses ATP and promotes the dissociation of both proteins from the DNA. In contrast, interaction with specific origin sequences inhibits ATP hydrolysis, stabilizing the interaction with the DNA. (Borlado and Méndez 2008) Subsequently, origin licensing during G1 phase culminates with the recruitment and loading of two CDT1-bound hexamers of MCM2-7 onto the replication origin by cyclin E in a CDK2 independent manner. (Caldon and Musgrove 2010; Geng et al. 2007) (Fig.1.2.) At the G1-S transition point, inactive MCM2–7 double hexamer (DH) recruits CDC45 and the four subunit GINS complex. DNA replication origin firing is mediated by multiple phosphorylation events carried out by DDK (DBF4-CDC7) and cyclin E/CDK2 within the MCM2–7 complex,

resulting in helicase activation and DNA unwinding. (Boos and Ferreira 2019; Borlado and Méndez 2008; Leman and Noguchi 2013; Speck et al. 2005) At this point, primase and DNA polymerases are loaded and the replisome machine executes DNA synthesis. (Yuan et al. 2017) (Fig.1.2.)

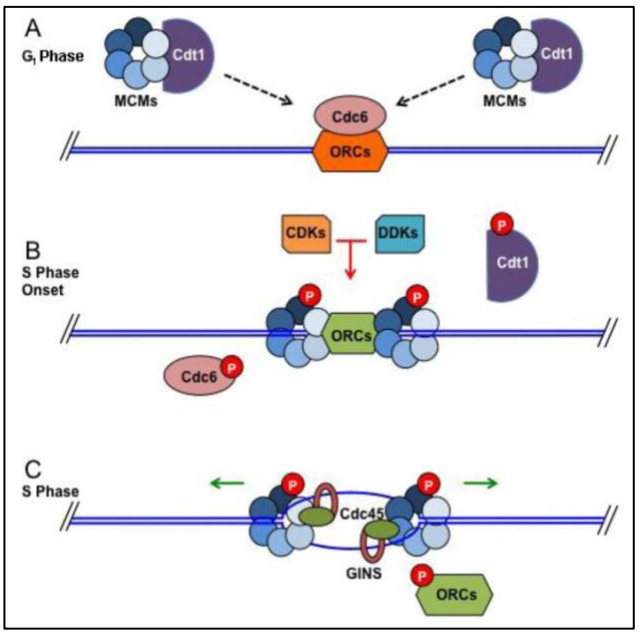


Figure 1.2. DNA replication origins during G1 and S phase. (A) CDC6 and ORC bind to DNA origin sequence and promote the recruitment of MCM-CDT1 complexes to replication origins.
(B) CDK/DDK-dependent phosphorylation of pre-replication complex (RC) components leads to CDC6 and CDT1 dissociation allowing replisome assembly and origin firing.
(C) MCMs and associated proteins (GINS and CDC45) unwind DNA to expose template DNA. At this point the replisome can be assembled and replication initiated.
(Modified from Leman and Noguchi, Genes, 2013)

Decline of cyclin E1 levels is tightly regulated by ubiquitin-mediated proteolysis. In the cell cycle, ubiquitination plays a central role in cell-cycle transitions and checkpoints by establishing the strict temporal control of proteins such as cyclins and cyclin-dependent kinase (CDK) inhibitors. The ubiquitin system requires the cooperation of three components that participate in a cascade of ubiquitin transfer reactions: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). E3 recognises target proteins with high specificity and therefore has a crucial role in controlling individual target protein abundance.

Several studies suggested that the complex of SCF^{fbw7} (SKP1/ CUL1/ F-box protein) complex regulates the stability of cyclin E1, promoting poly-ubiquitination and proteasomal degradation. The complex is formed by the F-box domain that interacts with SKP1, a

component of the SKP1-CUL1-F-box protein (SCF) ubiquitin ligase machinery. SKP1 binds one of many F-box proteins (69 in human) and also recruits CUL1 (and RBX1 with CUL1) to assemble a functional SCF complex. Fbw7 selectively recognises cyclin E1, and SCF^{fbw7} catalyses cyclin E1 ubiquitination *in vitro.* (Koepp et al. 2001) Fbw7 has been shown to act as a tumour suppressor. (D'Angiolella, Esencay, and Pagano 2013; Hao et al. 2007)

1.3. The impact of cyclin E1 overexpression

Loss of the cell cycle restriction point and deregulated control of G1-S phase transition are common features of cancer cells that can lead to uncontrolled cell proliferation. (Kotsantis, Petermann, and Boulton 2018) In particular, defects in RB pathway components may provoke oncogene-induced aberrant proliferation. Abnormal cyclin E1 expression is able to destabilise the highly conserved regulation of the cell cycle progression leading to genomic instability and cancer malignancy. (Tort et al. 2006) Unlike cyclin D or cyclin A, cyclin E1 remains essential for promoting G1-S phase transition in cells with an absent or defective RB pathway. (Ohtsubo et al. 1995) Additionally, constitutive expression of cyclin E1 bypasses pRB-mediated cell cycle arrest. (Alevizopoulos et al. 1997; Lukas, Herzinger, et al. 1997) These observations demonstrate the fundamental differences between cyclin E1 and other cyclins, strongly suggesting that cyclin E1 is a key player for S-phase entry. (Duronio et al. 1996) An aberrant expression of cyclin E1 protein could lead to genomic instability in human cancers due to its key regulatory role in G1/S phase transition and priming/firing origins. (Macheret and Halazonetis 2018a).

Indeed, ectopic increases in cyclin E1 protein levels lead to an accelerated G1-S phase transition in the cell cycle (Resnitzky et al. 1994) and chromosome instability. (Spruck, Won, and Reed 1999) A premature G1-S phase transition drives cells into S phase without completing the G1-dependent gene transcription programmes leading to a deregulated

replication process and an increase in replication-transcription conflicts. Interference between replication and transcription may enhanced depletion of replication building blocks such as deoxynucleotide pools (dNTPs), histones or RPA protein, thereby compromising efficient DNA replication. (Bester et al. 2011; Resnitzky et al. 1994)

In addition, pre-replication complex assembly and origin firing are also disrupted. (Ekholm-Reed et al. 2004) Constitutive activation of cyclin E/CDK2 results in unscheduled pre-RC initiation prior to S-phase entry. Early initiation of pre-RCs, with insufficient deoxynucleotide pools, would lead to inefficient DNA synthesis, DNA replication stress and genomic instability. (Ekholm-Reed et al. 2004; Jones et al. 2013) Moreover, cyclin E1 1overexpression intensifies the CDK2 kinase activity and induces a rise in replication origin firing density promoting uncoordinated replication fork progression and stalled replication forks. (Macheret and Halazonetis 2018a)

A recent study identified a novel set of DNA replication origins mapped within the highly transcribed genes under conditions of cyclin E1 overexpression. Origin sequences that are usually suppressed during G1 and activated in S phase entry, were triggered prematurely delineating a new oncogene activity implicated in inducing replication stress. (Macheret and Halazonetis 2018b)

Inefficient proteolytic processing of cyclin E1 can generate hyperactive low molecular weight isoforms (LMW-E) in tumours overexpressing cyclin E1. (Caruso et al. 2018) In particular, recent studies showed that LMW isoforms of cyclin E are hyperactive biochemically as their presence can increase cell transition into S-phase and can promote aberrant substrate phosphorylation through CDK2 interaction. Functions of LMW-E isoforms include hyperactivation of CDK2 in both the nucleus and in the cytoplasm, where

the LMW-E/CDK complex interacts with a novel sets of substrates not usually regulated by nuclear cyclin E/CDK2 complex. (Caruso et al. 2018; Delk, Hunt, and Keyomarsi 2009) LMW-E isoforms cannot be inhibited by p21/ p27 and are less likely to be degraded by the ubiquitin-proteasomal system. The proteolytic processing of cyclin E1 isoforms appears to occur exclusively in tumour cells and appears to be independent from the ubiquitination-proteasome pathway. Cleavage of cyclin E1 to LMW-E isoforms is considered more likely to be generated via amino terminus cleavage of the full-length cyclin E1 by serine protease or calcium dependent protease activity. (Porter et al. 2001)

Overall, accurate replication of the genome is essential to preserve genomic stability and cyclin E1 appears to have a critical role in regulating cell cycle progression. Unbalanced levels of cyclin E1 induce replication stress and DNA damage not only deregulating the timing of cell cycle progression but also by disrupting the regulation of DNA replication processes leading to genomic instability. (Fragkos et al. 2015) Consequently, DNA damage response signalling may be activated to provide a natural barrier to delay or prevent tumorigenesis and genetic instability. (Bartkova et al. 2006; Gaillard, García-Muse, and Aguilera 2015) In particular, it has been shown that oncogene-induced replication stress triggers activation of DNA damage response pathways that drive cells into senescence. (Di Micco et al. 2006)

1.4. The DNA damage response (DDR)

Thousands of DNA damage events occur each day in our cells. (Lindahl 1993) In consequence, cells have evolved a co-ordinated set of signalling responses – the DNA damage response (DDR) – in order to preserve genomic integrity. (Ciccia and Elledge 2011; Jackson and Bartek 2009) DNA damage can be caused by exogenous exposure to radiation or genotoxic agents. Additionally, DNA damage can be caused by endogenous factors such

as dNTP misincorporation during DNA replication, base depurination or deamination due to hydrolysis or interaction with reactive products of cellular metabolism such as reactive oxygen species (ROS) or formaldehyde. (Klungland et al, 1999) When DNA lesions remain unrepaired or aberrantly repaired, deleterious mutations can affect cell viability leading to senescence, cell death or mutations that may culminate in cancer development. (De Bont and van Larebeke 2004; Hoeijmakers 2019)

The DNA damage response (DDR) comprises a complex network of signalling pathways that are specialized to detect DNA alterations, signal their presence and facilitate their repair to ensure genomic stability and cell viability. (Ciccia and Elledge 2011; Jackson and Bartek 2009; Weber and Ryan 2015a) In particular, DNA damage signalling will determine whether cells will undergo senescence, transient growth arrest or apoptosis through cell cycle checkpoint activation. (Ciccia and Elledge 2011; Jackson and Bartek 2009)

Two key DDR signalling components are the protein kinases ATR and ATM. (Blackford and Jackson 2017; Ciccia and Elledge 2011; Lempiäinen and Halazonetis 2009) Both proteins are members of the phosphatidylinositol 3-kinase- related kinase (PIKK) family of serine/threonine protein kinases, which also includes DNA-dependent protein kinase catalytic subunit (DNA-PKcs/*PRKDC*). (Lempiäinen and Halazonetis 2009) The N-terminal region of PIKK family proteins appears to be important for mediating interactions with substrates and adapter proteins. (Fernandes et al. 2005) For instance, in ATR, the N-terminus contains the binding site for ATRIP, which mediates the localisation of ATR to sites of replication stress. (Cortez et al. 2001; Zou and Elledge 2003) The ATR-ATRIP complex is recruited by binding RPA at sites of single stranded DNA structures (ssDNA), which may arise e.g. at resected DNA double strands breaks (DSBs) or at stalled DNA replication forks. On the other hand, ATM activates the DDR in presence of DNA DSBs which may arise e.g.

following the collapse of stalled replication forks. Several studies have suggested that DDR pathways may limit cancer development in its early stages operating as a barrier for proliferation of aberrant cells (Bartkova et al. 2006; Gorgoulis et al. 2005) and specifically through the activation of the tumour suppressor protein p53. (Halazonetis, Gorgoulis, and Bartek 2008) Indeed, two of the most widely studied ATR/ATM targets are the protein kinases CHK1 and CHK2 respectively (together with ATR and ATM) restrict cyclin-dependent kinase (CDK) activity by various mechanisms, some of which are mediated by activation of the p53 transcription factor to induce cell cycle arrest and apoptosis. In particular, CHK2, a key downstream target of ATM (Matsuoka, Huang, and Elledge 1998) and mediator of ATM signalling, can interact directly with p53 and phosphorylate it on serine 20. (Chehab et al. 1999, 2000)

In conclusion, cancer cells lacking in G1-S cell cycle-checkpoints due to deficiencies in the pRB-E2F pathway and/or oncogene amplification such as cyclin E1 are characterized by high levels of replication stress and chromosomal instability. (Bartkova et al. 2006; Jones et al. 2013; Di Micco et al. 2006; Tort et al. 2006) However, DDR signalling induced by oncogene activation may also act to restrain cell proliferation driving cancer cells to senescence and provide an obstacle to cancer development. (Di Micco et al. 2006)

1.5. The role of ATR in response to DNA damage

The ATR signalling pathway is activated in response to DNA damage including DNA single strands breaks, DNA base adducts and DNA replication stress. (Ciccia and Elledge 2011; Cimprich and Cortez 2008b) In particular, ATR signalling pathway activation occurs when single-stranded regions occur on DNA. Replication protein A (RPA) coats ssDNA that has a 5′ double stranded DNA primer junction. RPA-coated DNA then recruits the ATRIP which is in a complex with ATR, thereby localising ATR to regions of ssDNA. (Costanzo et al. 2003; Fanning, Klimovich, and Nager 2006; Zou, Liu, and Elledge 2003) ATR signalling

activation depends on the co-localisation of ATRIP-ATR with the Rad9-Rad1-Hus1 (9-1-1) complex. Specifically, RPA recruits the RAD17/RFC-5 clamp loader complex that enables the loading of 9-1-1 complex onto the DNA. (Ellison and Stillman 2003; Zou, Liu, and Elledge 2003) Subsequently, the 9-1-1 complex mediates the recruitment of the main ATR activation protein: the DNA topoisomerase 2-binding protein (TOPBP1). (Delacroix et al. 2007; Lee, Kumagai, and Dunphy 2007) TOPBP1 loading depends on a recognition site mediated by a C-terminal phosphorylated tail in RAD9. (Lee, Kumagai, and Dunphy 2007) TOPBP1 is a molecular bridge that mediates the interaction between the independently recruited 9–1–1 and ATRIP–ATR complexes, leading to checkpoint activation. (Yang and Zou 2006)

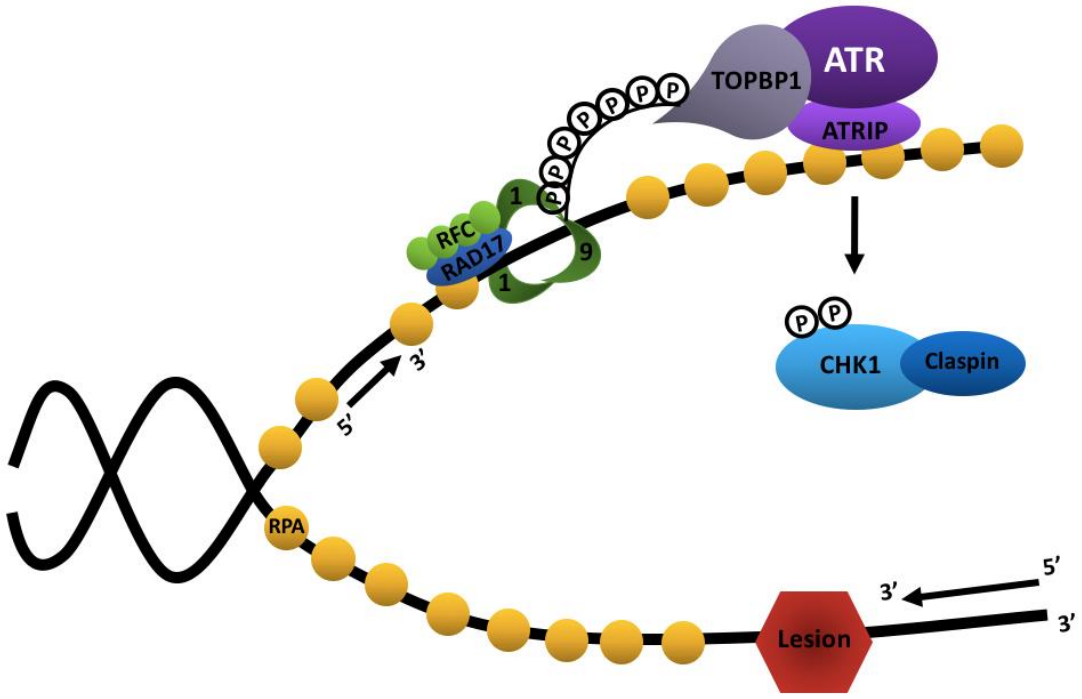


Figure 1.3: ATR is activated at sites of ssDNA. Black lines represent DNA. In this example, a DNA lesion (red) inhibits leading strand DNA synthesis and helicase activity continues to unwind double stranded DNA at the replication fork, creating long strands of ssDNA. ssDNA regions are coated by RPA (yellow) which can recruit ATRIP:ATR complexes (light purple:dark purple). RPA also recruits the RAD17/RFC2-5 clamp loader complex (blue and light green) that enables loading of the RAD17-RAD1-HUS1 (9-1-1) ring complex (dark green) onto the DNA. This complex subsequently recruits TOPBP1 (grey) which stimulates ATR activity. Claspin, recruited by RAD17, recruits CHK1, which is phosphorylated by ATR on two serine residues: ser345 and ser317. Once

activated, CHK1 dissociates and signals downstream responses. Directions of DNA replication are indicated. Modified from Wagner & Kaufmann, 2010.

Once the active ATRIP-ATR complex is assembled onto ssDNA e.g. at a stalled replication fork, it can phosphorylate downstream substrates to coordinate cell cycle arrest and stabilization of the stalled replication forks. The main downstream effector of ATR is thought to be CHK1 kinase activity. Claspin, an adaptor protein, is recruited by RAD17 and it is phosphorylated and activated by ATR. Claspin promotes the interaction between ATR and CHK1 proteins to facilitate CHK1 phosphorylation. (S. Liu et al. 2006) CHK1 is phosphorylated by ATR at serine 317 and 345 and can also be autophosphorylated at serine 296. Once phosphorylated, CHK1 is released from chromatin and phosphorylates CDC25 phosphatases. (Bartek, Lukas, and Lukas 2004) (Fig.1.4) CDC25 protein phosphatases regulate cell cycle transitions by dephosphorylating and activating CDK1 and CDK2 thereby promoting cyclin E/CDK2 and cyclin A/CDK2 dependent replication origin firing and cyclin B/CDK1 dependent G2-M phase progression. (Maya-Mendoza et al. 2007) (Fig.1.4) Phosphorylation of CDC25A or CDC25C by activated CHK1 leads to CDC25A ubiquitin-dependent proteolysis or 14-3-3 dependent nuclear export, respectively, (Boutros, Dozier, and Ducommun 2006; Jiang et al. 2003; Peng et al. 1997) thereby inhibiting both CDK1 and CDK2 activity (phosphorylated forms of CDK1 and CDK2 are inactive) leads to both inhibition of DNA synthesis (S phase arrest) and G2-M cell cycle progression (G2 phase arrest). (Fig.1.4) CDK1 and CDK2 are phosphorylated (inactivated) by WEE1. WEE1 kinase is activated by ATR-CHK1 signalling which further reinforces the effects of decreased CDC25 phosphatase activity on cell cycle arrest. (Boutros, Dozier, and Ducommun 2006)

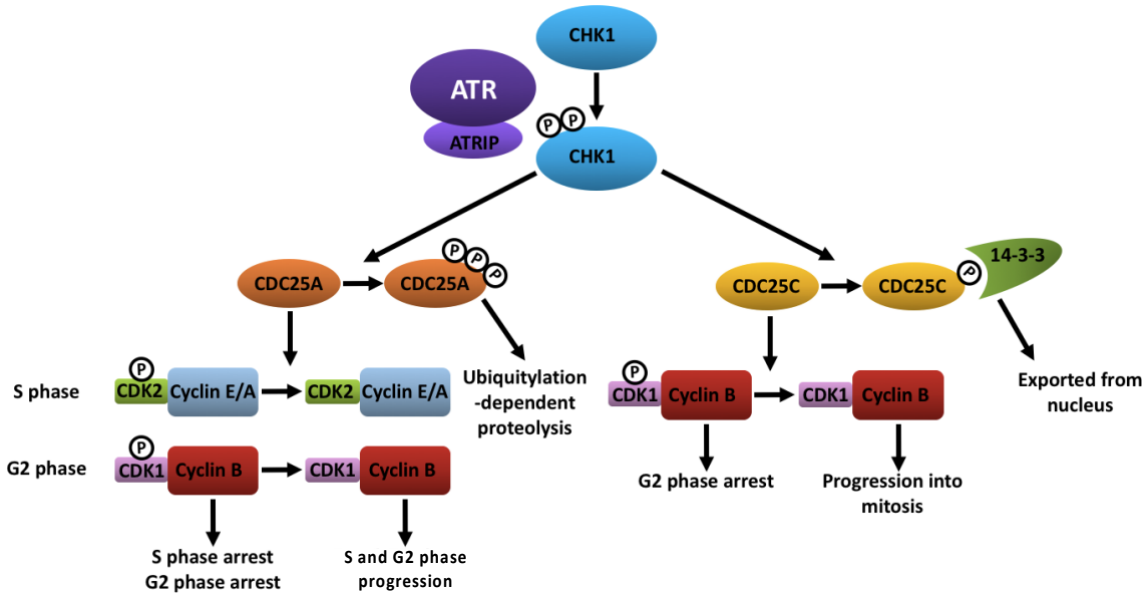


Figure 1.4: ATR-induced cell cycle arrest is mediated via CHK1 activity.
Modified from Wagner & Kaufmann, 2010.

1.6. Targeting the DDR for cancer therapy

ATR is an attractive anticancer target as it plays a key role in regulating the response to DNA replication stress, which is a common feature of cancer cells. (López-contreras and Fernandez-capetillo 2013) In particular, pharmacological inhibition of the DNA damage response could enhance cytotoxicity of standard chemotherapeutics and may provide an exciting opportunity to target cancer cells by exploiting genetic differences that exist between normal and tumour cells. (Hattinger et al. 2019) There is also the possibility that combinational therapies might have the potential to decrease pharmacological doses of standard chemotherapeutics that consequently would be less toxic for normal cells. (Weber and Ryan 2015b)

Some studies support the hypothesis that pharmacological inhibition of ATR might have consequences also in normal cells as ATR activity is required in all proliferating cells during S-phase to guarantee correct DNA replication and maintenance of genomic stability. Indeed,

it has been reported that depletion of ATR can lead to defects in tissue homeostasis, (Ruzankina et al. 2007) and can induce substantial replication stress causing severe side effects due to toxicity in highly proliferative normal tissues. (Murga et al. 2009)

However, research studies have shown that ATR inhibition can be selectively cytotoxic for cancer cells and it might represent a novel anticancer therapeutic approach. For instance, it has been shown that depletion of functional ATR increases genomic instability causing synthetic lethality in cancer cells characterised by oncogene-induced replication stress such as oncogenic RAS or MYC-driven tumorigenesis. (Gilad et al. 2010; Murga et al. 2016; Schoppy et al. 2012) Notably, inhibition of ATR signalling (reduced to 10% of normal levels) was sufficient to induce synthetic lethality in oncogenic RAS-driven tumours, while only marginally affecting normal bone marrow and intestinal homeostasis. (Schoppy et al. 2012) These findings suggested that partial ATR inhibition may be sufficient to sustain viability of highly proliferative human tissues and that complete inhibition of ATR kinase activity may not be required to produce selective toxicity in cancer cells.

It is also possible that genetic modifications in genes encoding enzymes involved in DNA damage repair pathways might also influence response to therapy for osteosarcoma, including ATR inhibition. (Hattinger et al. 2019) For instance, there is evidence indicating that gene polymorphisms, in ERCC (excision repair cross complementation group genes) or in nucleotide excision repair pathway may influence the prognosis for osteosarcoma patients. (Li et al. 2018; Zhao et al. 2013) Cancer cells with less efficient DNA repair pathways such as mutations in ATM or *PRKDC* are more likely to rely on ATR activity for survival due to their high burden of replication stress. (Kantidze et al. 2018) Further investigation of germline and somatic genetic changes is required to identify novel molecular biomarkers that could delineate which patients and tumours would show the greatest susceptibility to DDR-target agents. (Hattinger et al. 2019)

1.7. Development of ATR inhibitors in clinic

Several strategies have been used to inhibit ATR signalling in the preclinical setting. However, some show nonspecific inhibitory activity and will also inhibit ATM or other kinases. For example, caffeine sensitises tumour cells to ionizing radiation and genotoxic agents by inhibiting both ATR and ATM. (Powell et al. 1995; Sarkaria et al. 1999) Recent studies have also identified that Schisandrin B, isolated from the fruit of *Schisandra Chinensis*, appears to have specificity for ATR, although it affects also ATM at higher concentrations. It has also been found that Schisandrin B can increase sensitivity of non-small cell lung cancer (NSCLC) cells to UV radiation by abolishing the S and G2/M checkpoints and inhibiting CHK1 phosphorylation. However, it is only effective at high concentrations (30 μM) where it also inhibits ATM activity. (Nishida et al. 2009) Most recently NU6027, NVP-BEZ235, TORIN 2 and ETP-46464 were reported to be potent ATR inhibitors that sensitized tumour cells to a variety of genotoxic chemotherapeutics. (Q. Liu et al. 2011; Peasland et al. 2011; Toledo et al. 2016; Weber and Ryan 2015a) However, they can also inhibit CDK2, PKI3, mTOR and ATM.

The first potent and selective ATR inhibitor was disclosed by Vertex Pharmaceuticals in an high-throughput screen assay. (Prevo et al. 2012; Reaper et al. 2011) VE-821 is an ATP-competitive inhibitor of ATR kinase and exhibits low cross-reactivity to related PIKK family proteins such as ATM and DNA-PK. (Reaper et al. 2011). VE-821 inhibits ATR, reduces pCHK1 phosphorylation at Ser345 and showed synergetic effects when used in combination with genotoxic agents (gemcitabine, camptothecin and etoposide) as well as with ionising radiation (IR) and cross-linking agents (cisplatin and carboplatin). (Reaper et al. 2011) Additionally, it has also been reported that the combination of VE-821 and cisplatin sensitises ATM deficient cells. Importantly, this cytotoxic synergy with cisplatin has been observed in cancer cell lines but not in non-tumour cell lines which showed a transient and

reversible growth arrest. VE-821 has also been shown to increase sensitivity to radiotherapy in hypoxic cancer cells that have undergone replication arrest in S-phase. This result is of potential clinical significance as hypoxic cells are known to be more resistant to radiotherapy. (Pires et al. 2012; Prevo et al. 2012)

An analogue of VE821, VE-822 also developed by Vertex exhibited a greater potency and selectivity in inhibiting ATR as well as improved bioavailability, solubility and pharmacokinetic properties. (Charrier et al. 2011) It has also been shown to display cytotoxic synergy to radiation and gemcitabine in human pancreatic cancer cells, while maintaining low toxicity levels in non-tumour cells. (Fokas et al. 2012) Notably, VE-822 potentiates the effect of cisplatin in NSCLC cells *in vitro* and in human tumour xenografts. (Hall et al. 2014) Moreover, given its potency and selectivity, VE-822 was the first ATR specific inhibitor to enter in clinical trials to evaluate safety, pharmacokinetic and tolerability in combination with radiotherapy or chemotherapy. (ClinicalTrials.gov: NCT02157792, EUDRACT: 2012-003126-25)

Another ATR inhibitor currently in clinical trials studies is AZD6738, developed by AstraZeneca. AZD6738 is an analogue of AZ20, a potent and selective ATR inhibitor which showed tumour growth inhibition in LoVo human colorectal cancer tumour xenografts *in vivo* at well-tolerated doses. AZ20 was the first reported ATR inhibitor with the required potency, selectivity, and properties to explore ATR pharmacology *in vivo*. (Foote et al. 2013; Jacq et al. 2012) AZD6738 has significantly improved solubility, bioavailability and pharmacokinetic properties compared with AZ20 that make it suitable for oral dosing. (Charrier et al. 2011; Vendetti et al. 2015) AZD6738 is an ATP competitive ATR kinase inhibitor that selectively induces tumour cell death through inducing S-phase accumulation, impairment cell cycle progression and increasing DNA damage/replication stress. In

particular, it blocks the phosphorylation of the ATR downstream target CHK1, and increases ATM-dependent phosphorylation of ATM, CHK2 in H2AX *in vitro*. Furthermore, AZD6738 produces synthetic lethal effects in ATM deficient tumours. (Guichard et al 2013; Jones et al 2013) The synthetic lethality interaction between ATR and ATM signalling pathways might be due to the overlapping and cooperating roles of these pathways in regulating cell cycle progression with DNA repair. In addition, non-small lung cancer cells deficient in both ATM and p53 showed high sensitivity to ATR inhibition *in vitro*, suggesting that functioning-p53-protein might be important in this setting. (Anika M. Weber et al 2013) Indeed, AZD6738 enhanced the cytotoxic effect of cisplatin and gemcitabine in NSCLC cells with functioning ATM kinase activity, and synergised with cisplatin in ATM deficient NSCLC cells. Interestingly, daily administration of AZD6738 for 14 consecutive days is well tolerated in mice and enhances the therapeutic efficacy of cisplatin in tumour xenograft models. (Vendetti et al, 2015). In addition, the radiosensitising properties of AZD6738 in multiple cancer cell lines were independent of both p53 and BRCA2 status. In particular, AZD6738 combined with ionising radiation promotes an aberrant mitosis resulting in acentric chromosome fragment-containing dysfunctional micronuclei, indicating mitotic catastrophe. (Dillon et al. 2017)

AZD6738 clinical trials are still in early stage. However, after the recruitment of two patients, it was possible to investigate multiple ascending dose study to assess pharmacokinetic in CLL, PLL, and B cell lymphoma patients. (ClinicalTrials.gov: NCT01955668) A phase I clinical trial is investigating the safety of AZD6738 alone and in combination with radiotherapy in patients with solid tumours (NCT02223923; EUDRACT: 2013-003994-84 and 2014-002233-66) and another trial is evaluating the combination of AZD6738 with carboplatin, olaparib, or durvalumab, a monoclonal antibody against PD-L1. (NCT02264678)

A range of DDR modulators targeting ATR (AZD6738), CHK1 (CCT245737) and CHK1/2 (AZD7762) were of specific interest in this project and were also extended to include other classes of DDR modulators (such as ATMi, DNA-PKi, WEE1i). The DDRi used in this research project are shown in Table 1.1.

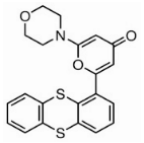
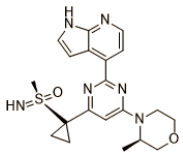
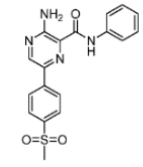
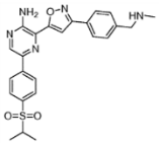
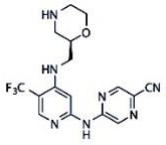
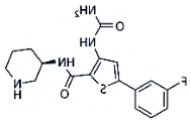
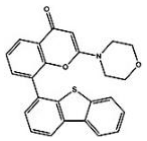
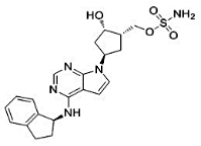
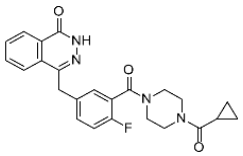
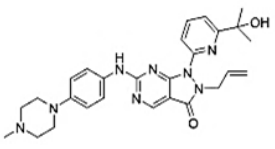
Target	Compound	Chemical Structure
ATM	KU-55933	
ATR	AZD6837	
	VE821	
	VE822	
CHK1	CCT-245737	
CHK1/2	AZD7762	
DNA-PK	NU7441	
NEDDYLATION	MLN-4924	
PARP	AZD2281	
WEE1	MK-1775	

Table 1.1: DDR inhibitors, their targets and their chemical structure

1.8. Aim of this study

We hypothesised that cyclin E1 overexpression could be an essential molecular feature determining sensitivity to DNA damage response modulators in osteosarcoma and other cancers.

AIM1: to establish the impact of cyclin E1 overexpression on sensitivity to DDR inhibitors in osteosarcoma cells.

AIM2: to examine the relationship between cyclin E1 expression and sensitivity to ATR inhibition in lung cancer cell lines.

CHAPTER 2

2. Material and Methods

2.1. Mammalian Cell Culture

2.2. Maintenance of cell lines

The cell lines used in this research study are listed in Table 2.1. U2OS human osteosarcoma cell line (U2OS-Cyclin E cell line; from T. Halazonetis, University of Geneva, Switzerland) were maintained in Advanced Dulbecco's Modified Eagle Medium (DMEM)/F12 (Life Technologies, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum (FBS; Sigma-Aldrich; St. Louis, MO, USA), 2mM GlutaMAX™ (Life Technologies), and 50 U penicillin / 50 µg/mL streptomycin (Life Technologies). The medium was supplemented with G418 400 µg/ml (Sigma, Cat. No. A1720), puromycin 1 µg /ml (Sigma, Cat. No. P8833) and tetracycline 2 µg /ml (Sigma, Cat. No. T7660) for the selection and maintenance of vector-containing cells. U2OS Cyclin E cell line was tested for the absence of mycoplasma contamination. PC9 and A549 cell lines were maintained in Advanced Dulbecco's Modified Eagle Medium (DMEM)/F12 (Life Technologies, Carlsbad, CA, USA) supplemented with 5% foetal bovine serum (FBS; Sigma-Aldrich; St. Louis, MO, USA), 2mM GlutaMAX™ (Life Technologies), and 50 U penicillin / 50 µg/mL streptomycin (Life Technologies). The cells were cultured in 25, 75 and 175 cm^2 tissue culture flasks (Corning Incorporated; Corning, NY, USA) and incubated in a standard humidified incubator at 37˚C and 5% CO2. The cells were passaged at 70-80% confluency. Passaging the cells involved aspiration of medium, washing with sterile PBS prewarmed to 37°C in water bath, trypsinisation with Trypsin/EDTA (Life Technologies) following incubation at 37°C for 5 min. Successively, prewarmed culture medium was added to

inactivate trypsin and the final solution mixed to form a single cell suspension. The cells were then split in 1:10 dilution.

Table 2.1: Cell lines used in this study

Cell Line	Disease	Obtained from
U2OS	Osteosarcoma	Kindly provided by Prof. Madalena Tarsounas, University of Oxford (UK)
U2OS Cyclin E	Osteosarcoma	Kindly provided by Prof. Thanos Halazonetis, Geneva University (Switzerland)
PC9	Non-small cell lung carcinoma	Kindly provided by Dr Kazuto Nishio, Kinki University, Osaka, Japan
A549	Non-small cell lung adenocarcinoma	ATCC

2.3. Cell counting and seeding

Cell density and viability were measured using the Countess® automated cell counter (Life Technologies). A 1:1 mixture of a cell suspension and 0.4% trypan blue solution (Life Technologies) was added to a Countess® cell counting chamber slide and inserted into the Countess® automated cell counter for standard counting. The desired number of cells were then seeded in culture media prior to any experiments. The U2OS Cyclin E cell line has a stably integrated TET-Off inducible system, which requires removal of tetracycline from the culture medium to allow gene transcription of the oncogene of interest (CCNE1). Three days before each experiment, U2OS Cyclin E cells were collected from the T175-culture flask and centrifuged at 561 g for 5 min, washed with sterile PBS followed by centrifugation at 561 g for 5 min. The final pellet was then seeded in culture media prepared by using with Advanced Dulbecco's Modified Eagle Medium (DMEM)/F12 (Life Technologies, Carlsbad, CA, USA) supplemented with 5% TET System approved foetal bovine serum

(FBS; Takara, Cat No 631106), 2mM GlutaMAX™ (Life Technologies), and 50 U penicillin / 50 µg/mL streptomycin (Life Technologies), supplemented with G418 400 µg/ml (Sigma, Cat. No. A1720), puromycin 1 µg/ml (Sigma, Cat. No. P8833). This media was used to induce CCNE1 expression condition for experiments.

2.4. Cryostorage and thawing of cells

For long term storage, cells were grown to 70-80% confluency, treated with Trypsin/EDTA (Life Technologies) as described above. The cells collected in culture medium were centrifuged (250 g for 5 min) and the pellet was re-suspended with culture medium containing 10% (v/v) DMSO. 1 ml aliquots were stored in cryogenic tubes within in an isopropanol Mr. Frosty™ Freezing Container (Thermo Scientific) at - 80°C and thereafter transferred to liquid nitrogen storage vessel for long term storage.

To recover cells from the liquid nitrogen storage, the cells were rapidly thawed in a 37°C water bath with gentle agitation, the contents of the vial was diluted in 10 ml prewarmed culture medium. After centrifugation (250 g for 5 min), the pellet was resuspended in 7 ml prewarmed culture medium and the cell suspension was transferred to a 75 cm^2 flask. After 24 h, depending on the confluency of cells, the medium was changed or cells were transferred into a T175-culture flask and incubated at 37°C to reach 70% cell confluency.

2.5. Drug Treatments

Stock solutions of KU55933 (TOCRIS Bioscience; Bristol, UK), and MLN-4924, (AdooQ® Bioscience; Irvine, CA, USA), AZD2281, AZD6738, AZD7762, (AstraZeneca; Macclesfield, UK), MK1775, NU7441, VE-821, VE-822 (Selleck Chemicals; Houston, TX, USA), were prepared in 100% DMSO at a concentration of 10 mM. All stock solutions were

aliquoted and stored at -20°C until use, and dilutions of the drugs for cells treatment were prepared using complete culture medium.

2.6. Cell Proliferation Assay with Resazurin

Resazurin-based cell viability assays were used to measure cell growth and to assess the effect of DNA damage response inhibitors and chemotherapeutic drugs on cell proliferation and viability. Cells were seeded in 180 µl culture medium in triplicate in 96-well plates (Costar, flat bottom; Corning Incorporated) at a density of 1000 cells/well and incubated overnight to allow re-attachment. After 24 hours, drugs and 0.01% DMSO (vehicle control) were added in 180 µl complete culture medium at the required concentration and cells incubated for a further 5 days. On the fifth day of treatment, 18 µl resazurin solution (0.125 mg/mL in dH2O; Sigma-Aldrich) per 180 µl media was added to each well, including media-only (blank) wells and the plates were incubated at 37°C for 2 h.

Only metabolically active cells are able to reduce resazurin to highly fluorescent resorufin. The fluorescence signal is directly proportional to the number of viable cells. Fluorescence intensity was detected by using POLARstar Omega plate reader (BMG Labtech GmbH; Ortenberg, Germany) with excitation at 544 nm, emission at 590 nm, and at a gain of 1000. The average of the values of the triplicate blank wells was subtracted from all other wells and the resulting data normalized to the average of the vehicle-treated wells.

2.7. PicoGreen Cell Proliferation Assay

The PicoGreen assay is a sensitive and specific assay for quantifying changes in double-stranded DNA (dsDNA) content. The PicoGreen cell proliferation assay was used to measure cell growth (determined by increased dsDNA content) over a period of 8 days. Cells were seeded in 180 µL complete culture medium in triplicate in 96-well plates (Costar, flat bottom; Corning Incorporated) at a density of 1000 cells/well and incubated overnight to

allow re-attachment. After 24 h, the medium in the first column (day 1) was aspirated and 100 µL of DNA lysis buffer (Table 2.2) was added per well. Every 24 h for eight days, the medium was aspirated in the appropriate column and 100 µL of DNA lysis buffer was added to each well. After DNA lysis buffer was added to the final wells, Quant-iT PicoGreen dsDNA reagent (Life Technologies) was diluted 1:200 in sterile PBS (Lonza; Basel, Switzerland). 100 µL of the resulting PicoGreen® solution was then added to each well, and the plates were incubated for 20 min. Fluorescence intensity was measured with a POLARstar Omega plate reader (BMG Labtech GmbH, Ex 480 nm, Em 520 nm, gain 10000). The average of the values of the triplicate blank wells (no cells) was subtracted from all other wells.

Table 2.2: DNA lysis buffer composition.

Component	Amount	Final Concentration
0.5 M EDTA. pH 8.0 [a]	2.5 mL	25 mM
Triton X-100 [b]	50 µL	0.1%
dH2O 47.45 mL	47.45 mL	

[a] AccuGENE, Lonza
[b] VWR International; Randor, PA, USA

2.8. Protein Detection by Western Blotting

2.8.1. Preparation of cell extracts, gel electrophoresis and transfer

Cells (5 x 10^5) were seeded in 100 cm^2 tissue culture dish (Corning Incorporated; Corning, NY, USA) incubated to reach 70% confluency. Media together with any floating cells was collected in a falcon tube. Attached cells were washed with sterile pre-warmed 1xPBS (Sigma-Aldrich) and treated with Trypsin/EDTA (Life Technologies) as described previously in Table 2.2. Culture medium was added to inactivate the trypsin activity and

detached cells were collected and added into the Falcon tube together with the media/floating cells. After centrifugation (5 mins at 561 g), the resulting pellet was resuspended in 1ml 1xPBS (Sigma-Aldrich) and transferred into microcentrifuge tubes (Sigma-Aldrich) and centrifuged again for 5 mins at 693 g. To lyse the cells, the cell pellet was treated with 40-100 µl lysis buffer (Table 2.3) supplemented with 1 mM sodium orthovanadate (Na_3VO_4; Sigma-Aldrich) and cOmplete™ protease inhibitor cocktail (Roche; Basel, Switzerland) and left on ice for 10 mins. Merck Novagen Benzonase Nuclease (Fisher Scientific 10349963) was added to the cell lysates (0.5 -1 µl nuclease for every 50-100 µl of lysate), then samples were vortexed and left them on ice for 5 min before 15 min centrifugation at 4°C. The tubes were taken gently from the centrifuge and placed on ice, the supernatant of each samples was transferred in fresh microcentrifuge tubes, and the pellets were discarded.

Protein concentrations were determined by using the Bradford protein assay, a colorimetric protein assay based on absorbance of the dye Coomassie Brilliant Blue G-250. The dye interacts with carboxyl groups in proteins by forming a non-covalent complex by Van der Waals force and electrostatic interactions. The reaction is dependent on the amino acid composition of the measured proteins. Bovine serum albumin (BSA) was used as a protein standard. The Bradford assay was performed in accordance with the manufacturer's instructions (BioRad, UK). A standard curve was constructed using BSA (Sigma) with concentrations ranging from 0 to 1 mg/ml. Absorbance readings were taken at 595 nm (POLARstar Omega plate reader BMG Labtech GmbH), and all standards and samples were measured in duplicate. Microsoft Excel 2013 and GraphPad Prism® 7 (GraphPad Software, Inc.; La Jolla, CA, USA) was used for computing the unknown samples concentrations and designed the standard curve.

Protein samples were diluted in distilled water in order to have equal protein concentrations in each sample. 4x Laemmli sample loading buffer (Sigma-Aldrich) was added and samples

were incubated at 95˚C for 5 mins in a dry heating block (AccuBlock™ Digital Dry Bath; Labnet International, Inc.; Edison, NJ, USA). 10-50 µg protein per lane was loaded onto a precast 4-20% gradient Mini-PROTEAN® TGX™ gel (BioRad; Hercules, CA, USA) alongside a Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (#1610375). The gel was run at 150 V for 60 min with Tris/Glycine/SDS running buffer (BioRad). Proteins were then transferred to nitrocellulose membranes (0.45 µm pore size Trans-Blot®; BioRad) in cold 1x Tris/Glycine buffer (BioRad) with 20% methanol (v/v; Sigma-Aldrich) at 100 V for 60 min with an ice pack cooler and magnetic stirrer.

Table 2.3: Lysis buffer stock components for western blot cell extract collection. Lysis buffer stock solution was aliquoted and stored at 4 ˚C.

Component	Amount	Final Concentration
1 M Tris-HCl, pH 7.614 [a]	1.25 mL	50 mM
NaCl [b]	200 mg	137 mM
(v/v) glycerol [b]	2.5 mL	10%
(v/v) Igepal [c]	25 µL	0.1%
10% (v/v) SDS [d]	250 µL	0.1%
2% sodium fluoride (NaF) [c]	2.626 mL	50 mM
dH2O	16.1 mL	

[a] Promega; Madison, WI, USA
[b] VWR
[c] Sigma-Aldrich
[d] AppliChem GmbH; Darmstadt, Germany

2.8.2. Immunoblotting and Protein Detection using LI-COR

Nitrocellulose membranes were stained by using Ponceau S solution (Sigma-Aldrich), carefully cut where appropriate, washed twice with PBS-T or TBS-T (Table 2.4) for 5 min then incubated in blocking buffer (1:1 solution of PBS or TBS and Odyssey® Blocking Buffer LI-COR) for 1 h at room temperature on a roller (Stuart® roller mixer SRT9D; Bibby Scientific; Staffordshire, UK). Diluted primary antibodies (Table 2.5) were added and the membranes incubated overnight at 4°C. After 24 h, membranes were washed (3 x 5 min) in PBS-T or TBS-T and incubated with the appropriate fluorophore conjugated secondary antibody (Table 2.6) diluted in 1:1 solution of PBS-T or TBS-T and Odyssey® Blocking Buffer for 60 min at room temperature. Membranes were then washed (3 x 5 min) in PBS-T or TBS-T, then once with PBS for 3 min. Proteins detection was by infrared scanning densitometry (Odyssey Infrared Detection System, LI-COR Biosciences) at 680 nm and 800 nm. Membranes were stored in 1x PBS at 4 ˚C or dried and stored at RT. Cyclophilin B (PPIB) was used as a loading control and (in cases where quantification was needed) for comparison of protein levels, where band intensities were normalised to loading control (PPIB) levels.

Table 2.4: Buffers and solutions used in immunoblotting.

Buffer	Composition
1x phosphate buffered saline (PBS)	5x PBS tablets [a] dissolved in 1 L dH2O
PBS-T	1x PBS, 0.1% (v/v) Tween-20 [a]
1x Tris-buffered saline (TBS)	1x pack OmniPur® TBS 20x Ready Pack Powder [b] dissolved in 20 L dH2O
TBS-T	1x TBS, 0.1% (v/v) Tween-20

[a] Sigma-Aldrich
[b] Calbiochem; Darmstadt, Germany

Table 2.5: Primary antibodies used for immunoblotting analysis

Specification (Name)	**Species/ clonality**	**Dilution**	**Manufacturer, catalogue number**
Total CDC6	Rabbit mAb	1:1,000 (PBS)	Cell Signalling, #3387
phospho-CDC6 Ser54	Rabbit mAb	1:2,000 (PBS)	Abcam, #ab75809
Cyclin A2	Mouse mAb	1:1,000 (PBS)	Abcam. #ab16726
Cyclin E1	Mouse mAb	1:1000 (PBS)	Cell Signalling, #4129S
Cyclin F	Rabbit pAb	1:5000 (PBS)	Bethyl Laboratories, #A303-406A
phospho-histone-H3 Ser10	Rabbit pAb	1:2,000 (PBS)	Merck Millipore, #06-570
CHK1	Mouse mAb	1:1,000 (PBS)	Santa Cruz, #SC-8408
phospho-CHK1 Ser317	Rabbit pAb	1:1000 (TBS)	Cell signalling, #2344
Phospho-CHK1 Ser345	Rabbit pAb	1: 500 (PBS)	Cell Signalling, #2341
PPIB (Cyclophilin B)	Rabbit pAb	1:2,000 (PBS)	Abcam, #ab16045
Retinoblastoma (pRB)	Mouse mAb	0.5 mg/ml	BD Pharmingen™, #554136
KAP1	Rabbit pAb	1:10,000 (PBS)	Bethyl Laboratories, #A300-274A
Phospho-KAP1 Ser824	Rabbit pAb	1:2,000 (PBS)	Bethyl Laboratories, #A300-767A
total RPA32	Mouse mAb	1:500 (PBS)	Abcam, #ab2175
phospho-RPA32 Ser4/Ser8	Rabbit pAb	1:2,000 (PBS)	Bethyl Laboratories, #A300-245A
phospho-RPA32 Ser33	Rabbit pAb	1:2,000 (PBS)	Bethyl Laboratories, #A300-246A

Table 2.6: Secondary antibodies used for two-colour detection with infrared fluorescence.

Specification (Name)	Species/ clonality	Dilution	Manufacturer, catalogue number
IRDye® 800CW	goat polyclonal	1:5,000	LI-COR, #926-32211
IRDye® 680RD	goat polyclonal	1:5,000	LI-COR, #926-68070

2.9. Cell Synchronisation

Cell synchronisation experiments were carried out by using the selective CDK1 inhibitor, RO3306 that reversibly arrests proliferating human cells at the G2/M phase border and provides a means to enrich cells in G2/M phase. Cells ($5 \times 10_5$) were seeded in 10-cm-culture dishes and incubated overnight to allow attachment. After 24 h, the cells were treated with 10 µM CDK1 inhibitor RO-3306 (Selleck chemical) and incubated for 16-24 h to allow cell cycle enrichment in G2 phase. After washing with pre-warmed PBS (37°C) to remove the CDK1 inhibitor, cells were then incubated in pre-warmed medium and collected at different time points. Collection of the cells was carried out after trypsin addition (as described in Chapter 2.2). Each cell suspension was transferred into a sterile centrifuge tube and spin down (250 x g, 4 min at RT) and the pellet was then resuspended in 1 ml PBS centrifuged again (250 x g, 4 min at RT). The supernatant was removed, and cell pellets were stored at -20°C until protein extraction and western blot analysis.

2.10. Flow Cytometry

To analyse DNA content by flow cytometry, cells were first seeded in 10 cm-culture dishes and grown until approximately 65% confluency. Cells were treated with 10 µM RO3306 (Selleck chemical) and incubated for 16-24 h to allow cell cycle synchronisation in G2 phase. Cells seeded in 10 cm-culture dishes at same cell density without RO-3306 were used as

controls for cell cycle synchronisation. After 24 h, media containing any floating/detached cells was retained and attached cells collected by using trypsin (Chapter 2.2). Both floating and attached cells were combined and pelleted by centrifugation (225 x g, 5 min). The supernatant was discarded and the cell pellets were fixed by dropwise addition of 1.5 mL ice-cold 70% ethanol (Sigma-Aldrich) while vortexing. Samples were then left for 30 min on ice before being stored at -20 °C for later analysis. For analysis, samples were washed with 2 mL 1xPBS and resuspended in PBS to rehydrate cells for 2 mins. Cells were then centrifuged (225 g for 5 min) and resuspended in 1xPBS. Propidium iodide (PI; Calbiochem) was added to a final concentration of 20 μg/mL. (Fig.2.1) Propidium iodide stains DNA stoichiometrically so that the intensity of the fluorescence signal is directly proportional to the amount of dsDNA contained in the cells. Propidium iodide fluorescence (dsDNA content of each cell) was measured using a BD FACSCalibur Flow Cytometer (BD Biosciences; San Jose, CA, USA) and BD CellQuest Pro Software. FlowJoTM FACS analysis software (BD, Becton, Dickinson and Company, USA) was utilised to determine the proportion of cells in G1, S or G2/M cell cycle phases. (Fig.2.1)

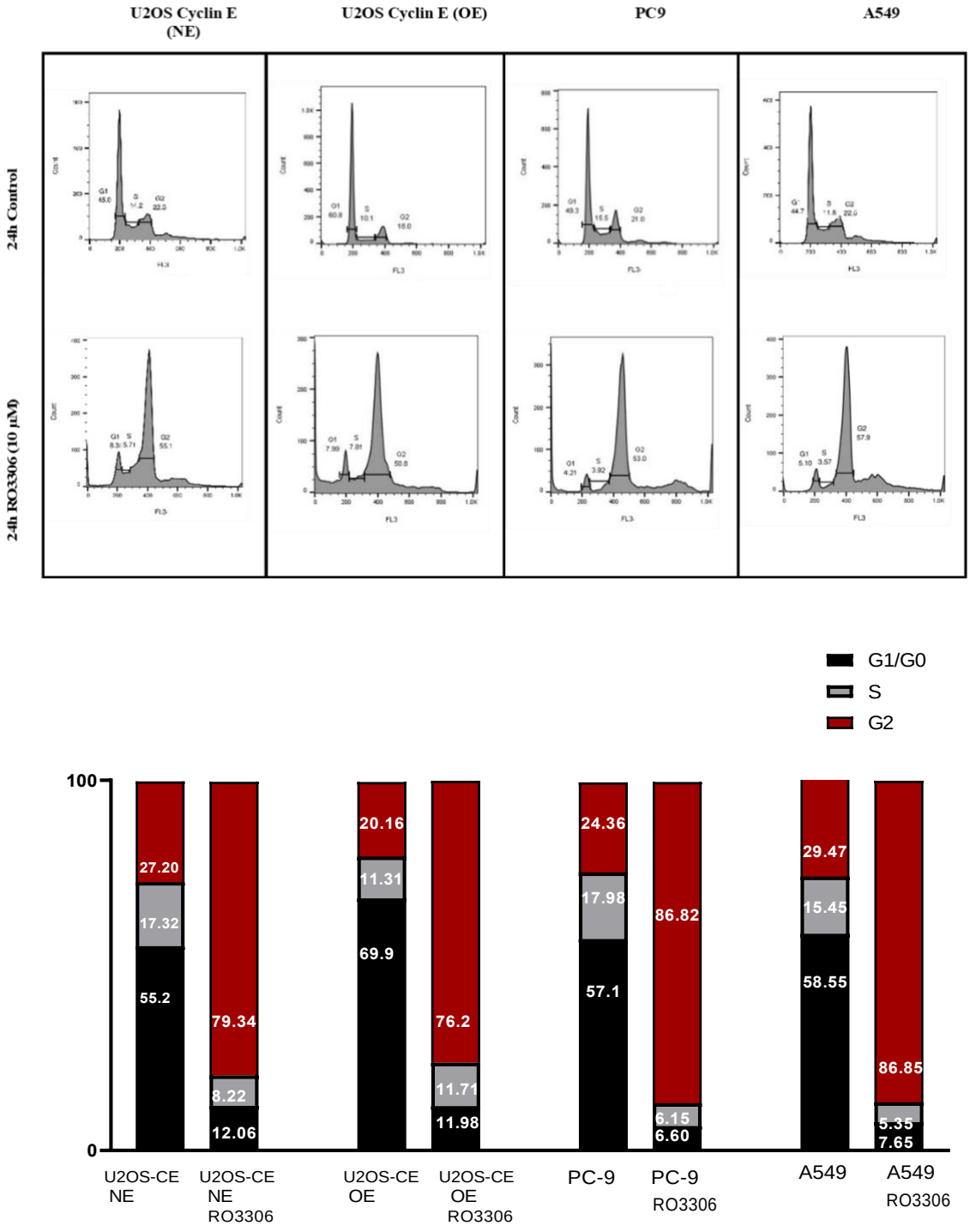


Figure 2.1. Cell Synchronisation Analysis with FACS – Flow Cytometry. RO3306 treatment leads to G2-phase accumulation in U2OS Cyclin E, PC-9 and A549 cell lines. U2OS Cyclin E with normal levels (NE,+TET) and with cyclin E1 overexpression (OE,-TET), PC-9 and A549 cells were treated with RO3306 for 24 h before harvesting and cell cycle distribution analysis were carried out by propidium iodide staining and flow cytometry analysis.

2.11. Data Analysis and Statistics

Microsoft Excel 2013 and GraphPad Prism® 7 (GraphPad Software, Inc.; La Jolla, CA, USA) was used for all statistical analyses, and all values are expressed as the mean ± standard error of the mean (SEM) of at least three independent experiments unless otherwise stated. GraphPad Prism® 7 (GraphPad Software, Inc.; La Jolla, CA, USA) was used for calculating IC_{50} values as the mean ± standard deviation (SD) of at least three independent experiments.

CHAPTER 3

3. The U2OS Cyclin E cell line (U2OS-CE)

3.1. The TET-off system in U2OS Cyclin E cell line

To study the effects of constitutive cyclin E1 expression on DNA replication and DNA damage response activation in mammalian cells, we used a U2OS human osteosarcoma cell line with a stably integrated TET-off inducible gene expression system. (Fig.3.1) The U2OS Cyclin E cell line was generated by the group of Jiri Lukas. (Santoni-Rugiu et al. 2000) Briefly, U2OS cells stably expressing tTA (TetR-VP16) were transfected with pBI-HA-pRBΔcdk/CycE vector. The pBI vector allows expression of two genes from a bidirectional tetracycline (TET)-repressible promoter. Stable transfection with the pBI-HA-pRBΔcdk/CycE vector allows expression of both cyclin E1 (*CCNE1*) and mutated retinoblastoma (pRB) proteins in tetracycline repressible fashion. (Fig.3.1) In the mutant pRB (pRBΔcdk) protein, eleven CDK phosphorylation sites that regulate pRB binding to E2F have been mutated to alanine so that E2F activity is inhibited when pRBΔcdk expression is induced. (Fig.3.1) (Lukas, Herzinger, et al. 1997; Resnitzky et al. 1994; Santoni-Rugiu et al. 2000)

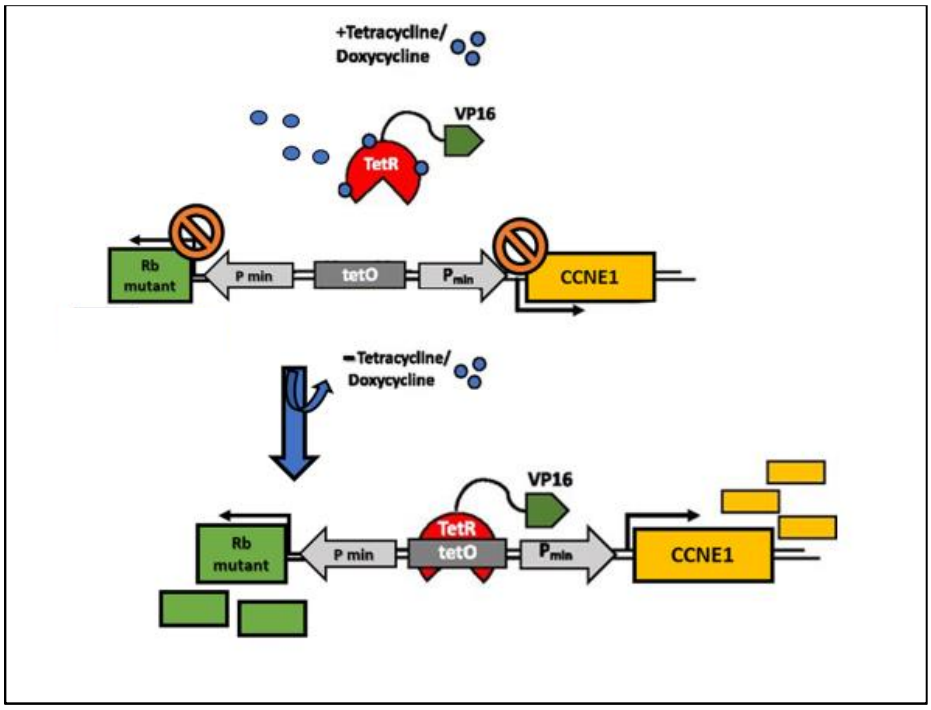


Figure 3.1. Schematic representation of the TET-OFF inducible system in U2OS-Cyclin E osteosarcoma cell line. In absence of tetracycline or doxycycline, U2OS human osteosarcoma cells with stably integrated TET-regulated transcriptional activator (tetR fused to the activating domain of VP16 of the herpes simplex virus) binds to a series of repeats of the tet-operator sequences (tetO) placed just up/down-stream of the minimal promoter (e.g. human cytomegalovirus minimal promoter, P min). Once Tet-VP16 is localised, it induces the transcription of the genes of interest in absence of the antibiotics in a precise dose-dependent manner. In the presence of tetracycline, tetracycline binds tetR and this precludes binding to the tetO DNA sequences. (Adapted from Gossen et al., 1995)

To confirm tetracycline dependent expression of retinoblastoma and cyclin E1 proteins, U2OS-CE cells were seeded in three different conditions: culture media with (1) tetracycline (2 μM), (2) doxycycline (0.165 μM) or (3) without antibiotics. Cells were collected following 3 days incubation at 37°C and the expression of both proteins was examined by using western blot analysis. U2OS-CE cells seeded in culture media without tetracycline (TET) or doxycycline (DOX) were expected to express increased levels of the pRB (pRBΔcdk) and cyclin E1. As it can be seen in figure 3.2. western blot analysis confirmed the synthetic TET/DOX-based gene expression system. U2OS-CE cells expressed increased levels of cyclin E1 protein when antibiotics were absent in the cell system, suggesting

ectopic expression. In repressive conditions (+TET or +DOX) expression of cyclin E1 could be detected as a double band at 45-50 kDa. On induction (no TET or DOX), cyclin E1 protein levels were increased and restricted mainly to five bands ranging from 45 to 33 kDa potentially corresponding to five tumour-specific low molecular weight isoforms (LMW-E) of cyclin E1. (Fig.3.2) The development of LMW-E isoforms are related to an aberrant degradation of cyclin E1 which has been related to protease activity of elastases or calpains. LMW-E isoforms have been described as a hyperactive biochemically provoking an increase of aberrant cyclin E/CDK2 complex activation. (Caruso et al. 2018; Porter et al. 2001) Under our growth condition, expression levels of pRB protein were not increased in the absence of TET or DOX. There was no evidence of phosphorylated (higher molecular weight) forms of pRB in any of the growth conditions. Previous data have reported that U2OS cells do not accumulate active pRB or respond with pRB activation after DNA damage suggesting U2OS cells are functionally pRB negative by an unknown mechanism (Broceño, Wilkie, and Mittnacht 2002) and therefore the impact of expressing pRBΔcdk would be expected to be minimal.

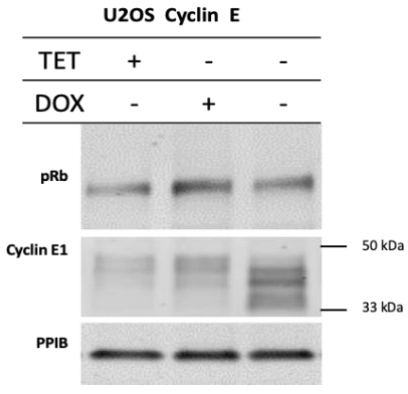


Figure 3.2. Characterisation of the system TET-off in U2OS Cyclin E cell line. U2OS-CE cells ($5x10_5$) with a stably integrated TET-Off inducible system were seeded in 10-cm-culture dishes with and without tetracycline (2 μM) or doxycycline (0.165 μM). Cell lysates were collected after 3 days induction and analysed by Western Blot analysis. Cyclophilin B (PPIB) was used as a loading control. The gel represents one independent experiment.

To confirm that levels of cyclin E1 protein were specifically due to induction under the TET-off system and to rule out other biological artefacts, cyclin E1 protein expression in U2OS-CE cells was compared with the U2OS parental cell line. Both U2OS-CE and U2OS parental cell lines were seeded in presence and absence of tetracycline and doxycycline. Cells were then collected following 3 days incubation and cell lysates were analysed by Western blot analysis. (Fig.3.3.) Endogenous expression of cyclin E1 in the U2OS parental cell line is comparable to U2OS-CE cells in the presence of TET or DOX. As an important control, the addition of TET or DOX does not affect cyclin E1 protein expression in the U2OS parental cell line. (Fig.3.3.) The increased levels of cyclin E1 protein in U2OS-CE cells in the absence of TET or DOX indicates this increase is the result of ectopic expression from the integrated pBI-HA-pRBΔcdk/CycE vector and furthermore that this expression is tightly regulated in the TET-off system. (Fig.3.3.)

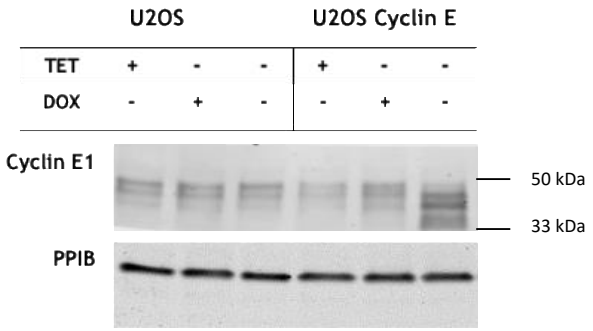


Figure 3.3. Evaluation of cyclin E1 overexpression in Parental U2OS and U2OS Cyclin E cell lines. U2OS-CE and parental U2OS cells were seeded in 10-cm-culture dishes at $5x10_5$ cells/dish in culture media with/without TET (2 μM). or DOX (0.165 μM). Cell lysates were collected after 3 days and expression of cyclin E1 was assessed by Western Blot analysis. Only U2OS-CE cells showed increased expression of cyclin E1 in the absence of antibiotics. Cyclophilin B (PPIB) was used as a loading control. The gel represents one independent experiment.

To further characterise the U2OS-CE cell line, we performed a time course experiment to evaluate changes in cyclin E1 expression after the removal of tetracycline. U2OS-CE cells

were cultured in media without TET or DOX and collected every 24 h for 4 days. Lysates were analysed by western blot analysis. (Fig.3.4) which revealed increased expression of cyclin E1 at 24 h which was sustained over at least 4 days. Interestingly, high levels of cyclin E1 were detected as a multiple group of bands ranging between 45-33 kDa revealing the presence of LMW isoforms of cyclin E1. For future experiments, we selected 3 days of induction as the time point for investigation, unless otherwise stated.

In conclusion, we confirmed that U2OS-CE cell line has a tightly regulated TET-off control of cyclin E1 expression. Upon removal of TET, ectopic cyclin E1 is expressed at high levels within 24 h, and this expression is sustained for at least 4 days. We were not able to demonstrate ectopic expression of pRB. The control of pRB activity in U2OS cells has been reported to be markedly deficient (Broceño, Wilkie, and Mittnacht 2002) with U2OS considered functionally null for pRB function despite expressing physiological levels of the protein.

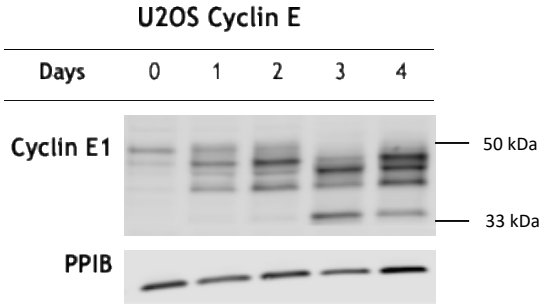


Figure 3.4. Time-course experiment to assess the constitutive expression of cyclin E1 in U2OS Cyclin E cell line. U2OS- CE cells were seeded in 10-cm-culture dishes at 5 x10_5 cells/dish in culture media without tetracycline (2 μM). Cell lysates were collected every day for 4 days and ectopic expression of cyclin E1 was assessed by Western Blot analysis. Cyclophilin B (PPIB) was used as a loading control. The gel represents one independent experiment.

The TET-off system worked with either or TET or DOX to suppress gene expression. For subsequent experiments, we chose to use TET since Doxycycline was reported to downregulate DNA-PK protein expression, a key DDR pathway. (Lamb et al. 2015)

3.2. Cell proliferation assays

We investigated whether increased cyclin E1 expression altered cell growth rate. We first measured cell proliferation using resazurin to evaluate metabolically active cells after 5 days. (Fig. 3.5a) Surprisingly, the data showed that U2OS-CE cells with normal expression levels of cyclin E1 (NE, +TET) or with overexpression of cyclin E1 (OE, -TET) had similar growth rates. (Fig.3.5a) We confirmed these findings using PicoGreen to determine dsDNA levels as a measure of proliferation. (Fig.3.5b) Indeed, these results indicate that ectopic expression of cyclin E1 does not increase tumour cell growth rate in U2OS cells suggesting that cyclin E1 protein levels are not rate limiting for cell growth or cell cycle progression.

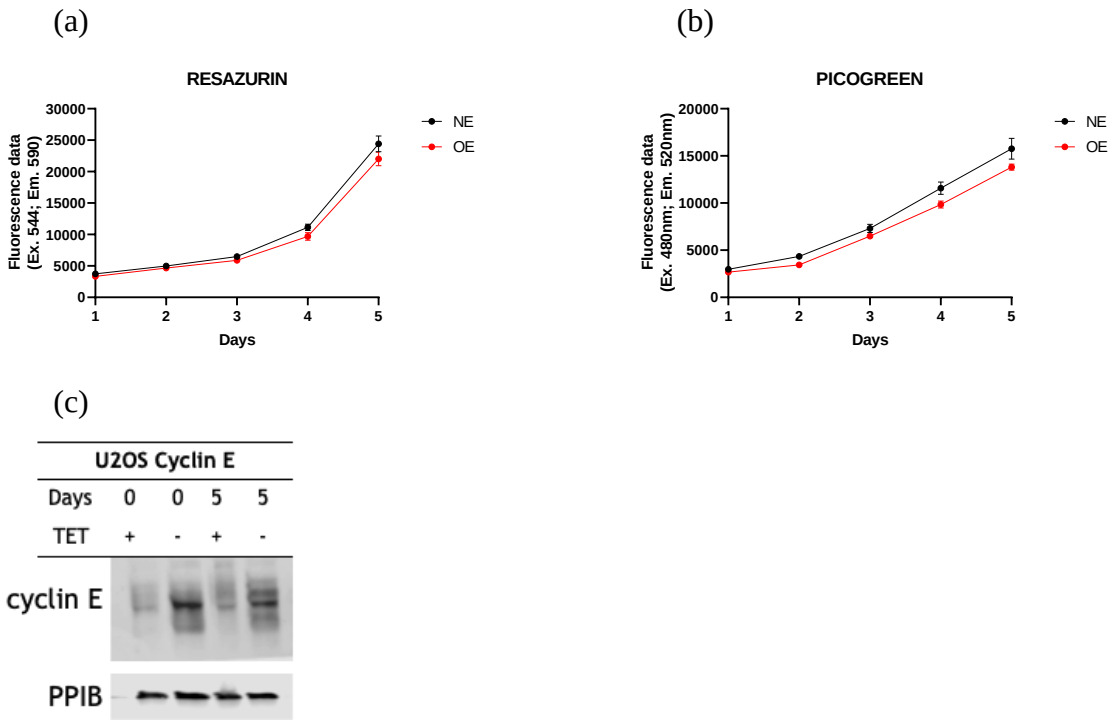


Figure 3.5. Cyclin E1 overexpression does not affect proliferation of U2OS Cyclin E cells *in vitro*. Cell proliferation in U2OS Cyclin E cell cultures was investigated with (a) resazurin (cell

metabolic activity marker) and (b) PicoGreen (dsDNA content marker), up to 5 day following induction of cyclin E1 overexpression (OE, -TET) or no induction (normal level, NE, +TET). Graphs represent the mean of three independent experiments each performed with triplicates (± SEM). (c) Western blot analysis confirm the cyclin E1 overexpression on day 0 (following 3 days induction) and on day 5 of each proliferation assay. Cyclophilin B (PPIB) was used as a loading control. Representative data of n = 3 experiments.

3.3. Cell Cycle Analysis: the impact of Cyclin E1 overexpression

To further evaluate the role of cyclin E1 overexpression in cell cycle kinetics, we carried out western blot analyses of synchronised cells. Although ectopic expression of cyclin E1 did not increase growth rate, we hypothesised that it might alter cell cycle distribution, for example by accelerating cells through the G1/S phase transition, but then leading to a prolonged S phase. We treated cells with the CDK1 inhibitor RO3306 at 10 μM for 24 h in order to reversibly arrest cell cycle at G2 phase to provide a highly enriched/synchronised G2 phase cell population. Cell synchronisation at G2 phase was verified by flow cytometry analysis. (Fig. 2.1, Chapter 2.10) Subsequently, we washed off RO3306 to release cells from the G2 phase block and collected at different time points thereafter to investigate cell cycle progression.

Following release of U2OS-CE cells with normal levels of cyclin E1 (NE, +TET), cyclin E1 expression coincided with cyclin A2 expression possibly in early S phase (6 h after release from RO3306). Expression of CDC6, one of the components of the pre-replication complex (pre-RC) appeared to be expressed throughout the cell cycle in U2OS-CE cells but pCDC6-S54, corresponding to DNA replication origin activation (a marker of late G1/early S phase), suggested S phase entry of the cells at 6 h time-point. (Fig.3.6a)

In contrast, synchronised U2OS-CE cells with ectopic expression of cyclin E1 (OE, -TET) showed a consistent high level expression of the protein, but also showed evidence of early DNA replication origin firing suggested by early expression of pCDC6-S54 expression 0-2 h after release from RO3306 treatment. In contrast, cyclin A2 expression was only increased

at the 24 h time point. (Fig.3.6b) On the other hand, U2OS-CE cells with normal levels (NE,+TET) or overexpression (OE,-TET) of cyclin E1 showed the highest levels of pHH3 S10 (a marker of mitosis) at 2 h after release, corresponding to the mitotic phase of the cell cycle.

The western blot analyses suggest that cells with high levels of ectopic cyclin E1 expression exhibit early S phase entry. We hypothesise that the early timing of S-phase may be due to a cyclin E1 dependent early priming and firing of DNA replication origins that could result in increased levels of DNA replication stress.

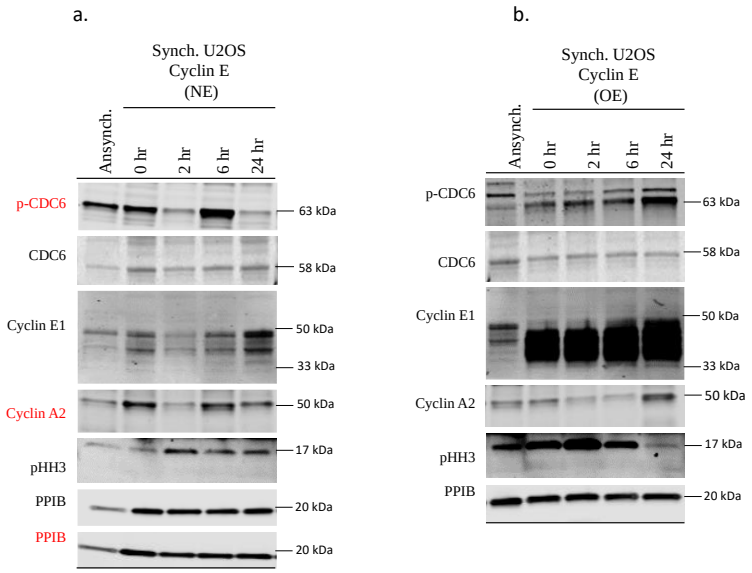


Figure 3.6. Western blot analysis of the cell cycle in normal and overexpressing cyclin E cells. U2OS-CE cells with (a) normal levels (NE, +TET) and (b) ectopic expression (OE, -TET) of cyclin E1 were synchronised using RO3306 (10μM). After 24 h, RO3306 was washed off and media with TET and without TET was replaced in cells with normal levels (NE, +TET) and ectopic expression of cyclin E1 (OE, -TET) respectively. Cell lysates were collected at different time points. Asynchronous cells served as controls. Protein levels were assessed by western blot analysis. Cyclophilin B (PPIB) was used as a loading control for each gel (red, black). Representative data of n = 3 experiments.

CHAPTER 4

4. Cyclin E1 overexpression and sensitivity to DDR inhibitors

4.1. Sensitivity to ATR inhibitors

We hypothesized that cyclin E1 overexpression could affect genomic stability and therefore have a role in modulating sensitivity to DNA damage response (DDR) modulators. Our data suggested that U2OS cyclin E overexpressing cells (OE, -TET) were sensitive to inhibition of ATR, a key mediator of the DNA-damage response (DDR). Pharmacological Inhibition of ATR pathway activation selectively induced cell death in cyclin E1 over-expressing cells.

U2OS-CE cells were seeded in cultured media with TET to maintain normal levels of cyclin E1 expression and in media without TET to allow cyclin E1 overexpression induction. After 3 days induction, U2OS-CE cells with background expression (NE, +TET) or ectopic expression (OE, -TET) of cyclin E1 were exposed to increasing concentrations of ATR inhibitors (ATRi). Additionally, we also tested CHK1 inhibition as CHK1 is considered to be a primary downstream effector of ATR signalling. After 5 days, cell proliferation/viability was assessed using resazurin.

Cells with ectopic overexpression of cyclin E1 (OE) were more sensitive to ATRi with AZD6738 showing the greatest differential between NE (IC_{50} = 0.7480 ± 0.3413, Table 2.7) and OE (IC_{50} = 0.13 ± 0.0326, Table 2.7) cells. (Fig.4.1a) In the absence of TET, U2OS-CE cells were also more sensitive to other ATRi (VE821, VE822) and a CHK1i (CCT245737). (Fig.4.1) In parallel, ectopic expression of cyclin E1 during the cell growth with inhibitors was confirmed by western blot analysis at day 0 (start) and day 5 (end) of the assay (Fig.4.1e).

Together the data indicate that high levels of cyclin E1 expression lead to increased sensitivity to ATR and CHK1 inhibitors, although the relative sensitivity to the highly potent VE822 was less than for the less potent compounds AZD6738 and VE821. Further studies are required to determine how the potency and/or selectivity of small molecule ATR inhibitors impacts on the relative sensitivity of cyclin E1 overexpressing cells.

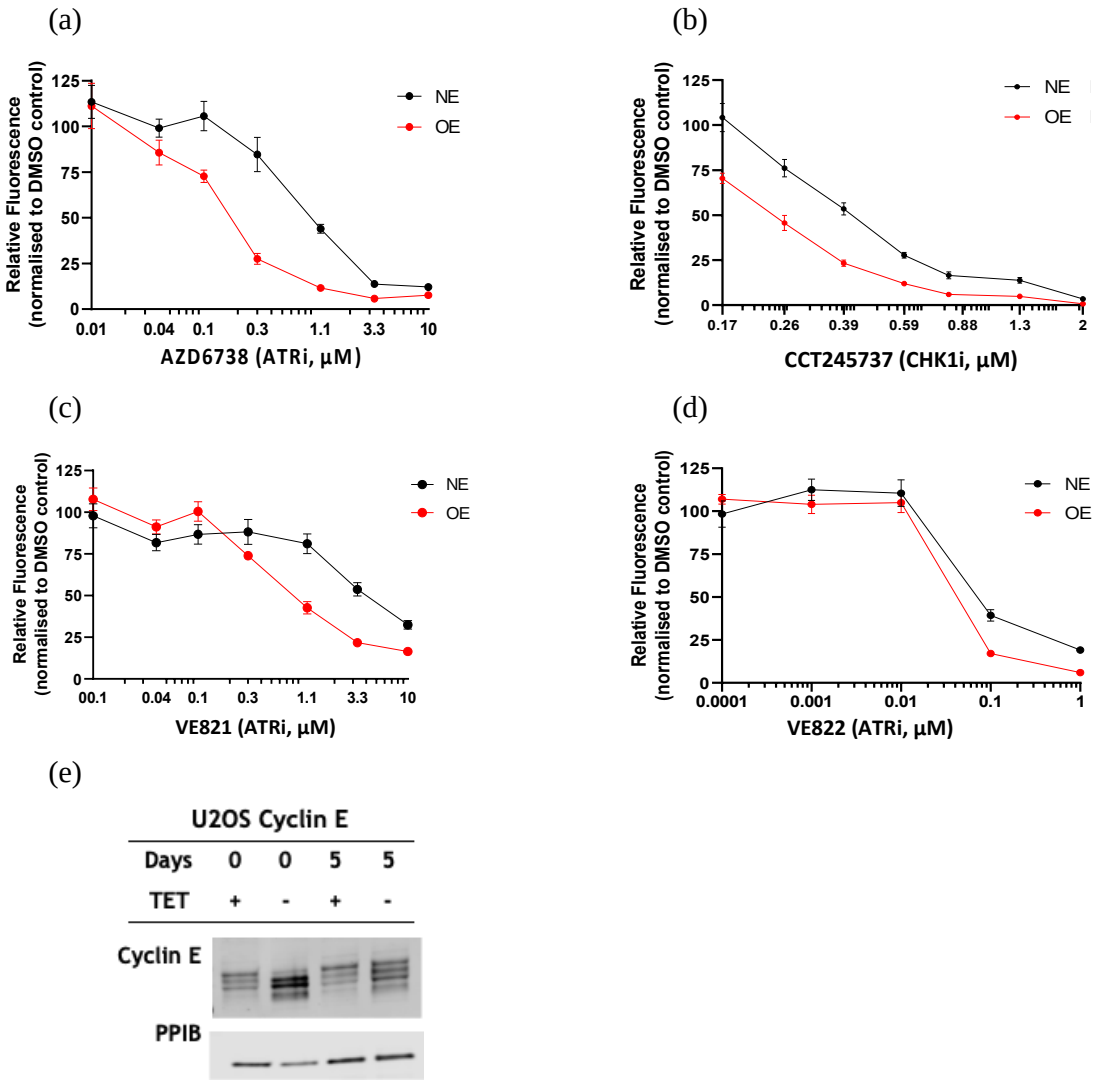


Figure 4.1. Sensitivity of U2OS Cyclin E cells to ATR and CHK1 inhibitors.
U2OS-CE cells with normal level (NE, +TET) and cyclin E1 overexpression (OE, -TET) were incubated for 5 days with increasing concentrations of (a) AZD6738, (b) CCT245737, (c) VE-821, (d) VE-822. Relative cell viability was assessed using the resazurin proliferation assay and normalised to DMSO treated controls. Graphs represent the mean of three independent experiments each performed with triplicates (± SEM). e. Western blot analysis confirm the cyclin E1 overexpression on day 0 (following 3 days induction) and on day 5 of each proliferation assay. Cyclophilin B (PPIB) was used as a loading control. Representative data of n = 3 experiments.

Target	Compound	IC_{50}	
		U2OS Cyclin E NE (μM)	U2OS Cyclin E OE (μM)
ATR	AZD6738	0.7480 ± 0.3413	0.13 ± 0.0326
	VE821	2.6280 ± 1.0741	0.7474 ± 0.6881
	VE822	0.0740 ± 0.0300	0.0584 ± 0.0194
CHK1	CCT245737	0.3079 ± 0.0719	0.2106 ± 0.0339

Table 2.7. IC_{50} values for the sensitivity of U2OS Cyclin E cell line to ATR and CHK1 inhibitors. IC_{50} values are presented as a mean and SD from three independent experiments, each performed in triplicate.

4.2. Sensitivity to other DNA Damage response modulators

To investigate further if cyclin E1 overexpression could modulate sensitivity to others DDR modulators, cells were exposed to different classes of DNA damage response inhibitors (ATMi, DNAPKi, PARPi, WEE1i, CHK1/2i, and neddylation inhibition). Alterations in cell viability were analysed in U2OS-CE cells with normal (NE) and overexpressing cyclin E1 (OE) cells by performing cell proliferation assays with resazurin after 5 days drug exposure. These experiments showed no marked differences in sensitivity between OE and NE conditions for U2OS-CE cells (Fig.4.2, Table 2.8), except for AZD7762 (a highly potent CHK1/2 inhibitor), although further experiments are required to better define the relative sensitivity due to the steep dose-response curve we observed (Fig. 4.2b). Since U2OS-CE cells were not sensitive to ATM inhibition the selective reduction of cell proliferation in OE cells due to AZD7762 treatment was most likely related to CHK1 inhibition.

Together our data suggest that selective sensitivity to DDR modulators in cyclin E1 overexpressing cells is restricted to inhibitors of the ATR-CHK1 pathway (Fig.4.2, Table 2.8).

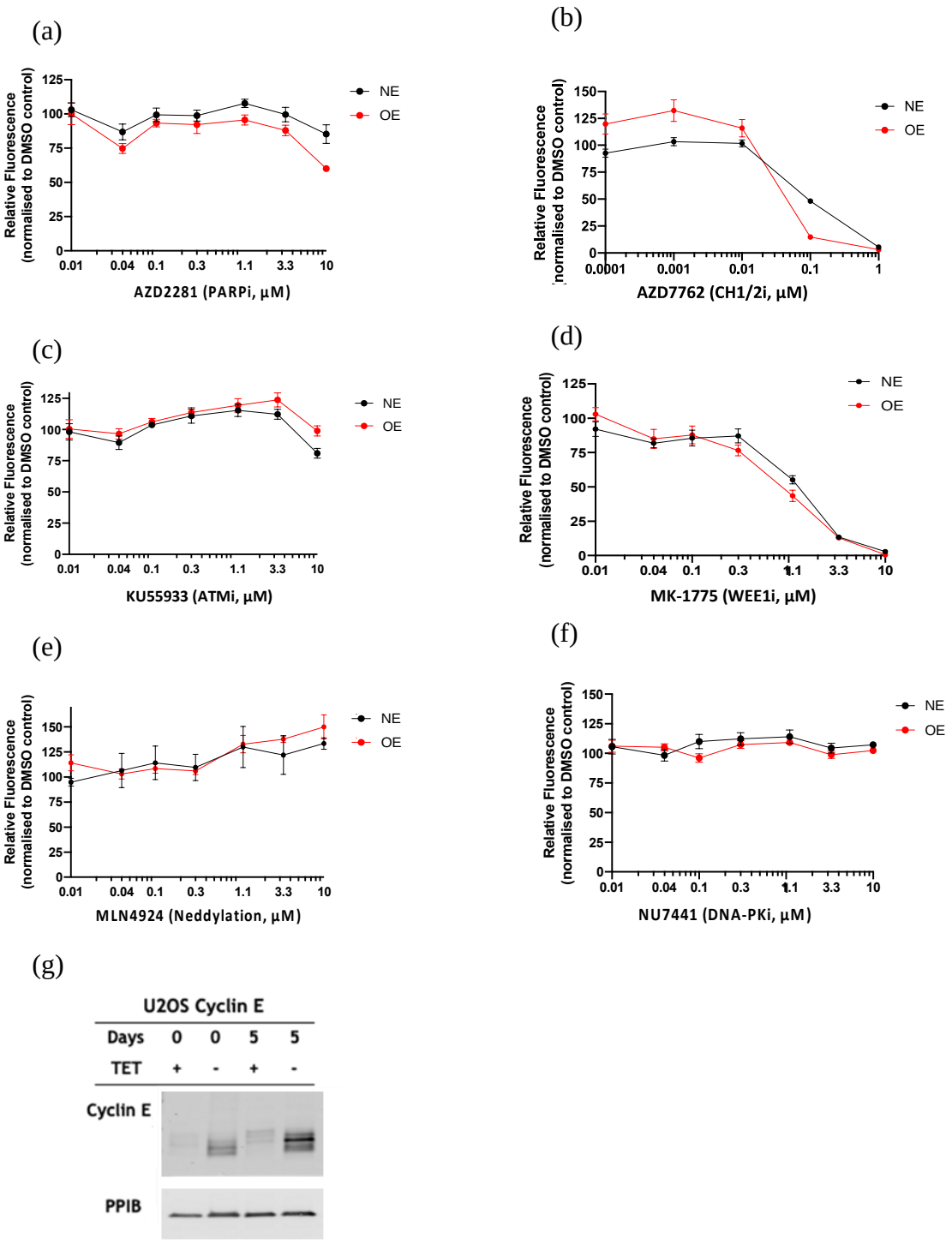


Figure 4.2. Sensitivity of U2OS Cyclin E cell line to different classes of DNA damage response modulators. U2OS Cyclin E cells with normal level (NE, +TET) and ectopic levels of cyclin E1 (OE, -TET) were exposed for 5 days to increasing concentrations of (a) AZD2281, (b) AZD7762, (c) KU55933, (d), MK-1775, (e) MLN4924, and (f) NU7441. Relative cell viability was assessed using the resazurin proliferation assay and normalised to DMSO treated controls. Graphs represent the mean of three independent experiments each performed with triplicates (± SEM). g. Western blot analysis confirm the cyclin E1 overexpression on day 0 (following 3 days induction) and on day 5 of each proliferation assay. Cyclophilin B (PPIB) was used as a loading control. Representative data of n = 3 experiments. h.

Target	Compound	IC_{50}	
		U2OS Cyclin E NE (μM)	U2OS Cyclin E OE (μM)
ATM	KU55933	> 10	> 10
CHK1/2	AZD7762	0.0969 ± 0.0104	0.0407 ± 0.0030
DNA-PK	NU7441	> 10	> 10
Neddylation	MLN4924	> 10	> 10
PARP	AZD2281	> 10	> 10
WEE1	MK-1775	1.372 ± 0.2288	1.3390 ± 0.4215

Table 2.8. IC_{50} values for the sensitivity of U2OS Cyclin E cell line to DDR modulators. IC_{50} values are presented as a mean and SD from three independent experiments, each performed in triplicate.

4.3. Signalling Responses: cell synchronisation and mechanistic studies

4.3.1. Replication stress analysis

To address whether DNA replication defects and DNA damage were induced by ectopic expression of cyclin E1, we performed immunoblot analysis of certain DNA damage response proteins. Cells with normal levels (NE, +TET) and with overexpression (OE, -TET) of cyclin E1 were enriched in G2 phase by incubating with a CDK1 inhibitor (RO3306 at 10 μM, 24 h). Synchronised cells with and without ectopic expression of cyclin E1 (NE, OE respectively) were released from the G2 cell cycle block by washing off RO3306; media was replaced for synchronised cells with normal levels (NE, +TET) and ectopic expression of cyclin E1 (OE, -TET) and then exposed to ATR inhibitor (AZD6738, 1 μM) or vehicle (DMSO, 0.01%) for different amounts of time, when cell lysates were prepared and analysed by western blot.

Levels of phospho-RPA(ser33), an important DNA replication stress biomarker of increased ssDNA that has been associated with ATR pathway activation, and phospho-CHK1 (ser317

and ser345) major downstream targets of ATR kinase activity were investigated under conditions of cyclin E1 over-expression (OE versus NE). However, induction of cyclin E1 did not increase pRPAser33 levels. (Fig.4.3)

In addition, levels of pCHK1ser317 or pCHK1ser345 were not reliably detected, therefore we were not be able to show ATR pathway activation on cyclin E1 overexpression.

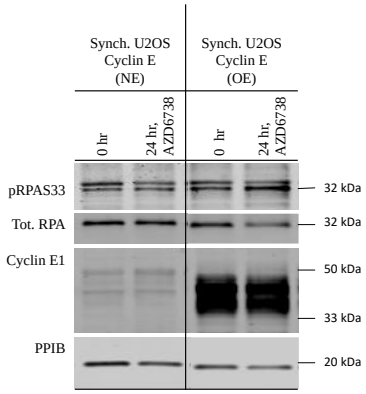


Figure 4.3. Replication stress analysis in U2OS Cyclin E cell line. U2OS-CE cells with normal levels (NE, +TET) and with ectopic expression (OE, -TET) of cyclin E1 were synchronised using RO3306 (10 μM). After 24 h, RO3306 was washed off and media with TET and without TET was replaced in cells with normal levels (NE, +TET) and ectopic expression of cyclin E1 (OE, -TET) respectively. Cell lysates were collected at different time points. Protein levels were assessed by western blot analysis. Cyclophilin B (PPIB) was used as a loading control for each gel. Representative data of one independent experiments.

However, to evaluate further the potential role of ectopic cyclin E1 expression in inducing DNA replication stress and in modulating sensitivity to ATR inhibition, we investigated the potential interaction between ATR and other DDR pathways such as ATM signalling. We hypothesised that sustained DNA replication stress and/or accumulation of DNA damage due to inhibition of ATR pathway signalling could trigger other DDR pathways.

4.3.2. ATM signalling pathway

ATM pathway activation may reflect sustained replication stress that leads to DNA replication fork collapse and the induction of DNA double strand breaks. To investigate in more details how the cells behaved upon ATR inhibitors treatment, we explored the ATM signalling pathway. Similarly to above, we synchronised cells in G2 using the CDK1 inhibitor RO3306 for 24 h then collecting cell lysate at different time points after release to verify cell cycle progression. Cells were exposed to AZD6738 (1 μM) or vehicle control (DMSO, 0.01%) and ATM signalling pathway activation (pKAP1, pRPAser4/8) was investigated by western blot analysis.

Vehicle treated U2OS-CE cells with endogenous levels of cyclin E1 (NE) revealed a G1/S phase entry corresponding to increased levels of cyclin E1 6 h after release from RO3306 treatment and did not show a clear increase in pRPAser4/8 levels at any of the time points. (Fig. 4.4a) Treatment of U2OS-CE cells under NE conditions with AZD6738 (1 μM) induced an increase in pRPAser4/8 at 6 h and 24 h, and a marked increase in pKAP1 levels, most notably after 24 h. The data suggest that in these cells, ATRi induced DNA replication stress (increased pRPAser4/8) and resulting in DNA replication fork collapse and ATM activation (increased pKAP1).

In contrast, vehicle treated cyclin E1 overexpressing cells (OE) exhibited higher levels of pRPAser4/8 irrespective of the time point suggesting that ectopic expression of cyclin E1 induces chronic DNA replication stress. The addition of AZD6738 (1 μM) to cyclin E1 overexpressing cells did not appear to further increase pRPAser4/8 levels, but there was a strong induction of ATM signalling after 24 h (increased pKAP1).

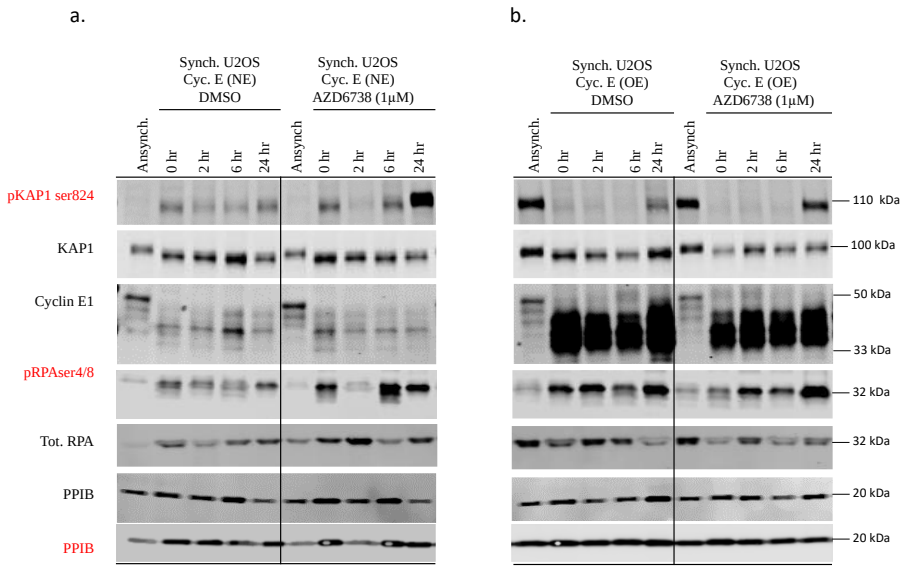


Figure 4.4. ATM signalling pathway in U2OS cyclin E cell line. U2OS-CE cells (a.) with normal levels (NE, +TET) and (b.) with ectopic expression (OE, -TET) of cyclin E1 were synchronised using RO3306 (10 μM). After 24 h, RO3306 was washed off and media with TET and without TET was replaced in cells with normal levels (NE,+TET) and ectopic expression of cyclin E1 (OE, -TET) respectively, and treated with either vehicle (DMSO, 0.01%) or AZD6738 (1 μM) for up 24 h at which time point lysates were collected. Asynchronous cells served as controls. Protein levels were assessed by western blot analysis. Cyclophilin B (PPIB) was used as a loading control for each gel (red, black). Representative data of n = 3 experiments.

4.4. Key Findings

In summary, the U2OS-CE cell line allowed us to investigate the role of cyclin E1 overexpression in modulating sensitivity to DDR modulators. Ectopic expression of cyclin E1 increased sensitivity to ATRi and CHKi and was associated with early entry into S-phase and increased origin firing. Although cyclin E1 overexpression was associated with increased levels of pRPAser4/8, suggesting increased levels of endogenous DNA replication stress, there was no clear effect on pRPAser33 indicating the relationship between phospho-RPA and DNA replication stress may be complex in this cell line. In both OE and NE conditions, prolonged (24 h) ATR inhibition led to increased levels of pKAP1, a sensitive substrate of ATM kinase activity, suggesting the presence of DNA replication fork collapse, or other form of induced DNA double-strand breaks, although this was not specific to cyclin

E1 over-expressing cells. Based on our data, we speculate that cyclin E1 overexpressing cells are more reliant on ATR pathway signalling, likely through CHK1, and are therefore more prone to cell death when exposed to ATR inhibitors. The mechanism of increased sensitivity to ATRi is not known but may involve an increased dependency on other DDR pathways in the presence of increased DNA replication stress.

Thus, DDR modulators represent a novel class of therapeutic agents that might be suitable for selectively targeting tumour cells with high levels of endogenous DNA replication stress due to oncogene activation (*CCNE1*).

CHAPTER 5

5. Cyclin E1 as a novel biomarker

5.1. Lung cancer cell lines

To further evaluate the role of cyclin E1 in modulating sensitivity on ATR inhibitors, our findings were extended to non-small cell lung adenocarcinoma cell lines (PC-9 and A549) which have similar growth profiles in 5 days proliferation assays. (Fig.5.1)

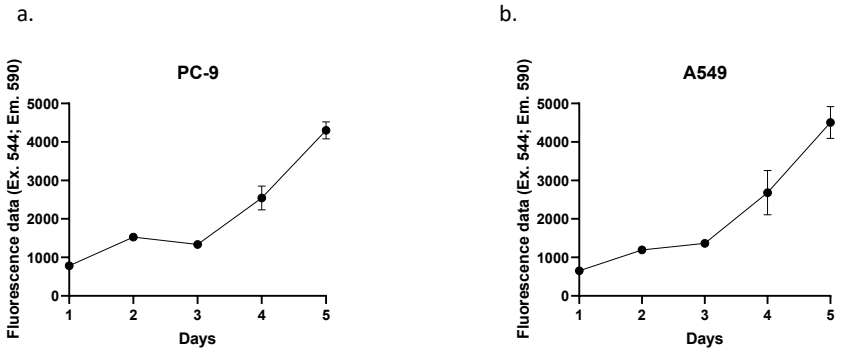


Figure 5.1. PC-9 and A549 cell proliferation. PC-9 (a) and A549 (b) cell proliferation was assessed using resazurin over 5 days. Graphs represent the mean of three independent experiments each done in triplicate (± SEM).

The effect of ATR and CHK1 inhibitors on PC-9 and A549 cell proliferation was tested in 5 days growth assays. (Figs. 5.2. and 5.3.) PC-9 cell line was more sensitive than A549 to growth inhibition by both ATR and CHK1 inhibitors (IC_{50} = 0.3821± 0.0959 and 0.0566 ± 0.0123 respectively, Table 2.9) and we hypothesised that this might be due to high levels of cyclin E1 expression analogous to U2OS-CE cells with induced cyclin E1 expression.

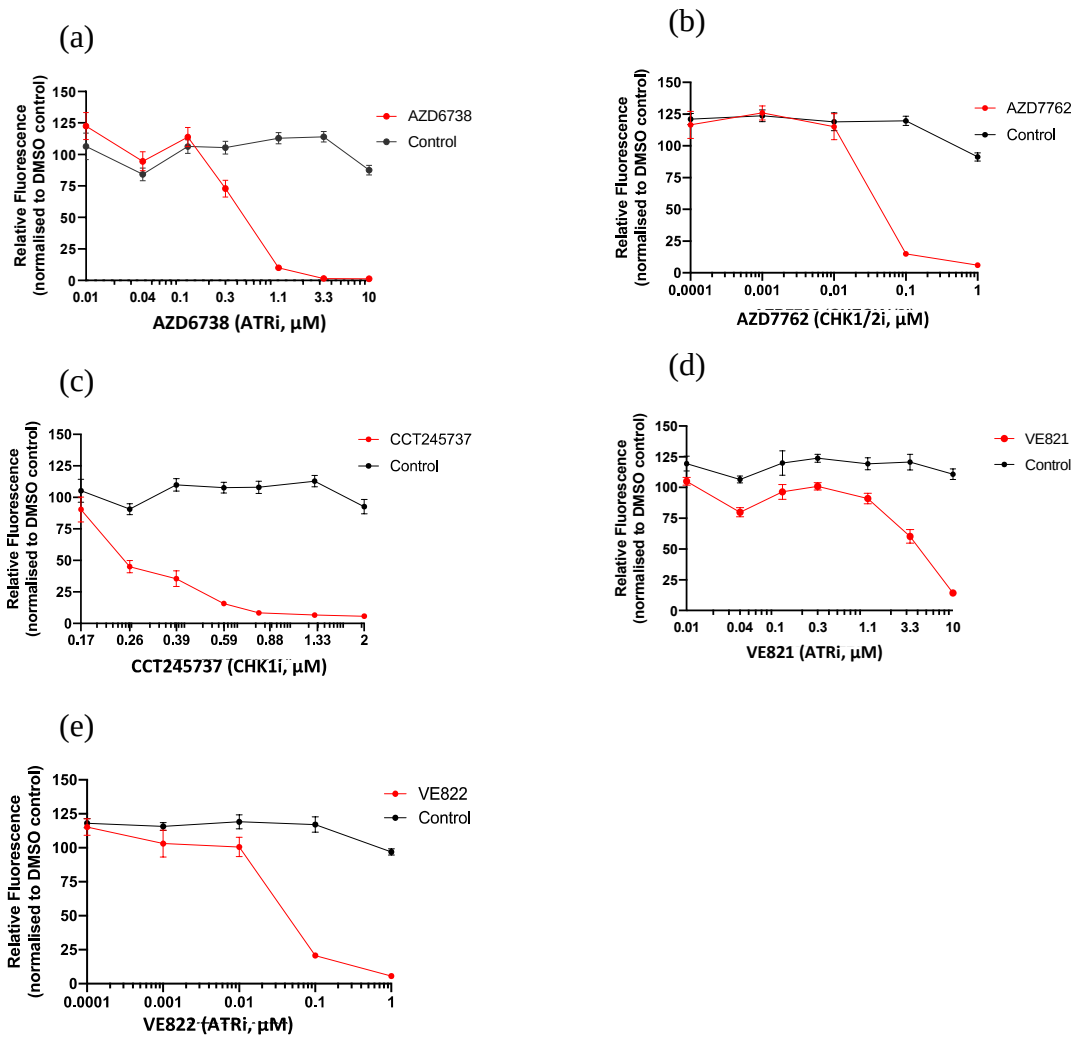


Figure 5.2. Sensitivity of PC-9 cell line to ATR and CHK1 inhibitors. PC-9 cell line was exposed for 5 days to increasing concentrations of (a) AZD6738, (b) AZD7762, (c) CCT245737, (d) VE821, (e) VE822. Relative cell viability was assessed using the resazurin proliferation assay and normalised to DMSO treated controls. Graphs represent the mean of three independent experiments done in triplicate (± SEM).

Target	Compound	IC_{50}	
		PC-9	Control (DMSO)
ATR	AZD6738	0.3821 ± 0.0959	> 10
	VE821	3.8710 ± 0.5728	> 10
	VE822	0.0505 ± 0.0228	> 1
CHK1	CCT245737	0.2303 ± 0.0523	> 2
CHK1/2	AZD7762	0.0566 ± 0.0123	> 1

Table 2.9. IC_{50} values for the sensitivity of PC9 cell line to ATR and CHK1 inhibitors. IC_{50} values are presented as a mean and SD from three independent experiments, each performed in triplicate.

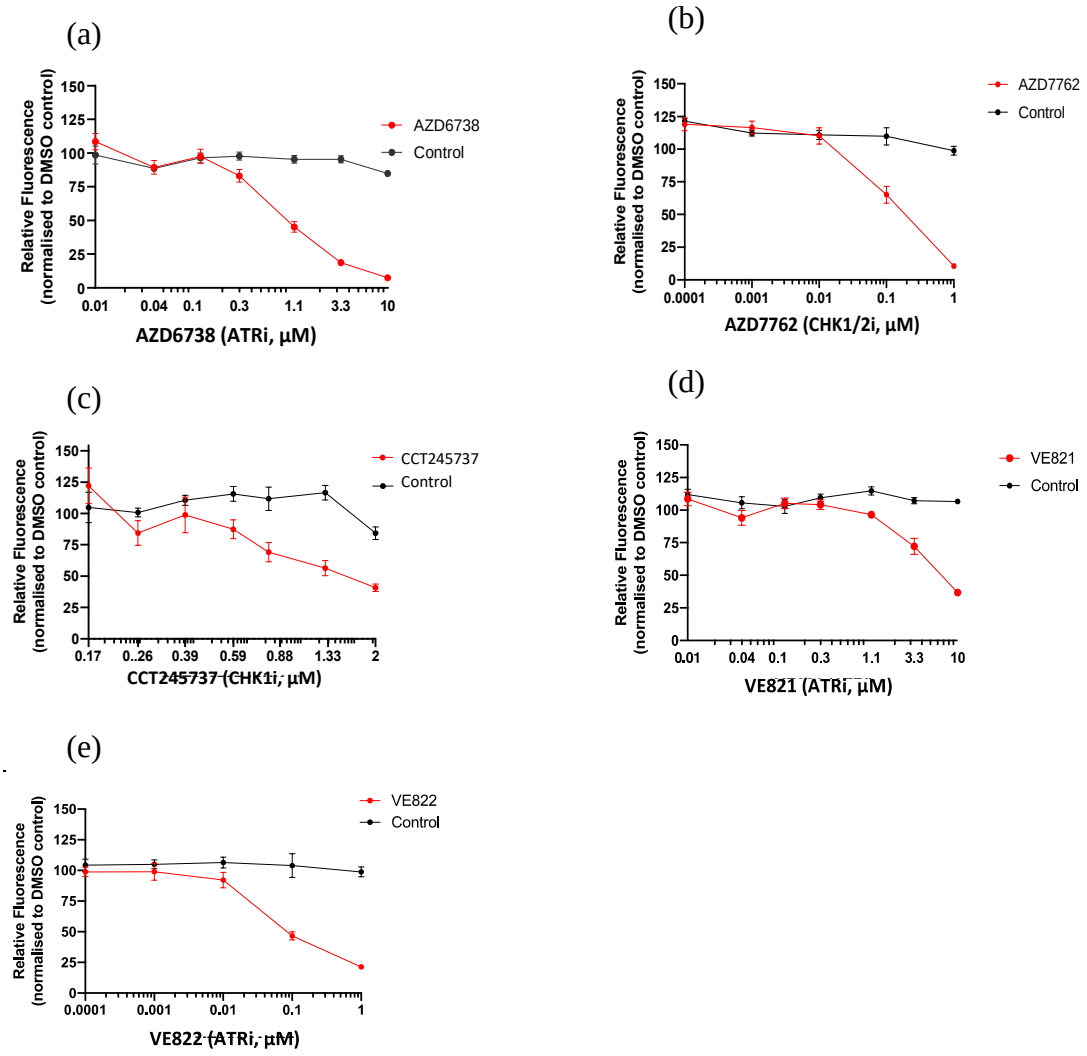


Figure 5.3: Sensitivity of A549 cell line to ATR and CHK1 inhibitors. A549 cell line was exposed for 5 days to increasing concentrations of (a) AZD6738, (b) AZD7762, (c) CCT245737, (d) VE-821, (e) VE822. Relative cell viability was assessed using the resazurin proliferation assay and normalised

to DMSO treated controls. Graphs represent the mean of three independent experiments each performed in triplicate. (± SEM)

Target	Compound	IC_{50}	
		A549	Control (DMSO)
ATR	AZD6738	1.0141 ± 0.4089	> 10
	VE821	4.9488 ± 5.1376	> 10
	VE822	0.4103 ± 0.4913	> 1
CHK1	CCT245737	> 2	> 2
CHK1/2	AZD7762	0.1919 ± 0.1317	> 1

Table 2.10. IC_{50} values for the sensitivity of A549 cell line to ATR and CHK1 inhibitors. IC_{50} values are presented as a mean and SD from three independent experiments, each performed in triplicate.

5.2. Signalling response in PC9 and A549 cells

We used RO3306 to enrich PC-9 and A549 cells in G2 phase using the same methodology as previously used for U2OS-CE cells. PC-9 and A549 cells were treated with CDK1 inhibitor RO3306 at 10 μM for 24 h and following release (washing off RO3306) cell lysates were collected at different time points and analysed by western blotting. (Fig.5.4)

In PC-9 cells high levels of cyclin E1 were apparent in G2 phase (0 h) and early after release (2-6 h), as well as at 24 h after release. Increased cyclin A2 occurred together with increased pCDC6-S54, after maximum levels of cyclin E1, and 24 h after release from the G2 block. (Fig 5.4a)

A549 cells exhibited a more complex expression profile for cyclin E1 which increased 6-24 h after G2 release and was possibly associated with increased pCDC6-S54 expression at 6 h. Multiple bands present for cyclin A in this cell line made interpretation although there was also a suggestion that cyclin A was increased 6 h after release from G2 phase. (Fig 5.4b)

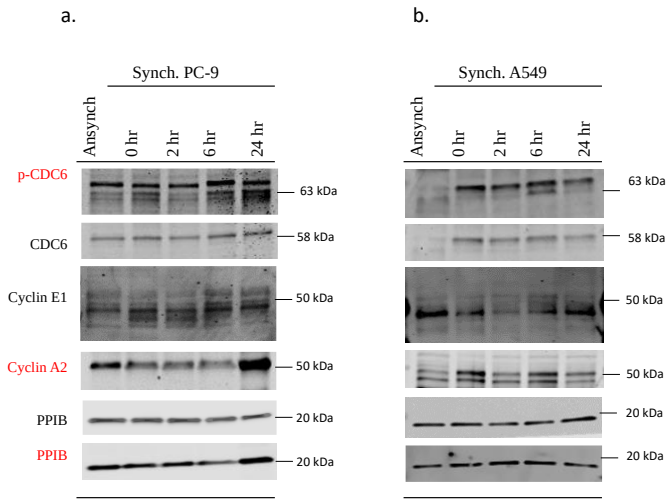


Figure 5.4. Western blot analysis of S phase cyclins (A and E) in PC-9 and A549 cells. PC-9 (a) and A549 (b) cells were synchronised using RO3306 (10, 24 hrs) and following release, cell lysates were collected at different time points. Asynchronous cells served as controls. Protein levels were assessed by western blot analysis. Cyclophilin B (PPIB) was used as a loading control for each gel (red, black). Representative data of n = 3 experiments.

A potentially important observation from the western blot analyses (Fig. 5.4) is that PC-9 cells appeared to exhibit an early expression of S phase cyclins compared with A549 cells. The early timing of cyclin E1 expression in PC-9 cell line may potentially explain its relatively higher sensitivity to ATRi and CHK1i compared with A549 cells. The addition of ATR inhibitor could increase DNA replication stress levels and induce cell death.

5.3. ATM signalling pathway

To determine whether cyclin E1 modulates the drug response in PC-9 and A549 cells in a similar fashion to cyclin E1 overexpression in the U2OS-Cyclin E cell line, we performed western blot analysis to investigate the ATM signalling pathway. A549 and PC-9 cells were synchronised with the CDK1 inhibitor RO3306 (10 μM, 24 h). Cells were exposed to

AZD6738 (1 μM) and compared with their vehicle control samples (DMSO, 0.01%). To be consistent with the earlier U2OS-CE cell studies, pRPAser4/8 was investigated as a marker of high levels of replication stress and pKAP1 to indicate ATM signalling pathway activation.

Vehicle treated G2 synchronised PC-9 cells showed detectable levels of pRPAser4/8 which were increased over the duration of the experiment up to 24 h after release from the G2 block. The expression of pRPAser4/8 after the addition of ATRi (AZD6738) did not appear markedly different from vehicle treated controls. (Fig 5.5a) In contrast, pKAP1 levels were marked higher in PC-9 cells 24 h after treatment with AZD6738 compared with the controls, suggesting active ATM signalling, possibly due to collapsed DNA replication forks or other modes of cell death that could induce DNA double strand breaks. Cyclin E1 levels were also highest 24 h after release from G2, perhaps indicating S phase accumulation or stalling in this experiment.

In contrast with the PC-9 cells, A549 cells showed broadly consistent unchanged levels of pRPAser4/8 at each of the time points tested, with no clear differences between vehicle or ATRi treated samples. (Fig 5.5b) Interestingly, pKAP1 was not detected in any of the samples over the time course of this experiment, potentially supporting the observation that A549 cells are less sensitive to the cytotoxic effects of ATRi than PC-9. Cyclin E1 levels were similar between the ATRi treated cells and the vehicle treated controls.

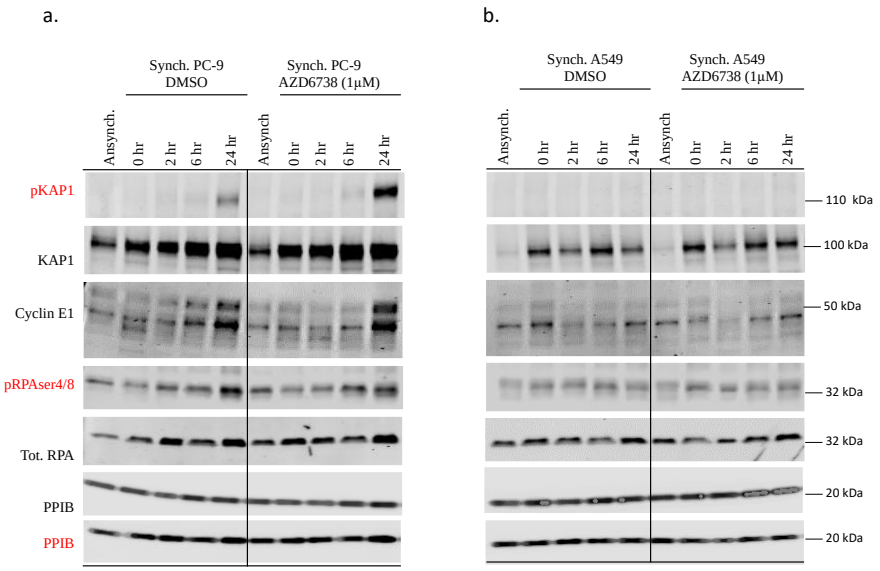


Figure 5.5. Western blot analysis of markers of DNA replication stress and ATM activity in PC-9 and A549 cells. PC-9 (a) and A549 (b) cells were synchronised using RO3306 (10, 24 hrs) and following release, treated with either vehicle (0.01% DMSO) or AZD6738 (1µM) and cell lysates were collected at time points up to 24 h. Asynchronous cells served as controls. Protein levels were assessed by western blot analysis. Cyclophilin B (PPIB) was used as a loading control for each gel (red, black). Representative data of n = 3 experiments.

We considered that this data provided some support for our hypothesis that early G1 phase cyclin E1 expression has the potential to increase sensitivity to the anti-proliferative effects of ATR inhibitors. Indeed, PC-9 cells reacts in a similar way to U2OS-CE overexpressing cells. However, future studies need to be carried out to understand the potential mechanism(s) as ATRi sensitivity and cyclin E1 overexpression do not seem directly related to the induction of DNA replication stress (at least as measured by phospo-RPA levels).

CHAPTER 6

6. Exploring a possible role for cyclin F

6.1. The Ubiquitin-proteolysis system

Surprisingly, inducing a G2 arrest with the CDK1 inhibitor RO3306 in U2OS-CE cells with ectopic expression of cyclin E1 (OE, -TET) resulted in very high levels of cyclin E1 protein compared with asynchronous cells (e.g. Fig 4.4.). Therefore, we hypothesised that CDK1 activity could have a role in controlling levels of cyclin E1 protein. As the expression of cyclin E1 protein is tightly controlled by ubiquitin-mediated proteolysis (Hao et al. 2007) we investigated whether cyclin F was involved. Cyclin F is the founding member of the F-box family of proteins, which are the substrate recognition subunits of SCF (Skp1-Cul1-F-box protein) ubiquitin ligase complexes. The $SCF_{cyclin\ F}$ mediates ubiquitylation and degradation of certain proteins, including several that are important for cell cycle progression and genome stability. (D'angiolella et al. 2012; D'Angiolella, Esencay, and Pagano 2013)

Cells were enriched in the G2 phase of the cell cycle by CDK1 inhibition (RO3306, 10 μM, 24 h). After release from the G2 cell cycle block cell lysates were collected at several time points. Asynchronous cells were used as controls. Protein levels were assessed by western blot analysis.

As seen in previous experiments, western blot analysis of U2OS-CE synchronised cells expressing ectopic cyclin E1 levels (OE, -TET) have markedly increased levels of cyclin E1 protein compared with unsynchronised cells (Fig 6.1). In contrast the levels of cyclin E1 protein were not markedly affected by G2 phase arrest in U2OS-CE cells with endogenous expression of cyclin E1 (OE, +TET). We examined whether cyclin F expression levels were

correlated with the levels of cyclin E1. Cyclin F levels steadily decreased over 8 h following release from the G2 block, in both U2OS NE and OE conditions, before increasing at 24 h. (Fig. 6.1.) Moreover, the levels of cyclin F were not markedly different between NE and OE growth condition (Fig. 6.1.) and there was no evidence for decreased cyclin F levels being associated with increased levels of cyclin E1.

The striking increase in the levels of ectopic cyclin E1 that we observed in G2 arrested U2OS CE OE cells in the presence of CDK1 inhibition warrants further investigation, but our data suggest that it this is not directly due to an effect though cyclin F. We speculate that cyclin E1 levels may not be directly regulated by CDK1 activity, since high levels of cyclin E1 are sustained for at least 24 h after removal of RO3306. (Fig. 6.1.)

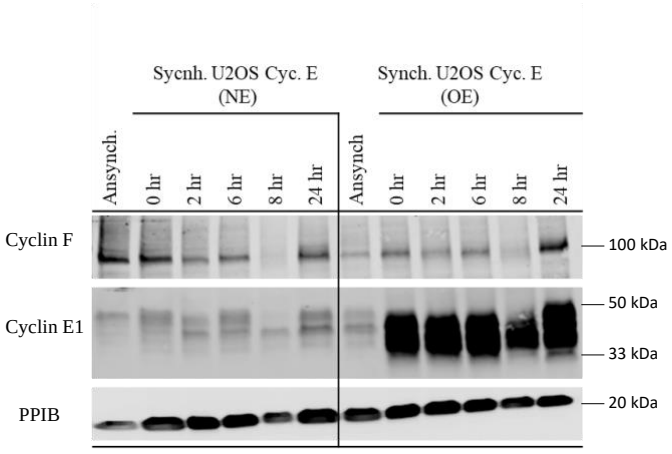


Figure 6.1. CDK1 inhibition and cyclin F protein levels. U2OS-CE with normal levels (NE, +TET) and with ectopic expression (OE, -TET) of cyclin E1 were arrested in G2 phase with RO3306 (10μM, 24 h) and cell lysates were collected at different time points after release. Asynchronous cells served as controls. Protein levels were assessed by western blot analysis. Cyclophilin B (PPIB) was used as a loading control for the gel. Representative data of one experiment.

CHAPTER 7

7. Discussion

Osteosarcoma is a genetically complex malignancy, predominantly afflicting the adolescent population and it is related still with relatively poor long-term outcomes. (Baumhoer, Amary, and Flanagan 2019; Longhi et al. 2006) Although there has been some improvement in the understanding of osteosarcoma biology, this has not yet translated into therapeutic advances. Current treatments involve genotoxic agents such as cisplatin, methotrexate and doxorubicin. However, the chance of recurrence of the disease remains high. Additionally, current standard chemotherapeutics used in clinic are accompanied by significant side effects. To improve survival and the quality of life of patients, new therapeutic alternatives need to be developed. The aim of this research project was to investigate the potential of using DNA Damage response modulators in cyclin E1 overexpressing tumour cells such as osteosarcoma. In addition, we proposed high levels of cyclin E1 expression as a potential biomarker for defining a suitable population to be efficiently treated with ATR inhibitors.

Here, we provide evidence for increased sensitivity of cyclin E1 overexpressing osteosarcoma cells to ATR inhibitors. We used an osteosarcoma cell line model (U2OS-CE) with a stably integrated TET-off system to produce tightly regulated induction of ectopic expression of cyclin E1. We used this cell line in a cell proliferation screen using DDR modulating agents. ATR inhibition, most notably with AZD6738, was 3-5-fold sensitive to cyclin E1 over-expressing cells. Western blot analysis suggested increased levels of endogenous DNA replication stress (using phospho-pRPA as a read out) in U2OS cells with ectopic cyclin E1 expression. We speculate that, as a consequence of increased cyclin E1-induced DNA replication stress, tumours may have become more dependent on a proficient ATR/CHK1 pathway for growth and survival.

Since ATR inhibitors exhibited the greatest effect in reducing viability of U2OS cells with cyclin E1 overexpression, we examined whether cyclin E1 protein levels were associated with sensitivity to ATRi in two lung cancer cell lines with differing sensitivity to ATRi. The more sensitive cell line (PC-9) appeared to have different levels of cyclin E1 compared with the less sensitive cell line (A549). A potentially novel suggestion from the current work is that early expression of cyclin E1 (in G1 phase) may be associated with sensitivity to ATRi. Early expression of cyclin E1 may be associated with early firing of DNA replication origins, contributing to increased levels of DNA replication stress. (Jones et al. 2013; Macheret and Halazonetis 2015, 2018)

In our studies, we made extensive use of the human osteosarcoma derived cell line (U2OS-cyclin E) that has an inducible TET-off system for tetracycline regulated ectopic expression of cyclin E1 under conditions where E2F1 activity is blocked by a constitutively active pRB (pRbΔcdk) mutant. Overexpressing cyclin E1 cells has the distinctive feature of independently promoting cell cycle progression which is not the case for overexpression of other cyclins such as cyclin D or cyclin A. (Lukas et al. 1997) In our studies, we observed similar cell proliferation rates and cell cycle distributions in U2OS-CE cells under NE (+TET) and OE (-TET) conditions. However, since U2OS has been reported to be functionally pRB-negative (despite expressing both total and phospho-pRB) (Broceño, Wilkie, and Mittnacht 2002) it seems likely that the CDK-insensitive form of mutant pRB (pRbΔcdk) did not impact on E2F1 function.

Others have reported that constitutive overexpression of cyclin E1 can result in an accelerated entrance in S phase as well described by Resnitzky et al (1994), indicating that cyclin E1 can be a rate-limiting positive regulator of the G1-S phase transition. Our data suggested premature origin firing is associated with overexpression of cyclin E1 potentially

through increased cyclin E/CDK2 activity. It is noteworthy to highlight that LMW-E (indicated by five bands ranging from 45 to 33 kDa) may also deregulate G1-S phase transition by affecting CDK2 activity. (Caruso et al. 2018) Since cell proliferation rates were similar in U2OS-CE cells with normal levels (NE,+TET) and with overexpression (OE,-TET) of cyclin E1, this suggests the possibility that increased cyclin E/CDK activity may be target one or more downstream proteins controlling the onset of DNA replication, rather than induce increased proliferation. (Lukas et al. 1997)

A study by Macheret & Halazonetis, (2018) described a model showing how increased cyclin E1 could lead to DNA replication stress. By mapping genome-wide DNA replication and transcription in U2OS cells, they found that oncogene-induced DNA replication stress involved activating a subset of novel intragenic origin of replication associated with a higher frequency of genomic rearrangements in cancer. They reported that with increased cyclin E1, G1 phase was shortened, and DNA replication origins were initiated in transcriptionally active regions of the genome, leading to DNA replication stress. Notably, inhibition of ATR signalling in oncogene expressing cells induced cell death by intensifying synergistically genetic instability/DNA damage which indicated that ATR signalling activity was required for tumour cells to tolerate the oncogene-induced origins of DNA replication found in transcriptionally active regions of the genome.

In our studies, sustained inhibition of ATR for 24 h was associated with increased ATM activity as measured by pKAP1. Although ATM and ATR activities can be triggered by different type of DNA damage and can activate distinct pathways, their downstream targets and the mediated responses can be partially overlapping and dependent on the type of genotoxic stress. (Helt et al. 2005) The ATM signalling pathway is generally accepted to be

a major mediator of the DNA damage-induced G1 cell cycle checkpoint, primarily acting through p53. In contrast, ATR activity induces intra S-phase and G2/M checkpoints. However, several studies demonstrated that ATM can also contribute to the activation and preservation of intra-S phase and G2/M cell cycle arrest, suggesting a functional overlap of ATM and ATR signalling checkpoint activation. (Abraham 2001; Matsuoka et al. 2007) Together, ATM and ATR signalling ensure genomic stability by coordinating cell cycle progression with DNA repair. (Abraham 2001; Cimprich and Cortez 2008) In our studies, ATM activity occurs only 24 h after treatment with ATRi, suggesting this may be a consequence of DNA double strand breaks occurring at collapsed DNA replication forks in both U2OS-CE cells in NE and OE growth conditions. A549 cells were more resistant to ATRi than PC9 cells and, in contrast to PC9 cells, ATM activity was not increased in A549 lung cancer cells following treatment with ATRi for 24 h, suggesting DNA replication fork collapse may contributes to ATRi-mediated tumour cell death.

CHAPTER 8

8. Conclusions

The research study provides evidences that cyclin E1 overexpression induces a distinctive 3-5-fold sensitivity to ATR/CHK1 inhibitors. These findings highlight the importance of investigating further cyclin E1 as a potential biomarker for the use of ATR inhibitor in osteosarcoma. Furthermore, we examined the relationship between cyclin E1 expression and sensitivity to ATR inhibition in lung cancer cell line and data suggest that early expression of cyclin E1 in G1 phase worth further investigation.

Pharmacological inhibition of DNA Damage repair pathways may provide an exciting opportunity to target genetic differences that exist between normal and tumour cells. Given the elevated levels of DNA damage in cancer cells compared to the normal cells, further increase of DNA damage by DDR depletion could selectively induce cancer cell death. The distinctive ATR sensitivity of osteosarcoma cells with the overexpression of cyclin E1 might have the potential to represent a novel therapy against OS. In addition, DDR modulators might be used in combination with chemotherapeutics improving the efficiency of standard therapies.

References

Abraham, R. T. 2001. 'Cell Cycle Checkpoint Signaling through the ATM and ATR Kinases'. *Genes & Development* 15(17): 2177–96

Alevizopoulos, Konstantinos, Jaromir Vlach, Silke Hennecke, and Bruno Amati. 1997. 'Cyclin E and C-Myc Promote Cell Proliferation in the Presence of P16(INK4a) of Hypophosphorylated Retinoblastoma Family Proteins'. *EMBO Journal* 16(17): 5322–33.

Antonescu, Cristina R. et al. 2000. 'Alterations of Cell Cycle Regulators in Localized Synovial Sarcoma: A Multifactorial Study with Prognostic Implications'. *American Journal of Pathology* 156(3): 977–83.

Bartek, Jiri, Claudia Lukas, and Jiri Lukas. 2004. 'Checking on DNA Damage in S Phase'. *Nature Reviews Molecular Cell Biology* 5(10): 792–804.

Bartkova, Jirina et al. 2006. 'Oncogene-Induced Senescence Is Part of the Tumorigenesis Barrier Imposed by DNA Damage Checkpoints'. *Nature* 444(7119): 633–37.

Baumhoer, Daniel, Fernanda Amary, and Adrienne M. Flanagan. 2019. 'An Update of Molecular Pathology of Bone Tumors. Lessons Learned from Investigating Samples by next Generation Sequencing'. *Genes Chromosomes and Cancer* 58(2): 88–99.

Benassi, M. Serena et al. 1997. 'Altered G1 Phase Regulation in Osteosarcoma'. *International Journal of Cancer* 74(5): 518–22.

Bester, Assaf C. et al. 2011. 'Nucleotide Deficiency Promotes Genomic Instability in Early Stages of Cancer Development'. *Cell* 145(3): 435–46.

Blackford, Andrew N., and Stephen P. Jackson. 2017. 'ATM, ATR, and DNA-PK: The Trinity at the Heart of the DNA Damage Response'. *Molecular Cell* 66(6): 801–17.

Blagosklonny, Mikhail V, Arthur B Pardee, and Arthur B Pardee. 2016. 'The Restriction Point of the Cell Cycle The Restriction Point of the Cell Cycle'. 4101(August).

De Bont, Rinne, and Nik van Larebeke. 2004. 'Endogenous DNA Damage in Humans: A Review of Quantitative Data'. *Mutagenesis* 19(3): 169–85.

Boos, Dominik, and Pedro Ferreira. 2019. 'Origin Firing Regulations to Control Genome Replication Timing'. *Genes* 10(3): 1–21.

Borlado, Luis R., and Juan Méndez. 2008. 'CDC6: From DNA Replication to Cell Cycle

Checkpoints and Oncogenesis'. *Carcinogenesis* 29(2): 237–43.

Boutros, Rose, Christine Dozier, and Bernard Ducommun. 2006. 'The When and Wheres of CDC25 Phosphatases'. *Current Opinion in Cell Biology* 18(2): 185–91.

Broceño, Cristina, Scott Wilkie, and Sibylle Mittnacht. 2002. 'RB Activation Defect in Tumor Cell Lines'. *Proceedings of the National Academy of Sciences of the United States of America* 99(22): 14200–205.

Caldon, C. E., and Elizabeth A. Musgrove. 2010. 'Distinct and Redundant Functions of Cyclin E1 and Cyclin E2 in Development and Cancer'. *Cell Division* 5: 1–13.

Caruso, Joseph A. et al. 2018. 'Low-Molecular-Weight Cyclin E in Human Cancer: Cellular Consequences and Opportunities for Targeted Therapies'. *Cancer Research* 78(19): 5481–91.

Chabes, Andrei, and Lars Thelander. 2000. 'Controlled Protein Degradation Regulates Ribonucleotide Reductase Activity in Proliferating Mammalian Cells during the Normal Cell Cycle and in Response to DNA Damage and Replication Blocks'. *Journal of Biological Chemistry* 275(23): 17747–53.

Charrier, Jean Damien et al. 2011. 'Discovery of Potent and Selective Inhibitors of Ataxia Telangiectasia Mutated and Rad3 Related (ATR) Protein Kinase as Potential Anticancer Agents'. *Journal of Medicinal Chemistry* 54(7): 2320–30.

Chehab, Nabil H., Asra Malikzay, Michael Appel, and Thanos D. Halazonetis. 2000. 'Chk2/HCds1 Functions as a DNA Damage Checkpoint in G1 by Stabilizing P53'. *Genes and Development* 14(3): 278–88.

Chehab, Nabil H., Asra Malikzay, Elena S. Stavridi, and Thanos D. Halazonetis. 1999. 'Phosphorylation of Ser-20 Mediates Stabilization of Human P53 in Response to DNA Damage'. *Proceedings of the National Academy of Sciences of the United States of America* 96(24): 13777–82.

Choong, Peter F.M. et al. 2011. 'The Molecular Pathogenesis of Osteosarcoma: A Review'. *Sarcoma* 2011. 959248

Ciccia, Alberto, and Stephen J. Elledge. 2011. 'ScienceDirect - Molecular Cell : The DNA Damage Response: Making It Safe to Play with Knives'. *Molecular Cell* 40(2): 179–204.

Cimprich, Karlene A., and David Cortez. 2008a. 'ATR: An Essential Regulator of Genome Integrity'. *Nature Reviews Molecular Cell Biology* 9(8): 616–27.

Cimprich, Karlene A, and David Cortez. 2008b. 'ATR: An Essential Regulator of Genome Integrity.1. Cimprich KA, Cortez D: ATR: An Essential Regulator of Genome Integrity. Nat Rev Mol Cell Biol 2008, 9:616–27.' *Nature reviews. Molecular cell biology* 9(8): 616–27.

Connell-Crowley, Lisa, J. Wade Harper, and David W. Goodrich. 1997. 'Cyclin D1/Cdk4 Regulates Retinoblastoma Protein-Mediated Cell Cycle Arrest by Site-Specific Phosphorylation'. *Molecular Biology of the Cell* 8(2): 287–301.

Cortez, D., S. Guntuku, J. Qin, and S. J. Elledge. 2001. 'ATR and ATRIP: Partners in Checkpoint Signaling'. *Science* 294(5547): 1713–16.

Costanzo, Vincenzo et al. 2003. 'An ATR- and Cdc7-Dependent DNA Damage Checkpoint That Inhibits Initiation of DNA Replication'. *Molecular Cell* 11(1): 203–13.

D'angiolella, Vincenzo et al. 2012. 'The Cyclin F-Ribonucleotide Reductase M2 Axis Controls Genome Integrity and DNA Repair'. *Cell* 149(5): 1023–34.

D'Angiolella, Vincenzo, Mine Esencay, and Michele Pagano. 2013. 'A Cyclin without Cyclin-Dependent Kinases: Cyclin F Controls Genome Stability through Ubiquitin-Mediated Proteolysis'. *Trends in Cell Biology* 23(3): 135–40.

Degregori, James, Alexander Miron, North Carolina, and Received July. 1995. 'Arrest'. : 2873–87.

Delacroix, Sinny et al. 2007. 'Clamp Activates Checkpoint Signaling via TopBP1'. *Genes & Development* 1(507): 1472–77.

Delk, Nikki A., Kelly K. Hunt, and Khandan Keyomarsi. 2009. 'Altered Subcellular Localization of Tumor-Specific Cyclin E Isoforms Affects Cyclin-Dependent Kinase 2 Complex Formation and Proteasomal Regulation'. *Cancer Research* 69(7): 2817–25.

Dillon, Magnus T. et al. 2017. 'Radiosensitization by the ATR Inhibitor AZD6738 through Generation of Acentric Micronuclei'. *Molecular Cancer Therapeutics* 16(1): 25–34.

Duronio, Robert J., Adam Brook, Nicholas Dyson, and Patrick H. O'Farrell. 1996. 'E2F-Induced S Phase Requires Cyclin E'. *Genes and Development* 10(19): 2505–13.

Dyson, Nicholas. 1998. 'The Regulation of E2F by PRB-Family Proteins'. *Genes and Development* 12(15): 2245–62.

Ekholm-Reed, Susanna et al. 2004. 'Deregulation of Cyclin E in Human Cells Interferes with Prereplication Complex Assembly'. *Journal of Cell Biology* 165(6): 789–800.

Ellison, Viola, and Bruce Stillman. 2003. 'Biochemical Characterization of DNA Damage Checkpoint Complexes: Clamp Loader and Clamp Complexes with Specificity for 5′ Recessed DNA'. *PLoS Biology* 1(2).

Fanning, Ellen, Vitaly Klimovich, and Andrew R. Nager. 2006. 'A Dynamic Model for Replication Protein A (RPA) Function in DNA Processing Pathways'. *Nucleic Acids Research* 34(15): 4126–37.

Fernandes, Norvin et al. 2005. 'DNA Damage-Induced Association of ATM with Its Target

Proteins Requires a Protein Interaction Domain in the N Terminus of ATM'. *Journal of Biological Chemistry* 280(15): 15158–64.

Ferrari, Stefano et al. 2005. 'Neoadjuvant Chemotherapy with High-Dose Ifosfamide, High-Dose Methotrexate, Cisplatin, and Doxorubicin for Patients with Localized Osteosarcoma of the Extremity: A Joint Study by the Italian and Scandinavian Sarcoma Groups'. *Journal of Clinical Oncology* 23(34): 8845–52.

Fischer, Martin, and Gerd A. Müller. 2017. 'Cell Cycle Transcription Control: DREAM/MuvB and RB-E2F Complexes'. *Critical Reviews in Biochemistry and Molecular Biology* 52(6): 638–62.

Fokas, E. et al. 2012. 'Targeting ATR in Vivo Using the Novel Inhibitor VE-822 Results in Selective Sensitization of Pancreatic Tumors to Radiation'. *Cell Death and Disease* 3(12): e441-10.

Foote, Kevin M. et al. 2013. 'Discovery of 4-{4-[(3R)-3-Methylmorpholin-4-Yl]-6-[1-(Methylsulfonyl) Cyclopropyl]Pyrimidin-2-Yl}-1H-Indole (AZ20): A Potent and Selective Inhibitor of ATR Protein Kinase with Monotherapy in Vivo Antitumor Activity'. *Journal of Medicinal Chemistry* 56(5): 2125–38.

Fragkos, Michalis, Olivier Ganier, Philippe Coulombe, and Marcel Méchali. 2015. 'DNA Replication Origin Activation in Space and Time'. *Nature Reviews Molecular Cell Biology* 16(6): 360–74.

Friend, Stephen H. et al. 1986. 'A Human DNA Segment with Properties of the Gene That Predisposes to Retinoblastoma and Osteosarcoma'. *Nature*. 16-22;323(6089):643-6

Gaillard, Hélène, Tatiana García-Muse, and Andrés Aguilera. 2015. 'Replication Stress and Cancer'. *Nature Reviews Cancer*. 15(5):276-89.

Geng, Yan et al. 2007. 'Kinase-Independent Function of Cyclin E'. *Molecular Cell* 25(1): 127–39.

Gilad, Oren et al. 2010. 'Combining ATR Suppression with Oncogenic Ras Synergistically Increases Genomic Instability, Causing Synthetic Lethality or Tumorigenesis in a Dosage-Dependent Manner'. *Cancer Research* 70(23): 9693–9702.

Gorgoulis, Vassilis G. et al. 2005. 'Activation of the DNA Damage Checkpoint and Genomic Instability in Human Precancerous Lesions'. *Nature* 434(7035): 907–13.

Halazonetis, Thanos D., Vassilis G. Gorgoulis, and Jiri Bartek. 2008. 'An Oncogene-Induced DNA Damage Model for Cancer Development'. *Science* 319(5868): 1352–55.

Hall, Amy B. et al. 2014. 'Potentiation of Tumor Responses to DNA Damaging Therapy by the Selective ATR Inhibitor VX-970'. *Oncotarget* 5(14): 5674–85.

Hao, Bing et al. 2007. 'Structure of a Fbw7-Skp1-Cyclin E Complex: Multisite-Phosphorylated Substrate Recognition by SCF Ubiquitin Ligases'. *Molecular Cell*

26(1): 131–43.

Hattinger, Claudia Maria et al. 2015. 'Advances in Emerging Drugs for Osteosarcoma'. *Expert Opinion on Emerging Drugs* 20(3): 495–514.

Hattinger Claudia Maria, et al. 2019. 15 Expert Opinion on Drug Metabolism and Toxicology *Current Understanding of Pharmacogenetic Implications of DNA Damaging Drugs Used in Osteosarcoma Treatment*. Taylor & Francis. 15(4):299-311

Heinsohn, Susanne et al. 2007. 'Determination of the Prognostic Value of Loss of Heterozygosity at the Retinoblastoma Gene in Osteosarcoma'. *International Journal of Oncology* 30(5): 1205–14.

Helt, Christopher E. et al. 2005. 'Ataxia Telangiectasia Mutated (ATM) and ATM and Rad3-Related Protein Exhibit Selective Target Specificities in Response to Different Forms of DNA Damage'. *Journal of Biological Chemistry* 280(2): 1186–92.

Hoeijmakers, Jan H J. 2019. 'DNA Damage, Aging, and Cancer'. : 1475–85.

Jackson, Stephen P., and Jiri Bartek. 2009. 'The DNA-Damage Response in Human Biology and Disease'. *Nature* 461(7267): 1071–78.

Jacq, Xavier et al. 2012. 'Abstract 1823: AZ20, a Novel Potent and Selective Inhibitor of ATR Kinase with in Vivo Antitumour Activity'. In American Association for Cancer Research (AACR), 1823–1823.

Jiang, Kecheng et al. 2003. 'Regulation of Chk1 Includes Chromatin Association and 14-3-3 Binding Following Phosphorylation on Ser-345'. *Journal of Biological Chemistry* 278(27): 25207–17.

Jones, R. M. et al. 2013. 'Increased Replication Initiation and Conflicts with Transcription Underlie Cyclin E-Induced Replication Stress'. *Oncogene* 32(32): 3744–53.

Kantidze, Omar L. et al. 2018. 'Synthetically Lethal Interactions of ATM, ATR, and DNA-PKcs'. *Trends in Cancer* 4(11): 755–68.

Koepp, D. M. et al. 2001. 'Phosphorylation-Dependent Ubiquitination of Cyctin E by the SCFFbw7 Ubiquitin Ligase'. *Science* 294(5540): 173–77.

Koff, Andrew et al. 1992. 'Formation and Activation of a Cyclin E-Cdk2 Complex during the G 1 Phase of the Human Cell Cycle'. *Science* 257(5077): 1689–94.

Kotsantis, Panagiotis, Eva Petermann, and Simon J. Boulton. 2018. 'Mechanisms of Oncogene-Induced Replication Stress: Jigsaw Falling into Place'. *Cancer Discovery* 8(5): 537–55.

Lamb, Rebecca et al. 2015. 'Doxycycline Down-Regulates DNA-PK and Radiosensitizes Tumor Initiating Cells: Implications for More Effective Radiation Therapy'.

Oncotarget 6(16): 14005–25.

Lee, Joon, Akiko Kumagai, and William G. Dunphy. 2007. 'The Rad9-Hus1-Rad1 Checkpoint Clamp Regulates Interaction of TopBP1 with ATR'. *Journal of Biological Chemistry* 282(38): 28036–44.

Leman, Adam R., and Eishi Noguchi. 2013. 4 Genes *The Replication Fork: Understanding the Eukaryotic Replication Machinery and the Challenges to Genome Duplication*. Genes (Basel). 2013 Mar; 4(1): 1–32

Lempiäinen, Harri, and Thanos D. Halazonetis. 2009. 'Emerging Common Themes in Regulation of PIKKs and PI3Ks'. *EMBO Journal* 28(20): 3067–73.

Leng, Xiaohong, Lisa Connell-Crowley, David Goodrich, and J. Wade Harper. 1997. 'S-Phase Entry upon Ectopic Expression of G1 Cyclin-Dependent Kinases in the Absence of Retinoblastoma Protein Phosphorylation'. *Current Biology* 7(9): 709–12.

Leone, Gustavo et al. 1999. 'Collaborative Role of E2F Transcriptional Activity and G1 Cyclin-Dependent Kinase Activity in the Induction of S Phase'. *Proceedings of the National Academy of Sciences of the United States of America* 96(12): 6626–31.

Li, Chunpu et al. 2018. 'Association between Common Polymorphisms in ERCC Gene and Prognosis of Osteosarcoma in Patients Treated with Chemotherapy: A Meta-Analysis'. *OncoTargets and Therapy* 11: 3495–3504.

Lindahl, Tomas. 1993. 'Instability and Decay of the Primary Structure of DNA'. *Nature* 362(6422): 709–15.

Liu, Qingsong et al. 2011. 'Discovery of 9-(6-Aminopyridin-3-Yl)-1-(3-(Trifluoromethyl)Phenyl)Benzo[h][1,6]Naphthyridin-2(1H)-One (Torin2) as a Potent, Selective and Orally Available MTOR Inhibitor for Treatment of Cancer'. *J Med Chem.* 54(5): 1473–80.

Liu, S. et al. 2006. 'Claspin Operates Downstream of TopBP1 To Direct ATR Signaling towards Chk1 Activation'. *Molecular and Cellular Biology* 26(16): 6056–64.

Longhi, Alessandra. 2001. 'Osteosarcoma in Blood Relatives'. *Oncology Reports* 8(1): 131–36.

Longhi, Alessandra et el. 2006. 'Primary Bone Osteosarcoma in the Pediatric Age: State of the Art'. *Cancer Treatment Reviews*.32(6):423-36

López-contreras, Andrés J, and Oscar Fernandez-capetillo. 2013. 'Europe PMC Funders Group The ATR Barrier to Replication-Born DNA Damage'. 9(12): 1249–55.

López-Guerrero, José Antonio et al. 2004. 'Deregulation of the G1 to S-Phase Cell Cycle Checkpoint Is Involved in the Pathogenesis of Human Osteosarcoma'. *Diagnostic Molecular Pathology* 13(2): 81–91.

Lukas, Jiri, Klaus Hansen, et al. 1997. 'Cyclin E-Induced S Phase without Activation of the PRb/E2F Pathway'. *Genes and Development* 11(11): 1479–92.

Lukas, Jiri, Thomas Herzinger, et al. 1997. 'Cyclin E-Induced S Phase without Activation of the PRb/E2F Pathway'. *Genes and Development* 11(11): 1479–92.

Lundberg, Ante S., and Robert A. Weinberg. 1998. 'Functional Inactivation of the Retinoblastoma Protein Requires Sequential Modification by at Least Two Distinct Cyclin-Cdk Complexes'. *Molecular and Cellular Biology* 18(2): 753–61.

Macheret, Morgane, and Thanos D. Halazonetis. 2018a. 'Intragenic Origins Due to Short G1 Phases Underlie Oncogene-Induced DNA Replication Stress'. *Nature*.

Macheret, Morgane, and Thanos D. Halazonetis.. 2018b. 'Intragenic Origins Due to Short G1 Phases Underlie Oncogene-Induced DNA Replication Stress'. *Nature* 555(7694): 112–16.

Marina, N. 2004. 'Biology and Therapeutic Advances for Pediatric Osteosarcoma'. *The Oncologist*. 9(4):422-41

Masuda, H. et al. 1987. 'Rearrangement of the P53 Gene in Human Osteogenic Sarcomas'. *Proceedings of the National Academy of Sciences of the United States of America* 84(21): 7716–19.

Matsuoka, Shuhei et al. 2007. 'ATM and ATR Substrate Analysis Reveals Extensive Protein Networks Responsive to DNA Damage'. *Science* 316(5828): 1160–66.

Matsuoka, Shuhei, Mingxia Huang, and Stephen J. Elledge. 1998. 'Linkage of ATM to Cell Cycle Regulation by the Chk2 Protein Kinase'. *Science* 282(5395): 1893–97.

Maya-Mendoza, Apolinar et al. 2007. 'Chk1 Regulates the Density of Active Replication Origins during the Vertebrate S Phase'. *EMBO Journal* 26(11): 2719–31.

Di Micco, Raffaella et al. 2006. 'Oncogene-Induced Senescence Is a DNA Damage Response Triggered by DNA Hyper-Replication'. *Nature* 444(7119): 638–42.

Morgan, David O. 1992. 'Cell Cycle Control in Normal and Neoplastic Cells'. *Current Opinion in Genetics and Development* 2(1): 33–37.

Murga, Matilde et al. 2009. 'A Mouse Model of ATR-Seckel Shows Embryonic Replicative Stress and Accelerated Aging'. *Nature Genetics* 41(8): 891–98.

Murga, Matilde, Stefano Campaner, Andres J Lopez-contreras, and Luis I Toledo. 2016. 'Europe PMC Funders Group Exploiting Oncogene-Induced Replicative Stress for the Selective Killing of Myc-Driven Tumors'. 18(12): 1331–35.

Nishida, Hiroshi et al. 2009. 'Inhibition of ATR Protein Kinase Activity by Schisandrin B in DNA Damage Response'. *Nucleic Acids Research* 37(17): 5678–89.

Ohtsubo, M et al. 1995. 'Human Cyclin E, a Nuclear Protein Essential for the G1-to-S Phase Transition.' *Molecular and Cellular Biology* 15(5): 2612–24.

Peasland, A. et al. 2011. 'Identification and Evaluation of a Potent Novel ATR Inhibitor, NU6027, in Breast and Ovarian Cancer Cell Lines'. *British Journal of Cancer* 105(3): 372–81.

Peng, Cheng Yuan et al. 1997. 'Mitotic and G2 Checkpoint Control: Regulation of 14-3-3 Protein Binding by Phosphorylation of Cdc25c on Serine-216'. *Science* 277(5331): 1501–5.

Pires, I. M. et al. 2012. 'Targeting Radiation-Resistant Hypoxic Tumour Cells through ATR Inhibition'. *British Journal of Cancer* 107(2): 291–99.

Porter, D. C. et al. 2001. 'Tumor-Specific Proteolytic Processing of Cyclin E Generates Hyperactive Lower-Molecular-Weight Forms'. *Molecular and Cellular Biology* 21(18): 6254–69.

Powell, Simon N. et al. 1995. 'Differential Sensitivity of P53(-) and P53(+) Cells to Caffeine-Induced Radiosensitization and Override of G2 Delay'. *Cancer Research* 55(8): 1643–48.

Prevo, Remko et al. 2012. 'The Novel ATR Inhibitor VE-821 Increases Sensitivity of Pancreatic Cancer Cells to Radiation and Chemotherapy'. *Cancer Biology and Therapy* 13(11): 1072–81.

Reaper, Philip M. et al. 2011. 'Selective Killing of ATM- or P53-Deficient Cancer Cells through Inhibition of ATR'. *Nature Chemical Biology* 7(7): 428–30.

Resnitzky, D, M Gossen, H Bujard, and S I Reed. 1994. 'Acceleration of the G1/S Phase Transition by Expression of Cyclins D1 and E with an Inducible System.' *Molecular and Cellular Biology* 14(3): 1669 LP – 1679.

Resnitzky, D, and S I Reed. 1995. 'Different Roles for Cyclins D1 and E in Regulation of the G1-to-S Transition.' *Molecular and Cellular Biology* 15(7): 3463–69.

Rickel, Kirby, Fang Fang, and Jianning Tao. 2017. 'Molecular Genetics of Osteosarcoma'. *Bone* 102: 69–79.

Roschke, Anna V. et al. 2015. 'Chromosomal Instability Is Associated with Higher Expression of Genes Implicated in Epithelial-Mesenchymal Transition, Cancer Invasiveness, and Metastasis and with Lower Expression of Genes Involved in Cell Cycle Checkpoints, DNA Repair, and Chromatin Mai'. *Neoplasia* 10(11): 1222-IN26.

Ruzankina, Yaroslava et al. 2007. 'Deletion of the Developmentally Essential Gene ATR in Adult Mice Leads to Age-Related Phenotypes and Stem Cell Loss'. *Cell Stem Cell* 1(1): 113–26.

Sajadi, Kaveh R. et al. 2004. ‘The Incidence and Prognosis of Osteosarcoma Skip Metastases’. *Clinical Orthopaedics and Related Research* (426): 92–96.

Santoni-Rugiu, E. et al. 2000. ‘Involvement of Myc Activity in a G1/S-Promoting Mechanism Parallel to the PRb/E2F Pathway’. *Molecular and Cellular Biology* 20(10): 3497–3509.

Sarkaria, Jann N et al. 1999. ‘Inhibition of ATM and ATR Kinase Activities by the Radiosensitizing’. : 4375–82.

Scholz, R. B. et al. 1992. ‘Studies of the RB7 Gene and the P53 Gene in Human Osteosarcomas’. *Pediatric Hematology and Oncology* 9(2): 125–37.

Schoppy, David W et al. 2012. ‘0Odphfojd Tusftt Tfotjuj [Ft Nvsjof Dbodfst Up Izqpnpsqijd Tvqqsfttjpo Pg " 53’. 122(1): 241–52.

Speck, Christian, Zhiqiang Chen, Huilin Li, and Bruce Stillman. 2005. ‘ATPase-Dependent Cooperative Binding of ORC and Cdc6 to Origin DNA’. *Nature Structural and Molecular Biology* 12(11): 965–71.

Spruck, Charles H., Kwang A. Won, and Steven I. Reed. 1999. ‘Deregulated Cyclin E Induces Chromosome Instability’. *Nature* 401(6750): 297–300.

Toguchida, Junya. 2016. ‘Genetics of Osteosarcoma’. *Osteosarcoma* 2012: 3–17.

Toledo, Luis I et al. 2016. ‘A Cell-Based Screen Identifies ATR Inhibitors1. Toledo LI, Murga M, Zur R, et Al. A Cell-Based Screen Identifies ATR Inhibitors with Synthetic Lethal Properties for Cancer-Associated Mutations. Nat Struct Mol Biol. 2016;18(6):721-727.

Tort, Frederic et al. 2006. ‘Retinoblastoma Pathway Defects Show Differential Ability to Activate the Constitutive DNA Damage Response in Human Tumorigenesis’. *Cancer Research* 66(21): 10258–63.

Vendetti, Frank P. et al. 2015. ‘The Orally Active and Bioavailable ATR Kinase Inhibitor AZD6738 Potentiates the Anti-Tumor Effects of Cisplatin to Resolve ATM-Deficient Non-Small Cell Lung Cancer in Vivo’. *Oncotarget* 6(42): 44289–305.

Weber, Anika Maria, and Anderson Joseph Ryan.. 2015b. ‘ATM and ATR as Therapeutic Targets in Cancer’. *Pharmacology and Therapeutics* 149: 124–38.

Weinberg, Robert A. 1995. ‘The Retinoblastoma Protein and Cell Cycle Control’. *Cell* 81(3): 323–30.

Yang, Xiaohong Helena, and Lee Zou. 2006. ‘Recruitment of ATR-ATRIP, Rad17, and 9-1-1 Complexes to DNA Damage’. *Methods in Enzymology* 409(05): 118–31.

Yuan, Zuanning et al. 2017. ‘Structural Basis of Mcm2-7 Replicative Helicase Loading by

ORC-Cdc6 and Cdt1'. *Nature Structural and Molecular Biology* 24(3): 316–24.

Zhang, Jinghui et al. 2015. 'Germline Mutations in Predisposition Genes in Pediatric Cancer'. *New England Journal of Medicine* 373(24): 2336–46.

Zhao, Yi Lei et al. 2013. 'The Association of XPG and MMS19L Polymorphisms Response to Chemotherapy in Osteosarcoma'. *Pakistan Journal of Medical Sciences* 29(5): 1225–29.

Zou, Lee, and Stephen J. Elledge. 2003. 'Sensing DNA Damage through ATRIP Recognition of RPA-SsDNA Complexes'. *Science* 300(5625): 1542–48.

Zou, Lee, Dou Liu, and Stephen J. Elledge. 2003. 'Replication Protein A-Mediated Recruitment and Activation of Rad17 Complexes'. *Proceedings of the National Academy of Sciences of the United States of America* 100(SUPPL. 2): 13827–32.