Identification of novel therapeutic approaches for the treatment of tumours characterised by mutations in the Retinoblastoma tumour suppressor gene (RB1)

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I, Caterina Mancusi confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signed  Date 09 / 09 / 2020
Abstract

Retinoblastoma protein (RB1) is an important tumour suppressor, which initially has been discovered for its crucial role in the control of cell growth and cell cycle progression. Since then, RB1 has been shown to regulate a plethora of cellular events, including the maintenance of chromosomal stability and DNA repair.

Current chemotherapeutic strategies are specifically effective in cancers harbouring deficiencies in DNA repair pathways exploiting a vulnerability of these cancers. In particular genotoxic agents have been shown to induce tumour cell death in DNA damage repair deficient cancers. Inhibitors of the ataxia telangiectasia and Rad3-related protein kinase (ATR) are currently being evaluated in clinical trials as monotherapy as well as in combination with inhibitors of poly(ADP-ribose) polymerases (PARPs), DNA-damaging chemotherapy or radiotherapy. It has been shown that tumours defective in ataxia-telangiectasia mutated (ATM) kinase, display an increased dependency on ATR signalling, and that a combined inactivation of ATM and ATR leads to synthetic lethality. This opens an important question of whether other molecular features in cancers could serve as predictive biomarkers for ATR inhibitor sensitivity.

The data presented herein shows that deleterious mutations of RB1 result in hypersensitivity to multiple clinically relevant ATR inhibitors. RB1-mutant cancer cell lines display a significantly enhanced cell death following ATR inhibitor treatment compared to RB1 wild type cancer cell lines. These results were confirmed in RB1 knockout cell lines, proving that RB1 deficiency is the cause of the observed ATR inhibitor sensitivity in RB1 defective cancers. Additionally, RB1-mutant cells display features of DNA replication stress, which were exacerbated following ATR inhibition and exhibit the inability to stabilise stalled replication forks under replication stress inducing agents. These observations could provide a mechanistic explanation for the observed ATR inhibitor sensitivity caused by RB1 loss.

Data obtained in this thesis documents a link between RB1 status and ATR inhibitor sensitivity and arguments that cancer types harbouring RB1 mutations should be considered in the design of clinical trials involving ATR inhibitors.
Impact Statement

In my thesis I have sought ways to identify new therapeutic approaches exploitable for the treatment of tumours characterized by mutations in the retinoblastoma tumour suppressor gene (RB1).

The RB1 pathway is one of the most important pathways deregulated in multiple cancers and is important for tumour formation and progression. In addition to the well-documented role of RB1 mutations in the initiation of retinoblastoma, inherited RB1 mutations are thought to increase the risk to several other tumours. The development of osteosarcomas, for example, is commonly observed in retinoblastoma survivors. Susceptibility to small cell lung carcinoma (SCLC) has also been attributed to germline RB1 mutations and retinoblastoma survivors. Other cancers display somatic loss of RB1 less frequently but still in significant proportions, including bladder, oesophageal, liver, brain, breast, and prostate cancers, as well as chronic myelogenous leukaemia.

In my PhD thesis I show that cancers characterized by inherited mutations in the RB1 gene are sensitive to multiple ataxia telangiectasia and Rad3-related (ATR) inhibitors. The role of RB1 in this hypersensitivity has been assessed using knockout as well as knockdown strategies of RB1, which demonstrated RB1 loss as the event responsible for driving sensitivity to ATR inhibitors.

ATR inhibitors are currently tested in clinical trials as monotherapy as well as in combination with DNA-damaging chemotherapeutic drugs, ionizing radiation or PARP inhibitors, in advanced solid tumours and hematological malignancies. Preclinical observations support synthetic lethality of ATR inhibitors in ataxia telangiectasia-mutated (ATM)-deficient cancers. Currently, there is a lack of data on whether the presence of oncogenes or the inactivation of tumour suppressors can serve as predictive biomarkers for ATR inhibitor monotherapy.

With my work I provide a model of mechanistic evidence that ATR inhibition in RB1-mutant cancers leads to a decrease in DNA replication speed progression. Moreover, RB1-
mutant cancers show difficulties to protect the replication fork under stress inducing agents, likely leading to DNA replication catastrophe and explaining the selective cell death and sensitivity in cancer cells with $RB1$ defect.

The discoveries made could aid patient stratification in clinical trials involving ATR inhibitors. By extension they propose selective use of such inhibitors in patients affected by cancers characterized by confirmed $RB1$ gene mutations, agnostic of the site and tissue of origin these cancers arise. My research can also provide a basis for further conceptual investigations into the molecular processes that safeguards accurate and faithful DNA replication and the management of replication complications in cells.
Acknowledgements

This work would not have been possible without funding from Cancer Research UK.

I would like to extend sincere thanks to my supervisor Professor Sibylle Mittnacht for her enthusiasm for science and her guidance throughout my PhD project. I also would like to thank Professor John Hartley for his support and advise about my PhD project.

I am grateful for the support and help I have received from present and past members of laboratory 103.

Finally, I would like to thank my family, friends and my husband Steve for the continuing encouragement, love and understanding they have shown me throughout the challenging past four years.
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### Abbreviations

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<td>Analysis of variance</td>
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<tr>
<td>aNHEJ</td>
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<td>APS</td>
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<td>ATM</td>
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<td>DDR</td>
<td>DNA damage response</td>
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# Abbreviations

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<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DMSO</td>
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<td>DNA replication ATP-dependent helicase/nuclease 2</td>
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<td>Double strand break</td>
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<td>HLTF</td>
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<td>KAP1</td>
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<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny broth</td>
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<td>LIG4</td>
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<td>mCRPC</td>
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<td>ORC</td>
<td>Origin recognition complex</td>
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<td>Poly [ADP-ribose] polymerase</td>
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<td>Short hairpin RNA</td>
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<td>siRNA</td>
<td>Small interfering RNA</td>
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CHAPTER ONE

Introduction
1 Introduction

1.1 The Retinoblastoma gene

The Retinoblastoma gene (*RB1*) is a tumour suppressor gene that was first identified in a childhood form of retinal cancer known as retinoblastoma.

In 1971, Alfred Knudson published his theory, also known as the two-hit hypothesis, where he explained that most tumour suppressor genes require both alleles to be inactivated to cause cancer (Knudson, 1971). Knudson performed a statistical analysis on cases of retinoblastoma, a tumour that occurs both as a sporadic or heritable disease. Interestingly, children with inherited retinoblastoma often developed the tumour in both eyes, suggesting an underlying predisposition. Knudson suggested that two “hits” or mutations to the DNA were necessary to cause the cancer. In children with inherited retinoblastoma, the first mutation in the *RB1* gene was inherited, the second one acquired. In non-inherited retinoblastoma instead, two mutations had to take place before the tumour could develop. Today this hypothesis serves as the basis for researchers understanding of how mutations in tumour suppressor genes drive cancer.

It was fifteen years later when the *RB1* gene was identified by Friend et al. in the q14 region of the human chromosome 13 and it was the first tumour suppressor gene to be cloned and characterised (Friend et al., 1986).

In 1986, Friend et al., cloned the *RB1* gene and this identification was of interest to Ed Harlow, who was trying to identify the cellular proteins that interact with the adenovirus E1A oncoprotein (DeCaprio, 2009). It had been found that E1A interacted with a cellular protein with mass identical to that of the *RB1* gene product (110 kDa) and generation of a RB1 specific antibody revealed that this was indeed RB1 (Whyte et al., 1988). Soon after this, it was reported that RB1 was able to interact with both oncoproteins from other DNA viruses, including the large T antigen of simian vacuolating virus 40 (SV40) and the E7 protein of human papillomaviruses. It was shown that variants of these viral oncogenes that were unable to interact with RB1 were in fact unable to transform cells. These observations led to the conclusion that transformation by these oncogenes involved RB1 binding and subsequent RB1 inactivation (DeCaprio et al., 1988) (Dyson et al., 1989). Studies addressing the interaction of the RB1 protein with viral oncoprotein have been instrumental in delineating RB’s structure and understanding its cellular function (Huang et al., 1990, Kaelin et al., 1990).
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Thus RB1 mutants with alterations in the site required for viral oncoprotein binding were found to be weakened in their ability to associate tightly with the nuclear structure (Mittnacht and Weinberg, 1991). This indicated the interaction site of RB1 to viral oncoproteins was shared by the host cellular machinery; direct competition for this binding site promoted the inactivation of RB1 function upon infection.

1.2 Cellular roles of RB1

*RB1* codes for a 928 amino acid multifunctional protein (RB1), now known to be involved in various cellular processes including: proliferation, development, senescence and apoptosis. Notably, RB1 is instrumental in controlling the progression from G1 into S phase of the cell cycle.

1.2.1 RB1 and cell cycle regulation

The cell cycle is composed of four phases, the gap before DNA replication (G1), the DNA synthetic phase (S), the gap after DNA replication (G2) and the mitotic phase, which terminates in cell division (M). The process of cell division is highly ordered and regulated by checkpoints that can become activated due to DNA damage, exogenous stress signals, defects during the replication of DNA, or failure of chromosomes to attach to the mitotic spindle. The final aim of these signals is the arrest of the cell cycle to provide time for repair. The tumour suppressor RB1 plays a critical role in each of these cell cycle checkpoints.

Furthermore, RB1 is precisely phosphorylated in synchrony with the cell cycle progression (Chen et al., 1989, DeCaprio et al., 1989, Buchkovich et al., 1989) suggesting that RB1 might be a general cell cycle regulator and phosphorylation is now understood to play a key role in regulating the activity of the RB1 protein.

1.2.2 RB1 regulation by phosphorylation

Transition of cells from G1 into S phase is dependent on RB1 phosphorylation. In G1, RB1 was originally thought to be hypo-phosphorylated and as cells enter S phase RB1 becomes hyper-phosphorylated. Once cells have progressed through mitosis RB1 becomes dephosphorylated again (Chen et al., 1989). It was determined that RB1 is phosphorylated by cyclin D/cyclin dependent kinases (CDK) 4/6 and cyclin E/CDK2
complexes (Harbour et al., 1999). In mammals, exist three cyclin D forms exist (D1, D2 and D3) which show tissue-specific expression, and there are two cyclin E forms (E1 and E2) which show overlapping expression patterns in mouse tissues (Geng et al., 2001). D type cyclins are regulatory subunits that activate CDK4/CDK6, the cyclin D/CDK4/6 complexes are short lived and form in G1 in response to mitogenic signals. E type cyclins bind to and activate CDK2 and shows their highest levels of expression at the G1/S boundary (Giacinti and Giordano, 2006).

During G1, cells respond to mitogenic stimuli and once the cell reaches a certain threshold, the restriction point, mitogenic signals are no longer required for cell cycle progression. After this point the cell is obligated to progressing into S phase and begin synthesising DNA. Phosphorylation of RB1 is thought to be the critical event permitting passage through the restriction point (Planas-Silva and Weinberg, 1997). RB1 contains 15 consensus cyclin/CDK dependent serine/threonine phosphorylation sites; there is evidence that cyclin D/CDK4/6 and cyclin E/CDK2 complexes phosphorylate an overlapping but distinct phosphorylation sites. However, whether these kinases do cooperate in their role in RB1 regulation, fulfil mutually exclusive roles or act redundantly is a subject to continued debate (Mittnacht, 2005). Cyclin D/CDK4/6 was thought to perform the initial phosphorylation step, blocking RB1 activity and facilitating progression of cells through mid to late G1. In late G1, cyclin E/CDK2 further phosphorylates RB1 which allows cells to progress into S phase (Harbour et al., 1999).

Until the end of the 1990s it was believed that cyclin D/CDK4/6 complex inactivates RB1 by progressive multi-phosphorylation called hyper-phosphorylation, resulting in the gradual release of E2F transcription factors that drives cells into the late G1 phase. Subsequent examinations by 2 D protein electrophoresis, revealed that cyclin D phosphorylation in cells generate RB1 monophosphorylated forms and that those forms remained active in the regulation of E2F, suggesting that cyclin D/CDK4/6 complex may not act to cause RB1 inactivation but could support RB1 activation. Instead, CDK2 complexes cause hyper-phosphorylation, where RB1 is phosphorylated to full stoichiometry on all or most of its CDK consensus sites (Narasimha et al., 2014). Thereby the activation of cyclin E/CDK2 complexes may be responsible for the inactivation of all forms of RB1 by hyper-phosphorylation at late G1 phase, whereas the maintenance of RB1 in the inactive hyper-phosphorylated state during S, G2 and M phase may be done by cyclin A/CDK2 and cyclin B/CDK1.
Figure 1.1: Revised model of G1 cell cycle progression
Growth factor signalling and DNA damage stimulate Cyclin D/CDK4/6 activation that mono-phosphorylates RB1. Activation of Cyclin E/CDK2 inactivates mono-phosphorylated RB1 via a hyper-phosphorylation at late G1 phase, restriction point. Cyclin A/CDK2 and Cyclin E/CDK2 maintain RB1 in an inactive state during S, G2 and M phase. Adapted from (Narasimha et al., 2014).
Furthermore, it was shown that RB1 is activated when DNA damage occurs and this activation also require RB1 mono-phosphorylation by cyclin D/CDK4/6 (Narasimha et al., 2014, Bertoli and de Bruin, 2014) (Fig. 1.1). RB1 can in fact be mono-phosphorylated at any of the 14 CDK phosphorylation sites during G1 phase, and those 14 sites coordinate RB1 interaction providing it with functional specificity (Sanidas et al., 2019). The RB1 mono-phosphorylation at serine 811 (S811) promotes its association with nucleosome remodelling and histone deacetylation (NuRD) complex. RB1 phosphorylation manages its interaction with the different proteome, chooses different targets and control different aspects of RB1 function (Sanidas et al., 2019).

1.2.3 RB1 and transcriptional repression

As already mentioned, mutations leading to the loss of the RB1 gene or deregulation of its gene product, RB1 protein, are associated with several forms of cancers. RB1 controls transcription and is a negative regulator of cell proliferation through repression of the E2F family of transcription factors (E2Fs). Transcriptional repression in this model occurs through the binding of RB1’s “pocket” domain to E2F’ C-terminal transactivation domain. In addition to its interactions with E2F, RB1 can further regulate transcription through the binding to chromatin remodelers (Lee et al., 2002).

While RB1 binding to E2F occurs at the pocket domain, RB1 interactions with epigenetically relevant proteins involve the LXCXE-binding domain. The availability of the LXCXE binding site, even when RB1 is bound to E2F, presents expanded functions of RB1 in the recruitment of chromatin remodelers and other protein complexes. Indeed, RB1 has been reported to interact with over 300 proteins and many protein interactions are, or are involved with, chromatin modifier proteins. RB1 therefore extends relevance into protein complexes beyond those within the canonical RB1/E2F pathway (Sanidas et al., 2019).

Several of RB1 functions, both within its canonical role in cell cycle control and other non-canonical cellular functions, are exerted through its interactions with epigenetic modifiers.

The intricacy of these multiprotein complexes and the cellular context under which they interact with RB1 depict the diversity of RB1 cellular roles and may explain why RB1 inactivation is sufficient to cause cancer in some tissues but not in others. Thus, understanding the context of these RB1 interactions with multi-protein complexes that
control nucleosome and chromatin modifications has the potential of providing alternative strategies for cancer therapy in a pathway that, at least to this day, remains largely undruggable (Guzman et al., 2020).

1.3 The pocket family proteins
The RB1 family members share significant sequence and structural homology with RB1 (Fig. 1.2). The most extensive sequence homology is located in the small pocket region, a well conserved structure which consists of A and B domains that are separated by a flexible spacer region and which contains the marked dox involved in E2F coordination and the site for viral oncoprotein binding.

Structurally the A and B domains represent a single cyclin fold domain and interact in a way that the small pocket is sufficient to form a transcription repressor on its own (Fig. 1.2A) (Classon and Dyson, 2001). Crystallography data revealed that the most well conserved features among the pocket family proteins is the LXCXE motif. A number of cellular proteins are reported to contain an LXCXE-like motif that allow them to interact with RB1, p107 (RBL1) and p130 (RBL2) (Dick, 2007).

The combination of the small pocket and the C-terminal domain has been called the large pocket and it is the minimal growth suppressing domain found in RB1 family proteins. The large pocket is sufficient to interact with E2F family transcription factors and suppress their transcription.

While the overall structure of the pocket domain is well conserved between the three proteins, p107 and p130 are more related to each other by sequence similarity then either is to RB1 (Classon and Dyson, 2001). RB1 sequence in fact possess few structural features that are missing in p107 and p130 structure. RB1 possess two unique structures like the docking site, used by the E2F1 transcription factor and a short peptide region in the C-terminus completely occupied by cyclin/CDKs or protein phosphatase 1 (PP1) (Fig. 1.2B).

As there are different sequence structures in RB1 that p107 and p130 do not have, there are a number of well-known features in p107 and p130 that are not present in RB1. Both p107 and p130 contain longer spacer regions than RB1, and those spacers allow the interaction with cyclin/CDKs complexes (Litovchick et al., 2004). Lastly, p107 and p130 contain a N-terminal region with evidence that it may serve to inhibit CDKs (Fig. 1.2B).
Figure 1.2: Schematic representation of RB1, p107 and p130 open reading frame

A) Pocket protein structure with LXCXE motif in the small pocket, the large pocket is the growth suppressing domain capable to bind E2F transcription factors. B) Comparison of open reading frame structure of each pocket proteins. All three proteins have an A and B domains, but the B pocket domain consists of two regions in both p107 and p130 compared to RB1. Within the spacer region of p107 and p130 lies a binding site with high affinity for cyclin A/CDK2 and cyclin E/CDK2. The pocket proteins both contain a CDK inhibitory domain.
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1.4 RB1 and cancer

The variety of human cancers in which RB1 is inactivated reflects both its importance for tumour suppression and the complexity of its protein interactions and cellular functions. Based on analyses of human tumours, it is evident that the RB1 pathway is inactivated by different mechanisms that include both RB1 gene mutation/deletion and enzymatic inactivation of retinoblastoma proteins.

In addition to the well-documented role of RB1 mutation in the initiation of retinoblastoma, inherited RB1 mutations are thought to increase the risk for several other tumour types. The development of osteosarcomas, for example, is commonly observed in retinoblastoma survivors (Chauveinec et al., 2001). Susceptibility to small cell lung
carcinoma (SCLC) has also been attributed to germline RB1 mutations in retinoblastoma survivors (Kaye and Harbour, 2004). Other cancers display somatic loss of RB1 less frequently than SCLC but still in significant proportions, including bladder, oesophageal, liver, brain, breast, and prostate cancers, as well as chronic myelogenous leukaemia (CML).

Increasing evidence documents RB1 loss as a late event in cancers, associated with tumour evolution and linked to therapy resistance. For example, RB1 loss has been demonstrated to facilitate lineage plasticity and metastasis in prostate adenocarcinoma initiated by PTEN mutation. Additional loss of Tp53 causes resistance to anti-androgen therapy (Ku et al., 2017). Further studies showed that loss of RB1 function alters cytoskeletal organization, induces epithelial-mesenchymal transition, and increases migration, invasion, and metastases (Thangavel et al., 2017).

Analysis of tumour samples and cell lines derived from resistant EGFR mutant non-small cell lung carcinoma (NSCLC) patients revealed that the 5-15 % of patient tumours undergo transformation to SCLC histology upon acquisition of resistance and RB1 is lost in 100 % of these SCLC transformed cases (Wikenheiser-Brokamp, 2006, Niederst et al., 2015).

### 1.5 RB1 loss and genome instability

The majority of cancers show chromosome instability (CIN). CIN is an increase in somatic losses and gains of chromosomal material manifesting as aneuploidy or polyploidy. CIN is a hallmark feature of nearly all solid tumours and adult leukaemia. This instability develops at early stages of cancer neoplasia and can be detected in premalignant lesions.

In a work from Bester et al., the authors studied the replication dynamics in cells with the RB1-E2F pathway aberrantly inactivated and demonstrated that the expression of human papillomavirus (HPV) E7 protein (associated with the development of cervical carcinoma and head and neck cancer), targets RB1, p107 and p130, alters S phase progression and promotes stalling of replication forks. The list of E2F-regulated genes that are deregulated following E7 expression includes several genes that are necessary for nucleotide synthesis.
Figure 1.3: Multiple functions of RB1
The tumour suppressor RB1 is involved in multiple processes including cell cycle regulation, apoptosis, maintenance of the mitotic fidelity and inducing cell proliferation.
Bester et al., proposed that the altered replication dynamics are mainly a consequence of suboptimal nucleotide pools. In support of this, the scientists showed that adding nucleotides to the cell culture media resulted in a dramatic suppression of the replication defects in E7-expressing cells (Bester et al., 2011). CIN is also seen in hereditary breast and ovarian cancers that are caused by inheritance of mutations in BRCA1 and BRCA2 (Kennedy and D'Andrea, 2006). Technological advances have furthered high throughput tumour sequencing and these profiling studies have revealed that mutations in DNA repair genes are rare in sporadic cancers, with the most common mutations residing in TP53, the ataxia telangiectasia mutated (ATM) and cyclin-dependent kinase inhibitor 2A (CDKN2A). Mutations in these proteins have the potential to disrupt DNA damage repair or lead to defects in DNA damage checkpoint activation (Negrini et al., 2010). One of the most important pathways deregulated in many cancers and important for tumour formation and progression is the retinoblastoma tumour suppressor pathway.

Beside the canonical functions of RB1 involving cell cycle regulation, RB1 has been reported to possess functions in suppressing CIN and aneuploidy, and therefore could also induce tumorigenesis by removing safeguards that limit oncogenic transformation (Coschi and Dick, 2012, Manning and Dyson, 2012, Dyson, 2016) (Fig. 1.3). The output of canonical RB1 regulation is the release of E2F transcription factors from RB1 upon CDK-mediated phosphorylation to activate transcriptional targets that advance the cell cycle. The non-canonical pathway includes RB1 being distributed across the genome, often in complex with E2F1, in a sequence-independent manner, where it can engage numerous effectors such as cohesin, condensin II (Cap-D3), BRG1, XRCC5 and XRCC6 (Manning et al., 2014, Velez-Cruz et al., 2016, Cook et al., 2015). These serve to repair DNA, replicate DNA and ensure that chromosomes are faithfully distributed to daughter cells. In addition, the physical association of RB1 with repeat elements and enhancers allows it to silence repeat expression and control the expression of genes involved in pluripotency to regulate lineage commitment of the cell (Dick et al., 2018). These observations show, that in addition to the RB1 canonical cell cycle regulation function, RB1 is distributed throughout the genome in a sequence-independent manner. RB1, in fact, takes part in many fundamental processes of genome maintenance including DNA-break repair, DNA replication, chromosome condensation and heterochromatin formation (Fig. 1.4).
Figure 1.4: Canonical and non-canonical functions of RB1
RB1 plays important roles in multiple processes that can be E2F dependent or independent.
1.6 The DNA damage response

The integrity of cellular DNA is under constant threat from external and intracellular factors such as radiation from the sun, natural and man-made mutagenic chemicals and reactive oxygen species (ROS) produced as a by-product of cellular metabolism (Fig. 1.5).

Specific types of DNA damage, mismatches due to replication, single strand DNA breaks (SSBs) or double strand DNA breaks (DSBs), result in the activation of specific signalling and repair cascades. Few specific lesions can also be removed by direct chemical reversal and interstrand crosslink (ICL). The DNA damage response (DDR) pathways mitigate DNA repair; consequently, deficiencies in these pathways result in the accumulation of SSBs and DSBs. Poly(ADP-ribose) polymerase (PARP) enzymes are key to activating a host of downstream repair mechanisms and are primary proteins involved in SSB repair or base-excision repair (BER). Incorrectly repaired DNA lesions can lead to mutations while unrepaired damage can result in cellular senescence or apoptosis (Welsh et al., 2004, Mendoza et al., 2013, Ciccia and Elledge, 2010). Dysregulation of DDR and repair systems can cause several human disorders that are associated with cancer susceptibility, accelerated aging and developmental abnormalities (Pan et al., 2016).

DSBs are more lethal than SSBs as both strands of DNA are damaged and accurate repair is a more complex affair. If cells are in S or G2 phase and have undergone DNA replication then sister chromatids may be available to act as a template. If this is the case, then repair in the form of homologous recombination (HR) can occur. In addition to HR, cells can also repair DSBs through non-homologous end joining (NHEJ), this pathway is considered to be active through the entire cell cycle, but some studies suggested that is predominant in G0 and G1, where there are not sister chromatids available to act as a template. As NHEJ lacks such template, HR is thought to be more faithful repair mechanism (Fig. 1.6).
Figure 1.5: DNA damaging agents and DDR pathways
Examples of cell stressors are shown, with resultant DNA damage. DNA repair proceeds through a conserved general mechanism of damage recognition, lesion excision and processing. Single-strand break can be resolved via Mismatch repair (MMR); Base excision repair (BER) and Nucleotide excision repair (NER). Double-strand break can be resolved via Non-homologous end joining (NHEJ) or Homology-directed repair (HDR).
Figure 1.6: DNA double-strand break repair pathways

A) Homologous recombination is utilised in S or G2 phase of dividing neuronal progenitors. MRE11-RAD50-NBS1 (MRN) complex recognizes and senses double strand breaks (DSBs). CtIP-mediated nuclease activity is required for the end resection from 5’ to 3’, which leads to the formation of single-strand DNA (ssDNA). The exposed ssDNA is coated with DNA replication protein A (RPA). BRCA2 catalyses the exchange of RPA for RAD51, thus enabling invasion of the sister chromatid and error-free repair.

B) Non-homologous end joining is utilised in G0 or G1. DNA ends are bound by KU70/80, leading to the recruitment of DNA-PKcs. End processing is carried out by various enzymes including PNKP and Artemis and then ends are ligated by LIG4-XRCC4. This form of repair preserves genomic integrity but can be error prone.

BRCA2, breast cancer type 2 susceptibility protein; CtIP, C-terminal binding protein 1 interacting protein; DNA-PKcs, DNA-dependent protein kinase, catalytic subunit; MRN complex; LIG, DNA ligase; PCNA, proliferating cell nuclear antigen; PNKP, polynucleotide kinase 3'-phosphatase; RPA, replication protein A; TDP1, tyrosyl-DNA phosphodiesterase 1; XLF, XRCC4-like factor; XRCC4, X-ray repair complementing 4. Adapted from (Massey and Jones, 2018).
The two pathways are in competition, usually the DNA resection step is the major determinant to regulates the pathways efficiency. If the DNA resection doesn’t occur, the DSB ends could be directly ligated and repaired by NHEJ. When there is an extensive DNA resection, the ssDNA tail could invade the homologous sequences and the DNA will be repair with HR.

Three members of a phosphatidylinositol 3-kinase-related kinases (PIKKs) family have the principal roles in activating the DDR. These kinases are ataxia-telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related protein (ATR) and DNA-dependent protein kinase (DNA-PK) and are the most upstream DDR kinases that respond to the sensors recognizing DNA damage (Marechal and Zou, 2013) (Fig. 1.7). DNA-PK is primarily involved in DSB repair having a more direct role, whereas ATR responds to a wide range of DNA lesions, especially those associated with DNA replication (Cimprich and Cortez, 2008). All three DDR kinases sense damage through protein-protein interactions that serve to recruit the kinases to damage sites. Once localized, posttranslational modifications and other protein-protein interactions fully activate the kinases to initiate a cascade of phosphorylation events. Indeed, each kinase requires a specific protein co-factor for stable recruitment to DNA damage sites: ATM requires NBS1 (Falck et al., 2005), ATR requires ATRIP (Zou and Elledge, 2003) and DNA-PK requires Ku70 and Ku80 (Gell and Jackson, 1999).
Figure 1.7: ATM, ATR and DNA-PK signalling pathways
A) Ku70/80 bind to DNA DSBs and recruit DNA-PKcs. Upon DNA binding, autophosphorylation of DNA-PKcs induces a conformational change that destabilizes the NHEJ core complex, causing sliding of Ku inward on the DNA and enabling access of end-processing and ligation enzymes to DNA ends and facilitation of repair. B) Following DSBs ATM is predominantly activated through interactions with NBS1 of the MRN complex. Activated ATM phosphorylates numerous effector proteins such as H2AX on serine 139 (known as γH2AX). MDC1 (mediator of DNA-damage checkpoint protein 1) directly binds γH2AX and potentiates DNA-damage signalling leading to spreading of γH2AX. This in turn promotes recruitment and retention of DNA-damage mediator proteins such as 53BP1. CHK2 is a well-studied ATM substrate. C) ATR is primarily activated at regions of ssDNA which are first coated by RPA. ATR is then recruited to RPA-coated ssDNA via its interacting protein ATRIP. The recruitment of TOPBP1 to the ssDNA region is responsible for the activation of ATR which subsequently phosphorylates effector proteins such as CHK1 and p53. The ATR–CHK1 signalling cascade activates the G2–M checkpoint, promotes replication fork stabilization, and slows DNA replication by suppressing origin firing. Adapted from (Brown et al., 2017).
1.6.1 RB1 and DNA damage

Various lines of evidence indicate that RB1 status influences the activation of DNA damage checkpoints and DNA damage repair.

In human cells RB1 inactivation leads to accumulation of DSBs (Pickering and Kowalik, 2006). Depletion of RB1 in human osteosarcoma cell line (U2OS), showed higher numbers of γH2AX (H2A histone family member X) foci, indicative of DSBs (Rogakou et al., 1998). Interestingly, the number of γH2AX foci was elevated by inducing ionizing radiation. Importantly, γH2AX foci persisted longer in RB1 depleted cells. In both osteosarcoma and breast cancer cell lines, RB1 depletion led to lower survival in response to other DSB inducing drugs like etoposide and camptothecin (Velez-Cruz et al., 2016).

These observations supported the hypothesis that RB1 may regulate and actively contribute to the DSB repair pathways. Indeed, RB1 has been reported to regulate both HR and NHEJ. RB1 was found to recruit another protein BRG1 to DSB to induce DNA resection and in doing so initiating HR (Velez-Cruz et al., 2016). BRG1 belongs to the SWI/SNF family of ATPases, which could remodel chromatin structure to undergo DNA resection. Importantly, the recruitment of RB1 and BRG1 proteins to DSBs requires the ATM-mediated phosphorylation of E2F (Velez-Cruz et al., 2016). These observations suggest that RB1 participates in HR. In addition to HR, RB1 was also reported to play important roles in NHEJ (Cook et al., 2015). In RB1 depleted cells, the NHEJ efficiency was reduced and the radiation induced chromosomal aberration was elevated. This is thought to be a result of the protein interaction between RB1 and core members of the NHEJ pathway such as XRCC5 and XRCC6 (Cook et al., 2015). Furthermore, Arabidopsis Retinoblastoma Related (RBR), the plant homolog of the mammalian retinoblastoma tumour suppressor protein, has been described to transcriptionally regulate DNA damage response and cell death pathways, and through cooperating with the repair factor BRCA1, RBR maintains genome integrity after damage (Horvath et al., 2017). RBR, together with E2FA, frequently accumulates on γH2AX-labelled damage foci during DNA damage responses, and it physically interacts with AtBRCA1 (BRCA1 gene homolog in Arabidopsis) and co-localises at a subset of γH2AX-labelled nuclear foci (Horvath et al., 2017). More recently, Marshall et al. have shown how the levels of DNA damage and genome instability can be increased by inducing RB1 gene deletions via CRISPR/Cas9. They also showed higher level of sensitivity to peroxide and cisplatin,
suggesting that oxidative damage may cause sporadic DNA damage, as levels of ROS are elevated in RB1-deficient cells (Marshall et al., 2019).

1.6.2 RB1 loss and cancer therapy

In the past years there has been increasing evidence for these non-canonical roles of RB1 alongside its canonical function. Whereas the canonical model refers to the regulation of E2F transcription factors to regulate cell cycle, the non-canonical pathway refers to the pathways in which RB1 promotes histone modification and regulates the chromosome structure in a distinct manner, and in part mechanistically independent from cell cycle regulation. Importantly, these non-canonical functions seem to provide explanations for differences in therapy response and targeted anticancer therapies of RB1-defective cancers.

Defective RB1 function can influence cellular response and the response to a variety of cancer treatment approaches (Knudsen and Knudsen, 2008). Among these, agents that serve to block cell proliferation, such as hormone receptor antagonist, depend on functional RB1 for efficacy. To the same degree, multiple reports provide evidence that RB1-deficient cells are more sensitive to DNA-damaging agents (Mayhew et al., 2004, Zagorski et al., 2007).

Analysis of NSCLC provided a detailed understanding into the contribution of RB1 pathway components in cancer treatment response. Deregulated proliferation is influenced by mutations in genes encoding CDKN2A, cyclin D, CDK4/6 and RB1, and rapid proliferation induces sensitivity to DNA damage agents (Branzei and Foiani, 2008). RB1 loss itself, correlates with improved response to DNA damage agents (Zagorski et al., 2007) and longer patient survival (Cecchini et al., 2015, Ludovini et al., 2004).

Moreover, it has been shown that loss of RB1 expression, identified by immunohistochemistry, was strongly associated with improved response to neoadjuvant chemotherapy in estrogen receptor (ER)-positive and ER-negative breast cancers (Witkiewicz AK, 2012).

Molecular profiling of prostate cancer (PC) and metastatic PC have defined a spectrum of recurrent genomic alterations that include loss of TP53 and RB1. Importantly, the combination of TP53 and RB1 loss is seen in nearly 100 % of neuroendocrine PC that lack androgen receptor (AR) activity. Consequently, these tumours do not respond to AR-
targeted therapy (Ku et al., 2017, Mu et al., 2017). In a recent study, it has been shown, that cells which adapted to survive without RB1 and TP53 did not express neuroendocrine genes and the AR program remained active, though the overall AR transcriptional output was diminished (Nyquist et al., 2020).

The tumour suppressor roles of TP53 and RB1 are well-known to be affected across a wide range of human malignances and the key mechanisms responsible for suppressing tumourigenesis have been well characterized. However, it is not clear if the general function of TP53 or RB1 are responsible for PC progression and treatment resistance. In fact, while PC tumour cells with combined loss of TP53 and RB1 appeared resistant to a large number of therapeutic agents, they were sensitive to a combination of a PARP and ATR inhibitors. Previous studies have shown synergistic effects of PARP and ATR inhibition in the context of homology-directed DNA repair deficiency and the potential for ATR inhibition to overcome resistance to PARP inhibition (Wengner et al., 2020). The combined loss of TP53 and RB1 promotes unrestricted proliferation, loss of G1/S checkpoint controls, and replication stress. Recent studies suggest that PARP trapping onto damaged DNA and the further induction of replication stress may contribute to the cytotoxic activity of specific PARP inhibitors, and in this context, abrogating ATR function may be particularly effective (Maya-Mendoza et al., 2018). Here, the combination therapy repressed the tumour growth in vivo and in vitro (Nyquist et al., 2020). The potential vulnerabilities determined by the combined loss of TP53 and RB1 might confer an attractive therapeutic approach for clinical evaluations (Fig. 1.8).
Figure 1.8: Cancer types harbouring alterations in the RB1 gene TCGA dataset (cBioPortal https://www.cbioportal.org).
1.7 ATR, the essential kinase involved in DNA replication stress

ATR is the apical DNA replication stress response kinase, phosphorylating many substrates in response to DNA damaging agents such as UV (Matsuoka et al., 2007). Unlike ATM and DNA-PKcs, ATR is essential for proliferating cells, which made it difficult to study its functions until the recent development of selective small molecule ATR kinase inhibitors (Fokas et al., 2014).

In contrast to ATM and DNA-PKcs, which respond primarily to DSBs, ATR is activated by a much wider range of genotoxic stresses. This is because ATR is recruited via its partner protein ATR-interacting protein (ATRIP) to extended tracts of ssDNA coated with the ssDNA-binding protein complex RPA (replication protein A) (Zou and Elledge, 2003). RPA-coated ssDNA is generated by nucleolytic processing of various forms of damaged DNA (Rundle et al., 2017) or by helicase-polymerase uncoupling at stalled replication forks (Byun et al., 2005).

ATR recruitment to RPA-ssDNA is not sufficient for optimal activation but requires the presence of an ATR activator, TopBP1 (DNA topoisomerase 2-binding protein 1). TopBP1 has an ATR-activation domain that stimulates ATR kinase activity (Kumagai et al., 2006) through contacts with both ATRIP and the C-terminal PRD (PIKK regulatory domain) in ATR (Mordes et al., 2008). One important interaction of TopBP1 is its binding to the C-terminal tail of the RAD9 subunit of the RAD9-RAD1-HUS1 (9-1-1) complex (Delacroix et al., 2007) The 9-1-1 complex has a ring structure that is loaded onto RPA-ssDNA/dsDNA junctions by the RAD17-RFC complex (Ellison and Stillman, 2003).

A key ATR function is to phosphorylate and activate the protein kinase CHK1 (Guo et al., 2000), which in turn phosphorylates and inhibits CDC25A (Bartek et al., 2004).

The CDC25 phosphatase family has three members: CDC25A, CDC25B and CDC25C. Although the catalytic domains of these phosphatases are well conserved, their regulatory domains are greatly diverse. While CDC25B and CDC25C promote G2/M progression by primarily dephosphorylating CDK1, CDC25A plays more extensive role in assisting both G1/S and G2/M progression by dephosphorylating CDK4, CDK6, as well as CDK2 and CDK1. More importantly, overexpression of CDC25A has been frequently documented in multiple cancer cell lines, which is highly associated with the malignancy and poor prognosis in cancer patients.
Chapter one: Introduction

Figure 1.9: ATR activation after different stress induced factors
Exogenous and endogenous factors trigger formation of single-stranded DNA (ssDNA) breaks and/or stalling of replication forks, and recruit replication protein A (RPA), which commence the replicative stress response. RPA-coated ssDNA recruits the assembly of ataxia telangiectasia and RAD17 protein complexes. Ataxia telangiectasia and Rad3 related (ATR) kinase phosphorylates checkpoint kinase 1 (CHK1), which prevents G1/S and G2/M transition through suppression of cell division cycle 25 (CDC25A) and activation of cyclin dependent kinase (CDK1/2), respectively, leading to cell cycle arrest. CHK1 and CHK2 phosphorylate and stabilize p53, p21 and Rb, which maintain cell cycle arrest. Furthermore, CHK1 phosphorylates and activates the negative regulator of CDK1/2, WEE1-like protein kinase (WEE1). Adapted from (Ngoi et al., 2020).
The importance of the ATR-CHK1-CDC25A axis for cell survival is highlighted by the fact that lethality associated with ATR or CHK1 inhibition can be overcome by CDC25A inhibition (Ruiz et al., 2016) (Fig. 1.9).

ATR lies upstream of CHK1 and phosphorylates numerous factors including Werner syndrome ATP-dependent helicase (WRN), SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A-like protein 1 (SMARCAL1), and Fanconi anemia complementation group I (FANCI), which together help preserve replication fork stability, control cell cycle progression and facilitate the removal of DNA adducts that block fork progression (Pugliese et al., 2019, Yeom et al., 2019). Other direct substrates of ATR include RPA, MCM2, p53 and many other factors that play roles in replication fork progression, DNA repair and control of the cell cycle. In summary, ATR has a crucial role in preserving genomic integrity throughout the cell cycle and is therefore essential for cell survival (Gamper et al., 2013). ATR controls cell cycle arrest throughout S phase of the cell cycle (Reinhardt and Yaffe, 2009), in addition to playing a role in the G2/M phase checkpoint (Min et al., 2017) (Fig 1.9).

ATR dysregulation disrupts a broad range of cellular processes. Recent reports suggest that cells with impaired HR activities, mutations in TP53 gene or other DNA repair proteins are specifically sensitive to ATR inhibitors (Reaper et al., 2011).

### 1.7.1 ATR inhibitors

The broad role of ATR in DNA damage sensing, fork protection, and the DNA damage restriction checkpoint lends to its immense potential for inhibition in cancer therapy. Four potent and highly selective ATR inhibitors (M6620, AZD6738, BAY1895344, and M4344) are currently in clinical development.

Berzosertib (M6620, formerly VX-970, Merck) is a first-in-class ATR inhibitor. In a phase I study of advanced solid tumours treated with berzosertib ± carboplatin, berzosertib monotherapy was well tolerated with no high-grade toxicities or dose-limiting toxicities (DLT) (Yap et al., 2020). However, when combined with chemotherapy, frequent haematological cancers DLT necessitated dose interruptions and reductions. Berzosertib treatment was associated with a reduction in phosphorylated-CHK1 levels on paired tumour biopsies pre- and post-treatment, confirming its expected pharmacodynamic effect. Although limited monotherapy activity was reported (objective response rate of 9 %), complete response exceeding 19 months was noted in a colorectal cancer patient with ATM loss, and a durable partial response was seen in a patient
with BRCA1-mutant but platinum- and PARP inhibitors-resistant high grade serous carcinoma harbouring somatic mutation in TP53 gene. In another phase I study of berzosertib plus veliparib and cisplatin, a durable partial response occurred in a BRCA-wildtype platinum-resistant patient with ovarian cancer. Currently, combinatorial approaches with chemotherapy are under investigation in high grade serous carcinoma. Recently, a randomized phase II study of gemcitabine ± berzosertib in platinum-resistant high grade serous carcinoma has reported improvements in progression-free survival for the combination arm. Interestingly, progression-free survival benefit occurred only in the subgroup of patients who had a short platinum-free interval of less than three months; no progression-free survival benefit was seen in patients with a longer platinum-free interval (Konstantinopoulos et al., 2020).

Ceralasertib (AZD6738, AstraZeneca) is a selective and potent oral ATR inhibitor. As monotherapy, ceralasertib showed an objective response rate of 7% in a phase I study of advanced solid tumours; however, 48% of patients achieved stable disease, with durable responses noted (NCT02223923). Continuous daily dosing was not tolerated due to cumulative myelosuppression, thus different dosing schedules are being explored in future expansion cohorts which will focus on selecting patients with known homologous recombination deficiency. Study 4 was a phase I trial combining ceralasertib with carboplatin or olaparib in advanced solid tumours. Unsurprisingly, the combination of ceralasertib with carboplatin was not well tolerated due to myelosuppression. In this cohort, 3/37 responses were seen, including one patient with ATM-mutant clear cell ovarian cancer (NCT02264678) (Ngoi et al., 2020).

BAY 1895344 is a new and potent ATR inhibitor developed by Bayer. BAY 1895344 has been extensively studied in cancers with DNA repair deficiency. The in vivo anti-tumour efficacy of BAY 1895344 in combination with carboplatin was investigated in the IGROV-1 ovarian cancer model (Wengner et al., 2020). BAY 1895344 is currently under clinical investigation in patients with advanced solid tumours and lymphomas (NCT03188965).
## Table 1.1: ATR inhibitor-based clinical trials

<table>
<thead>
<tr>
<th>ATRi</th>
<th>Target cancer</th>
<th>Treatment</th>
<th>Phase</th>
<th>Biomarker selection</th>
<th>Trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>M6620 (formerly VX-970)</td>
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<td>Alone or with carboplatin</td>
<td>I</td>
<td></td>
<td>NCT03309150</td>
</tr>
<tr>
<td></td>
<td>Advanced solid tumour</td>
<td>Gemcitabine, cisplatin, etoposide or carboplatin</td>
<td>I</td>
<td>TP53 mutation of ATM loss</td>
<td>NCT02157792</td>
</tr>
<tr>
<td></td>
<td>Advanced solid tumour</td>
<td>Irinotecan</td>
<td>I</td>
<td></td>
<td>NCT02595931</td>
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<tr>
<td></td>
<td>Small-cell cancers</td>
<td>Topotecan</td>
<td>I/II</td>
<td>DDR pathway mutations</td>
<td>NCT02487095</td>
</tr>
<tr>
<td></td>
<td>Urothelial carcinoma</td>
<td>Cisplatin or gemcitabine</td>
<td>I/II</td>
<td>P53, p21 and ERCC2 mutations</td>
<td>NCT02567409</td>
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<tr>
<td></td>
<td>Ovarian cancer</td>
<td>Carboplatin + gemcitabine</td>
<td>I/II</td>
<td>DNA damage assay, HRR mutations</td>
<td>NCT02627443</td>
</tr>
<tr>
<td></td>
<td>Ovarian cancer</td>
<td>Gemcitabine</td>
<td>II</td>
<td></td>
<td>NCT02595892</td>
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<tr>
<td></td>
<td>mCRPC</td>
<td>Carboplatin ± docetaxel</td>
<td>II</td>
<td></td>
<td>NCT03517969</td>
</tr>
<tr>
<td></td>
<td>Advanced solid tumour</td>
<td>Cisplatin + veliparib XRT</td>
<td>I</td>
<td>DNA damage and apoptotic assay</td>
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<tr>
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<td>Cisplatin + XRT</td>
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<td>DNA damage assay</td>
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<td></td>
<td>Brain metastases</td>
<td>Whole brain XRT</td>
<td>I</td>
<td>ATR, CHK1, RAD51, cyclin E, DNA-PK assay</td>
<td>NCT02589522</td>
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<td>M4344 (oral)</td>
<td>Advanced solid tumour</td>
<td>Carboplatin, gemcitabine or cisplatin</td>
<td>I</td>
<td></td>
<td>NCT02278250</td>
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<tr>
<td>AZD6738 (oral)</td>
<td>CLL, PLL or B-cell lymphoma</td>
<td>Alone</td>
<td>I</td>
<td>ATR targeted inhibition biomarker</td>
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<tr>
<td></td>
<td>NHSCC</td>
<td>Alone</td>
<td>I</td>
<td>TH1/INFγ gene and TIL state</td>
<td>NCT03022409</td>
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<tr>
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<td>Refractory CLL</td>
<td>Acalabrutinib</td>
<td>I</td>
<td></td>
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<td></td>
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<td>Paclitaxel</td>
<td>I</td>
<td></td>
<td>NCT02630199</td>
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<tr>
<td></td>
<td>Advanced solid tumour</td>
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<td>I/II</td>
<td>ATM deficiency</td>
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<td>Olaparib</td>
<td>II</td>
<td>HRR mutations</td>
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<td></td>
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<td>Olaparib</td>
<td>II</td>
<td></td>
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<td>SCLC</td>
<td>Olaparib</td>
<td>II</td>
<td></td>
<td>NCT03428607</td>
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<tr>
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<td>Olaparib</td>
<td>II</td>
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<tr>
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<td>I</td>
<td></td>
<td>NCT02223923</td>
</tr>
<tr>
<td>BAY1985344</td>
<td>Solid tumour and lymphoma</td>
<td>Alone</td>
<td>I</td>
<td></td>
<td>NCT03188965</td>
</tr>
</tbody>
</table>

1.8 Mechanisms for stalled replication fork stabilisation

During proliferation, the key task of a cell is to duplicate its genome and pass it to the two daughter cells. In human cells the DNA has to be completely and accurately replicated during each cell cycle which requires proper function of every replication fork travelling along the template DNA.

Even under normal conditions, this process is very vulnerable and is often challenged by endogenous DNA lesions (Ying et al., 2016). These impairments to replication progression lead to fork slowdown and/or stalling, termed replication stress, threaten a faithful genome replication (Cortez, 2015). When the replication stress is prolonged, stalled replication forks can undergo irreversible fork breakage, which eventually results in genome instability (Elvers et al., 2011, Petermann et al., 2010).

Cells have developed a multitude of fork protection mechanisms to reduce the genotoxic effects by stabilizing, repairing and restarting stalled forks (McGrail et al., 2018). A simple model to rescue a stalled fork consists in two stages: fork stabilization and fork restart.

Fork stabilization prevents forks collapsing into DSBs, thus increasing their chance to recover. Stabilised forks sequentially undergo RPA mediated ssDNA protection, leading to ATR-mediated checkpoint activation as previously described (section 1.7), then RAD51 mediated fork reversal and suppression of nucleolytic fork degradation (Petermann et al., 2010). Replication checkpoint activation acts as a regulator of many cellular events that are required for fork stabilization.

1.8.1 ssDNA protection

Stalled replication forks are characterised by extensive ssDNA, generated by helicase uncoupling; ssDNA is very unstable and therefore needs to be protected. The first responder to ssDNA is RPA, which is an ssDNA-binding protein (Fan and Pavletich, 2012). RPA has a higher ssDNA affinity and abundance compared to other ssDNA-binding proteins like RAD51, therefore its recruitment to ssDNA occurs earlier than these other proteins (Fan and Pavletich, 2012). Similarly, at stalled replication forks, RPA is quickly loaded on the ssDNA to prevent formation of secondary structures that may block further fork processing (Fan and Pavletich, 2012). Another main function of RPA is to send out stress signals by activating the replication checkpoint through recruiting ATR. Furthermore, RPA binding to ssDNA recruits the fork remodelling protein SMARCAL1,
which reverts stalled replication forks avoiding fork collapse (Ciccia et al., 2009). Since the intracellular RPA pool is finite, ssDNA protection also relies on maintaining the RPA pool by limiting formation of ssDNA itself. The ATR/CHK1-dependent replication checkpoint that leads to global inhibition of origin firing is the major pathway that regulates this task. Inhibition of either ATR or CHK1 causes sustained origin firing and prevents recovery of DNA replication progression (Petermann et al., 2010). In the context of replication stress, ATR inhibition is devastating for ongoing origin firing producing excessive ssDNA that reduces the intracellular RPA pool, leaving stalled replication forks unprotected and eventually leads to fork collapse (Toledo et al., 2013).

In brief, ssDNA protection by RPA is the prerequisite for stabilization of stalled replication forks, which largely depends on checkpoint activation and suppressed origin firing to preserve the RPA pool. However, RPA protection is not sufficient to rescue stalled forks, as it was shown that RPA overexpression only delays and does not prevent fork collapse in cells under hydroxyurea treatments (Dungrawala et al., 2015).

1.8.2 Protective effects of fork reversal

Replication fork reversal mostly has three protective effects: first, backtracking and annealing of the nascent DNA strands, preventing replication fork progression across DNA lesions and consequently avoiding replication fork collapse (Ray Chaudhuri et al., 2012). Second, replication impediments can be repositioned back onto the double-strand template DNA after fork reversal, allowing extra time and room for the repair machineries to remove those impediments (Neelsen and Lopes, 2015). Third, fork reversal creates a Holliday junction (HJ) with a one-ended DSB, which can be recognised by HR resolvases such BLM (Bloom syndrome protein) (Larsen and Hickson, 2013) and by DSB repair factors like BRCA2 (Ying et al., 2012) and DNA-PKcs. Recruitment of these proteins is essential for fork stabilisation and restart however they do not necessarily carry out the same functions as they do in DSB or in HJ resolution.

*In vitro* studies have revealed the ability of two recombinase proteins, RAD51 and RAD54, to reverse modelling of stalled replication forks (Bugreev et al., 2011). In a recent study, RAD51 has been demonstrated as an *in vivo* mediator of fork reversal in response to a range of genotoxic treatments (Zellweger et al., 2015).

The SNF2 family proteins, including SMARCAL1, ZRANB3 and HLTF, are also important fork reversal enzymes in human cells. They promote fork reversal to stabilise stalled replication forks, preventing genome instability (Ciccia et al., 2012).
Figure 1.10: Replication fork remodelling and restart during replication stress

A) Following fork stalling and uncoupling, the ssDNA-binding replication protein A (RPA) is partially replaced by the recombinase RAD51, leading to the formation of a metastable RAD51 filament (short stretches of RAD51 monomers, separated by residual RPA molecules). Along with PCNA polyubiquitylation (PolyUb) and recruitment of the specialized DNA translocases SMARCAL1 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A-like 1), ZRANB3 and helicase-like transcription factor (HLTF), these metastable RAD51 filaments promote fork reversal by yet-elusive mechanisms. Following reversal, stable RAD51 filaments on the regressed arm protect reversed forks from nucleolytic degradation. RAD51 binding upstream and downstream of fork reversal is modulated by the anti-recombinase activities of RecQ-like helicase 5 (RECQ5) and F-box helicase 1 (FBH1), which have different effects on fork remodelling. The accumulation of ssDNA also leads to ataxia telangiectasia and Rad3-related (ATR) activation, which locally limits SMARCAL1-mediated fork reversal, but globally allows for fork remodelling and slowing also at unchallenged forks.

B) Following the repair of lesions on the template strand, reversed forks can be restarted by RECQ1-mediated reversed-branch migration, which is transiently inhibited by poly(ADP-ribose) polymerase 1 (PARP1)-mediated parylation. Alternatively, reversed forks can be restarted by DNA2–WRN-mediated DNA unwinding and controlled resection. DNA-directed primase/polymerase protein (PrimPol) may promote DNA synthesis restart. Adapted from (Mijic et al., 2017).
Although replication fork reversal applies protective effects on stalled forks, it also carries numerous risks. In fact, regressed forks are the entry points for various cellular nucleases that mediate stalled fork degradation (Mijic et al., 2017). Limited resection of regressed forks does not have pathological consequences but promotes HR-dependent fork recovery (Thangavel et al., 2017). However, when the controlling mechanisms are compromised, excessive nuclease activities will cause genotoxic consequences leading to chromosome aberrations and cell death (Mijic et al., 2017) (Fig. 1.10).

1.8.3 Protection against nucleolytic degradation
Stalled replication forks are characterised by exposed DNA ends in the form of ssDNA or dsDNA, which makes them vulnerable to various cellular nucleases including MRE11, CtIP, DNA2 and EXO1 (Kolinjivadi et al., 2017). It is established that MRE11 and CtIP cooperate to perform short-range resection, while EXO1 and DNA2 act independently in 5’-3’ long-range processing (Symington, 2016). All these nucleases are important players in generating HR substrates during DSB repair and they are tightly regulated to determine the repair pathway choice between HR and NHEJ (Tomimatsu et al., 2014). Similarly, the nuclease activities at the replication stress sites are also severely controlled to prevent excessive fork degradation that will destabilise the stalled forks. Multiple pathways take part in this field, including BRCA2-RAD51 axis and the Fanconi anaemia (FA) pathways, loss of which will cause fork breakage and genome instability (Michl et al., 2016).

1.9 The ATR-CHK1 signalling axis
As mentioned previously, once activated, the ATR and CHK1 kinases phosphorylate a multitude of substrates, together they cause a wide range of DNA metabolism alterations and blocking the cell cycle progression. The ATR/CHK1 checkpoint is highly conserved and is activated upon replication stress to preserve genome instability at stalled replication forks (Iyer and Rhind, 2017).

The activation of the ATR/CHK1 pathway has already been discussed in section 1.4.3. Once activated, the ATR/CHK1 checkpoint modulates both replication and transcription programmes.

In human cells ATR activity is needed for efficient transcription factor E2F1-dependent expression of ribonucleoside-diphosphate reductase subunit M2 (RRM2) (D’Angiolella et al., 2012). ATR-mediated RRM2 expression may be especially important in the early
The stages of S phase, where loss of ATR leads to the formation of greater levels of ssDNA. ATR also boosts ribonucleotide reductase (RnR) activity when DNA damage levels (and consequently the need for dNTPs) are high by preventing CDK and cyclin F-dependent RRM2 degradation (Saldivar et al., 2017).

Although transcription regulation is fundamental for stalled fork stabilization, it takes effect in a delayed manner.

For timely protection of stalled forks, posttranslational modification is also employed by the ATR/CHK1 checkpoint. First of all, ATR/CHK1 signalling regulates origin firing. In proliferating cells, replication origins are licensed during G1 phase. During unperturbed S phase, only 10% of licensed origins fire to initiate DNA replication, while the bulk remain dormant through S phase and are replicated passively by other forks (Ge and Blow, 2010). This tight control on origin firing is mediated by ATR/CHK1 signalling, which promotes replication progression by balancing the number and the velocity of replication fork progression (Petermann et al., 2010). Under replication stress, the regulation of origin firing becomes more critical, because excessive or continued origin firing will generate an excess of ssDNA, exhausting the RPA pool, which eventually will cause replication fork collapse (Toledo et al., 2013). Remarkably, and whilst suppressing global origin firing, the replication checkpoint seems to promote local origin firing in the vicinity of stalled forks, which probably allows the completion of replication by fork conjunction.

Second, ATR/CHK1 signalling controls DNA remodelling. As mention in section 1.5.2, an important configurational change to stalled replication forks is fork reversal, which prevents DSB formation caused by replication runoff or endonuclease cleavage (Ray Chaudhuri et al., 2012). However, excessive fork remodelling can also lead to DSBs due to aberrant nucleolytic processing. The most studied fork remodeler regulated by checkpoint activation is SMARCAL1, which has been shown to regress stalled forks both in vivo and in vitro. At the stalled forks ATR regulates SMARCAL1 activity by installing both inhibitory phosphorylation on Ser652 and stimulatory phosphorylation on Ser889 (Carroll et al., 2014). In fact, abrogating phosphorylation of either site causes genome instability (Carroll et al., 2014).

Third, ATR/CHK1 signalling might maintain replisome stability. Replisome stability can be described as the stable association of the replisome components with the stalled fork. Since the final goal of fork stabilization is to restore replisome integrity and function, it is reasonable to assume that the replication checkpoint plays a role in maintaining the
replisome stability. Some studies report decreased abundance of replisome components at stalled forks when the replication checkpoint in not active, while others show that replisome stability was not regulated by the checkpoint. The inconsistency is most likely to refer to different methodologies used to analyse replisome proteins associated with stalled forks (Iyer and Rhind, 2017, Cortez, 2015). Early studies using ChIP-PCR were designed to focus on proximal regions of early origins, not considering that early firing forks still progress a distance from their origins under replication stress. This might explain the reason for reduced replisome components in the absence of checkpoint activity. While the role of the replication checkpoint in replisome stability is still debated in yeast, in human cells evidence favours the hypothesis that the replication checkpoint does not affect replisome stability at stalled replication forks, as shown in a recent study using the iPond (isolation of proteins on nascent DNA) technique where no significant change was found in replisome protein abundance after fork stalling (Dungrawala et al., 2015).

To conclude, replication checkpoint activation induces an emergency mode for cells under replication stress that promotes stabilization of stalled replication forks to preserve genome stability.

1.10 Aims of this research

In previous published work it has been shown that RB1 loss drives the accumulation of DSBs, leading to the hypothesis that RB1 may regulate the DSB repair pathways. Indeed, RB1 has been reported to initiate both HR and NHEJ repair (Cook et al., 2015, Velez-Cruz et al., 2016, Horvath et al., 2017).

Cells that are unable to conduct repair via a specific repair route exhibit higher vulnerability towards inhibition of alternative DSB repair routes. This could be exploited when designing novel therapies, including opportunities for personalised, genotype-informed treatment.

Work undertaken in this thesis seeks to investigate whether repair hyper-reliance exists in cancers with RB1 loss towards inhibition of DDR pathways. The overarching hypothesis is that existing repair defects and liabilities in RB1-deficient cells might cause inability to cope with cell inherent genomic stress, and that increased need for DNA repair enzymes may exist in these tumours.
This project gathers mechanistic information relating to ATR pathway inhibition in *RB1*-mutant tumours, arguing that *RB1* mutation status should be considered in the design of trials involving ATR inhibitors.
CHAPTER TWO

Material and Methods
2 Material and Methods

This section contains all the methods used for the experiments within the thesis and the materials required to carry them out. Buffers and solutions are referred to by their names only in the methods sections, details of their constituents can be found at the end of this chapter. All the methods were carried out at room temperature unless otherwise stated.

2.1 Cell culture

2.1.1 Maintenance of cell lines

All cell lines used in this thesis were bought from ATCC and Mycoplasma certified. Cells were grown in different culture media (see Table 2.1) in 10 cm dishes at 37 °C in 5 % CO₂ in a humidified incubator. When cells reached ~ 80 % confluency, they were trypsinised and split 1:3 into a new dish. Cells were passaged in this way no more than twenty times after which they were disposed of and new cells were brought out from liquid nitrogen stored stocks.

2.1.2 Freezing-down cells

To maintain stocks of low-passage cells, batches of early passage cells were frozen. Cells were expanded especially for this purpose, for one passage, then trypsinised in 1 ml of trypsin for 3 minutes at 37 °C. Four cell volumes of media were added to deactivate the trypsin and cell number was determined using a haemocytometer. Cells were subsequently transferred to a 15 ml Falcon tube. Cells were then centrifuged for 5 minutes at 1200 rpm. Cell pellets were resuspended in FCS containing 10 % dimethyl sulfoxide (DMSO) (SIGMA) at a concentration of 1-2 million cells/ml. 1 ml of cell solution was transferred into cryovial tubes (SIGMA), placed at -80 °C and 24 hours later was transferred to liquid nitrogen for long term storage.

2.1.3 Cell counting

Cells numbers were determined using a haemocytometer. The number of cells in four quadrants was counted and an average generated. This average was multiplied to give the total number of cells/ml.
### Table 2.1: Cell lines used

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Medium</th>
<th>Cells seeded per 96-well</th>
<th>Tumour type</th>
<th>RB1 status</th>
<th>TP53 status</th>
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<td>RPMI</td>
<td>3000</td>
<td>Lung (NSC)</td>
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<td>mutated</td>
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<td>3000</td>
<td>Brain (GS)</td>
<td>mutated</td>
<td>mutated</td>
</tr>
<tr>
<td>BT-549</td>
<td>DMEM</td>
<td>3000</td>
<td>Breast</td>
<td>mutated</td>
<td>wild type</td>
</tr>
<tr>
<td>SF295</td>
<td>DMEM</td>
<td>3000</td>
<td>Brain (GS)</td>
<td>wild type</td>
<td>wild type</td>
</tr>
<tr>
<td>HCC70</td>
<td>DMEM</td>
<td>3000</td>
<td>Breast</td>
<td>wild type</td>
<td>mutated</td>
</tr>
<tr>
<td>DMS 114</td>
<td>Waymout</td>
<td>4000</td>
<td>Lung (SC)</td>
<td>wild type</td>
<td>mutated</td>
</tr>
<tr>
<td>HCT-116</td>
<td>DMEM</td>
<td>3000</td>
<td>Colorectal</td>
<td>wild type</td>
<td>wild type</td>
</tr>
</tbody>
</table>

#### 2.1.4 Cell Seeding in 6-well plates

When cells were required for protein analysis, siRNA or shRNA transfection or clonogenic assays, they were trypsinised and counted as described in section 2.1.3. A cell count of $2 \times 10^5$ cells per ml of media was made, 1 ml of cell solution was transferred in one well and 1 ml of media was added to have 2 ml total each well of a 6-well plate(s).
Chapter Two: Material and Methods

2.1.5 Cell seeding in 96-well plates

When cells were required for viability assay or cell fate assay cells were trypsinised and count as described in section 2.1.3. A cell suspension of 3000 to 7000 cells (see Table 2.1) per 150 µl of media was made and 150 µl of cell solution was pipetted into wells of a 96-well plate(s). If cells were to be siRNA transfected, the oligo complex would be added to 96-well plates before cells were added, see section 2.2.2.

Table 2.2: RB1 mutations

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Zygosity</th>
<th>†Gene sequence</th>
<th>†Protein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCI-H2009</td>
<td>homozygous</td>
<td>c.1696_2787del1092</td>
<td>p.?</td>
</tr>
<tr>
<td>NCI-H446</td>
<td>homozygous</td>
<td>c.940-2A&gt;T</td>
<td>p.?</td>
</tr>
<tr>
<td>NCI-H82</td>
<td>homozygous</td>
<td>c.980-2A&gt;T</td>
<td>p.?</td>
</tr>
<tr>
<td>NCI-H69</td>
<td>homozygous</td>
<td>c.2242G&gt;T</td>
<td>p.E748</td>
</tr>
<tr>
<td>NCI-H146</td>
<td>homozygous</td>
<td>c.2548C&gt;T</td>
<td>p.Q850</td>
</tr>
<tr>
<td>SaOS-2</td>
<td>homozygous</td>
<td>c.2212_2787del576</td>
<td>p.?</td>
</tr>
<tr>
<td>SF539</td>
<td>homozygous</td>
<td>c.346_349del</td>
<td>p.T116Lfs*8</td>
</tr>
<tr>
<td>BT-549</td>
<td>homozygous</td>
<td>c.265_607del343</td>
<td>p.?</td>
</tr>
</tbody>
</table>

†For a description of the sequence variation nomenclature please refer to: (den Dunnen and Antonarakis, 2000).

2.1.6 Drug treatment

Appropriate concentration for all the inhibitors used in this study (see Table 2.2) were determine experimentally. Drugs were dissolved in DMSO and stored as 10 mM stock at -80 °C in aliquots. Immediately prior to use they were diluted in warm medium to generate a 10 X concentrated working stock and to be added to the cells as appropriate. In all experiments, a vehicle control was generated using a final concentration of 0.1 % of DMSO without drug diluted in warm medium.
Table 2.3: Compounds used

<table>
<thead>
<tr>
<th>Compound</th>
<th>Company</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZD6738</td>
<td>BioVision</td>
<td>ATR</td>
</tr>
<tr>
<td>KU60019</td>
<td>Selleckchem</td>
<td>ATM</td>
</tr>
<tr>
<td>Olaparib</td>
<td>APExBIO</td>
<td>PARP</td>
</tr>
<tr>
<td>NU7441</td>
<td>TOCRIS</td>
<td>DNA-PK</td>
</tr>
<tr>
<td>VE821</td>
<td>Cayman</td>
<td>ATR</td>
</tr>
<tr>
<td>VE822</td>
<td>Selleckchem</td>
<td>ATR</td>
</tr>
<tr>
<td>BAY1895344</td>
<td>Selleckchem</td>
<td>ATR</td>
</tr>
<tr>
<td>Hydroxyurea</td>
<td>SIGMA</td>
<td>ribonucleoside diphosphate reductase</td>
</tr>
</tbody>
</table>

2.2 siRNA transfection of cells

Lyophilised siRNA oligonucleotides (oligos) (Table 2.3) were purchased in tube from Dharmaco. Tubes were spun in a centrifuge at 5000 rpm for 5 minutes to collect the content at the bottom of the tube. 1 X siRNA suspension buffer was added to generate a final concentration of 20 µM siRNA. siRNA oligos were stored at -80 °C.

2.2.1 siRNA transfection of cells in 6-well plates

This transfection was carried out the day after the cell seeding, see section 2.1.4. For one well of a 6-well plate, 2.5 µl of 20 µM siRNA, 9 µl of Hiperfect lipid transfection reagent (QIAGEN) and 488.5 µl of serum-free media was mixed and left at room temperature for 20 minutes to form siRNA-lipid complexes. The 500 µl of media and siRNA-lipid mix was then added to the well containing cells in 2 ml of medium, resulting in a final siRNA concentration of 2 nM. Cells were incubated in siRNA for 24 or 48 hours before further treatment.
2.2.2 siRNA reverse transfection of cells in 96-well plates

This transfection was carried out on the same day as cells were seeded and cells were seeded on top of oligo-liquid complexes. Essentially as described in section 2.2.1, a mix of serum-free medium was prepared with 2 nM final concentration of siRNA, a part was prepared a mix of serum-free medium with Hiperfect. First was added 10 µl of the mix with oligos in each well of the 96-well plate then on top was added 40 µl of the mix with Hiperfect and incubated for 20 minutes. A mix of cells (60 µl) to be transfected were added on top of the lipid-oligo complex and incubated for 36 hours before further treatment.

2.3 Plasmids involved

The plasmids involved in this study include lentiviral construct expression shRNA (Table 2.2).

<table>
<thead>
<tr>
<th>Gene name (oligo number)</th>
<th>Gene accession number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>*GIPZ Human RB1 clone #1</td>
<td>V2LHS_130606</td>
<td>TAAGTTACATGTCCTTTTC</td>
</tr>
<tr>
<td>*GIPZ Human RB1 clone #2</td>
<td>V2LHS_130608</td>
<td>TTAATCTGAAATGAAATCAC</td>
</tr>
<tr>
<td>*GIPZ Human RB1 clone #6</td>
<td>V2LHS_340825</td>
<td>TAAATCTGACATAATGCA</td>
</tr>
<tr>
<td>^SMARTpoolsiRNA RB1</td>
<td>D-003295-05</td>
<td>GAAAGGACAUGUGAAGCUUA</td>
</tr>
<tr>
<td>^SMARTpoolsiRNA RBL1</td>
<td>D-003298-05</td>
<td>CAAGAGAGUUUGUGGCAUA</td>
</tr>
<tr>
<td>^SMARTpoolsiRNA RBL2</td>
<td>D-003299-05</td>
<td>GAGCAGAGCUUAAUCGAAU</td>
</tr>
</tbody>
</table>

* shRNA constructs
^ siRNA oligos
2.3.1 Short hairpin constructs and viral infection
The lentiviral vector DNA was transfected into HEK293 packaging cells ($1 \times 10^6$ cells). Cells were transfected with psPAX2 (gag-pol expressor), pMDG (VSV-G expressor) and a lentiviral construct using calcium phosphate (Promega, Southampton, UK), accordingly to the manufacturer's instruction. Cells were maintained in DMEM (Gibco-Thermo Fisher Scientific) supplemented with 10 % FBS in an incubator with 5 % CO$_2$ at 37 °C. Retrovirus containing supernatants were harvested 48 and 72 hours after transfection, pooled, filtered through a 0.45-μM membrane, and mixed with polybrene (SIGMA) to yield a final concentration of 4 %. Viral preparations were stored at -80 °C or used to infect cell lines of interest.

For lentiviral transduction, H1299 and DMS 114 were plated at $1 \times 10^5$ cells per 6 well plate 24 hours. For infection, the culture medium was replaced by polybrene-containing viral supernatant. Cells were incubated at 37 °C for 8 hours at which time a second aliquot of virus was added for a further 8 hours. After 16 hours, infected cell populations were subjected to selection for virus uptake. Cells were cultured in the presence of 2 μg/mL puromycin (SIGMA) for vectors encoding puromycin resistance. shRNA constructs used are from Dharmacon, Horizon. Cells were frozen after knockdown verification via immunoblotting. For each single experiment a new vial of cells was thawed.

2.4 Cell viability assay
To assay for cell viability following drug treatments of cells, the Resazurin (2.5 mM final concentration) (SIGMA) was used. Resazurin is a phenoxazine dye that is fluorescent, cell-permeable and redox-sensitive. Resazurin has a blue to purple colour (at pH > 6.5) and is used in microbiological, cellular and enzymatic assays because it can be irreversibly reduced to the pink-coloured and highly fluorescent Resofurin. Resofurin can be detected by fluorimetry with an excitation maximum at 530-570 nm and an emission maximum at 580-590 nm. Resazurin is reduced to Resofurin by aerobic respiration of metabolically active cells and it can be used as an indicator of cell viability. This assay was used to measure effects of different drug treatments of cells.
2.4.1 Obtaining cell viability values using Resazurin reagent

Cells were seeded with or without siRNA oligos depending on the experiment, see section 2.8.3. A plate containing 3000 to 7000 cells per well (see Table 2.1) were treated the day after the cell seeding with scaling concentration of different inhibitors. The drug dilutions were carried out in a new plate, the drugs were diluted accordingly to use in fresh medium and diluted 1:3 ratio in series dilutions. To treat the cells 10 µl of drug-medium mix was added to each well of the 96-well plate and incubated for 96 hours in a humidified incubator. At the end of the treatment time, 20 µl of Resazurin was added to the cells and incubated at 37 °C for 4 hours. The luminescence signal per well was recorded using a Varioscan Lux (ThermoFisher Scientific) plate reader. The luminescence signal per well was normalised on the luminescence signal of vehicle treated wells and IC_{50} values were calculated using GraphPad-Prism software.

2.5 Time-lapse microscopy with IncuCyte Zoom

To assess cell fate following drug treatments, cells were subjected to time-lapse microscopy using IncuCyte Zoom system (Essen BioScience, GmbH). To detect cell death SYTOX™ Green Nucleic Acid Stain (1 µM final concentration) (Thermo Fisher Scientific) or Propidium iodide (PI) (40 µg/ml) (SIGMA) were used. SYTOX™ Green is a dead cell stain which enters the cells upon loss of membrane integrity and binds the DNA, thereby acting as a counterstain that can be analysed when excited at 488 nm (green) and the emission captured at a peak of 523 nm. PI is a fluorescent agent which can stain cells and nucleic acids. PI binds the DNA when cells undergo cell death and has a fluorescent excitation maximum of 493 nm (blue-green) and an emission maximum of 636 nm (red). After binding DNA, the quantum yield of PI is enhanced 20-30 fold and the excitation/emission maximum of PI is shifted to 535 nm (green) / 617 nm (orange-red). The IncuCyte live cell imager was used to detect SYTOX™ Green fluorescence emission signal and the IncuCyte Zoom (Sartorius) was used to detect PI red fluorescence emission signal.
2.5.1 Obtaining cell death values using SYTOX™ or Propidium Iodide nucleic acid stain

Cells were seeded with or without siRNA oligos depending on the experiment, (see section 2.2.2). A plate containing 3000 to 7000 cells per well (see Table 2.1) were seeded in FluoroBrite DMEM media (Gibco-Thermo Fisher Scientific) and SYTOX™ Green or PI was added to the media (see section 2.4). Drugs treatments were carried out as described in section 2.3.1 and cells were observed using an IncuCyte live cell imager for 5 days (see section 2.4). The IncuCyte measures mean fluorescence intensity per image and values were compared per treatments and normalised on confluence percentage per image and plotted.

2.6 Clonogenic assay

To assess the proliferation capacity of cells after drug treatments, clonogenic assays were performed. Cells were seeded in 6-well plates (see section 2.1.4). 24 hours after the seeding cells were treated with drugs or vehicle (see section 2.6.6). After 48 hours of treatments the medium was replace with fresh medium and cells were left to grow for 10-12 days in the incubator. The cells were then fixed with 37 % formaldehyde and stained with 2 ml of crystal violet solution for 30 minutes and then washed by running water. The stained plates were allowed to dry for 24 hours then scanned. To quantify the colonies in each well, 1 ml of destain solution was used for 30 minutes in shaking. The coloured solution was then measured by absorbance at 590 nm. The data were analysed with Excel worksheet and plotted with GraphPad Prism.

2.7 DNA fibre technique

This technique allows the labelling of active DNA replication forks in vivo and the sequential incorporation of the two different halogenated nucleotides IdU (5-Iodo-2’-deoxyuridine) and CldU (5-Chloro-2’-deoxyuridine) into the nascent DNA strands, provides information about replication directionality.

Cell lines were plated 48 hours prior to treatment. To measure the replication rates, labelling with 25 μM CldU (SIGMA) was performed in media for 20 minutes. CldU media were then replaced with media containing 250 μM IdU (SIGMA) with or without drug treatments for 20 minutes. Subsequently, cells were washed and then scraped into
ice cold phosphate-buffered saline (PBS), counted, and diluted to a concentration of 5 \times 10^5 cells/ml. A drop of cell suspension (2 μl) together with spreading buffer (see Materials section 2.11) (7 μl) was put on a microscope slide (SuperFrost® Plus, VWR) and incubated 6 minutes to lyse the cells. The slide was then tilted so that DNA fibres could spread over the slide. Slides were air dried and the fibres were fixed on the slides with methanol/acidic acid (3:1) at room temperature. Slides were incubated with 2.5 HCl for 1 hour and then incubated with blocking solution (1 % BSA in PBS with 0.1 % Tween (SIGMA)) for 1 hour. Subsequently slides were stained with polyclonal rabbit anti BrdU (see Table 2.6) for CldU and monoclonal mouse anti BrdU (see Table 2.6) for IdU in blocking buffer over night at 4 °C. Primary antibodies were fixed with 4 % paraformaldehyde (ThermoFisher Scientific) for 10 minutes to avoid washing them away during washes. Secondary antibodies were goat anti-rabbit AlexaFluor 594 and goat anti-mouse AlexaFluor 488 incubated in blocking buffer for 2 hours at room temperature. Slides were mounted using Slow Fade mounting solution (Invitrogen) using large coverslips (VWR). DNA fibres were imaged using a high-resolution widefield microscope (DeltaVision). The lengths of CldU and IdU tracks were measured using the Fiji software (ImageJ) and micrometre values were converted into kilobase pairs using the conversion factor 1 μm = 2.59 kb.

2.8 Protein Analysis

2.8.1 Protein extraction from cells
Cells lysates were generated using Laemli sample buffer (see Material section 2.11) pre-heated at 100 °C, this procedure was carried out after siRNA transfection or drug treatments. Cell monolayers were washed with PBS, then 100 μl of lysis buffer was added to each well and cells were scraped using a plastic scraper. Hot laemli lysate buffer releases chromosomal DNA producing a viscous lysate. Therefore, it is necessary to remove the DNA. To do this, lysates were spun over a layer of glass wool packed into a 1.5 ml Eppendorf tube. Specifically, two Eppendorf tube were placed on top of each other, the top Eppendorf tube was to receive the glass wool plug while the bottom Eppendorf would serve to collect the lysate as it passes through the glass wool and a small hole created in its tip on the top tube. The viscous lysate containing DNA was transferred into their corresponding top Eppendorf tube and were spun 3 times in a centrifuge. The bottom
Eppendorf tube with the fluid lysate were stored in -80 °C and the top Eppendorf were disposed of. The protein concentration in the lysates was estimated by BCA assay (Fisher Scientific) and associated protein were analysed by immunoblotting.

2.8.2 Chromatin fractionation

Cells lysates were generated using Cytoskeletal (CSK) buffer (see Material section 2.11), this procedure was carried out after drug treatments. To obtain the Soluble fraction, cell monolayers in dishes were washed with PBS and kept on ice, then 400 µl of CSK buffer was added to each dish and cells were scraped using a plastic scraper. Samples were centrifuged 4 °C, 3000 rpm for 3 minutes and the supernatant collected in a new Eppendorf tube. The remaining pellet was washed with CSK buffer and centrifugated at 4 °C, 3000 rpm for 3 minutes then the supernatant was discarded, and the pellet lysed with SDS buffer to obtain the Chromatin fraction. To obtain the Total fraction cell monolayers were washed with PBS, then 400 µl of SDS buffer was added. The Eppendorf tubes with the fluid lysate were stored in -80 °C and the proteins were analysed by immunoblotting.

2.8.3 SDS-Polyacrylamide gel electrophoresis

Protein samples were prepared for resolution by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), by mixing 20 µg of each sample with 6X SDS loading buffer. Samples were supplemented with an appropriate volume of 1X SDS protein samples buffer and the appropriate volume was loaded onto a gel. In order to determine the molecular weights of proteins, molecular weight marker (Dual Colour Precision Plus Protein Standards from BioRad) was prepared in a similar way and 3µl of maker was loaded as well. Depending on the protein to detect, gels (see Table 2.4) at different percentage of acrylamide were cast in Mini-PROTEAN® glass plates (BioRad). The resolving gel was poured and overlaid with water-saturated butanol and allowed to set. The butanol was washed off before the staker gel was poured in and a 15-well comb was inserted to create wells in the staker gel as it sets. The gel was secured into a Mini-PROTEAN® Tetra System (BioRad) running apparatus and the area behind and under it filled with gel-running buffer (10% protein electrophoresis buffer, 10 % SDS in deionised water). Protein samples were loaded into each well of the gel, flanked by wells containing
molecular weight markers. A current of 150 V and 20 mA was passed through the gel until the smallest of the molecular weight markers reached the bottom of the gel.

### 2.8.4 Immunoblotting

Resolved proteins were transferred from the SDS-PAGE gel onto Nitrocellulose Blotting Membrane (Amersham™ Protran™ from GE healthcare) for immunoblot analysis. The membrane was soaked in transfer buffer and overlaid with the gel. The gel and membrane were sandwiched between two pieces of Whatman paper 3 M and porous pads, which had also been soaked in transfer buffer, and then placed into a transfer cassette. The cassette was placed into a transfer tank filled with transfer buffer and current of 100 V was passed through the membrane for 1.5 hours until proteins had transferred from the gel to the nitrocellulose membrane. Membranes were placed into blocking buffer for 30 minutes, probed with primary antibody in blocking buffer over night (see Materials 2.11), then washed three times in TBST washing buffer for 10 minutes. Following this, membranes were probed for 1 hour with Horse Radish Peroxidase (HRP) conjugated antibody (Invitrogen) or IRDye 680- or IRDye 800CW-conjugated (LI-COR) in blocking buffer, then washed three times for 10 minutes in TBST washing buffer. To detect the HRP-bound antibodies, membranes were developed using enhanced chemiluminescence (ECL) substrate mix (SuperSignal™ West Pico Chemiluminescent Substrate, Thermofisher Scientific) with detection of the emitted chemiluminescence using X-ray films (Hyperfilm™ MP, Amersham). Imaging and quantification were obtain using Odyssey near-infrared (NIR) imaging system (LI-COR Bioscience).
Table 2.5: Recipe for various SDS-PAGE gel percentage

<table>
<thead>
<tr>
<th>Components</th>
<th>16 %</th>
<th>14 %</th>
<th>12 %</th>
<th>10 %</th>
<th>Staker</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 M Tris, pH 8.8</td>
<td>3.75 ml</td>
<td>3.75 ml</td>
<td>3.75 ml</td>
<td>3.75 ml</td>
<td>-</td>
</tr>
<tr>
<td>Acrylamide (30 % w/v)</td>
<td>8 ml</td>
<td>7 ml</td>
<td>6 ml</td>
<td>5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>SQ Water</td>
<td>3.25 ml</td>
<td>4.25 ml</td>
<td>5.25 ml</td>
<td>6.25 ml</td>
<td>3 ml</td>
</tr>
<tr>
<td>20 (w/v) SDS</td>
<td>75 µl</td>
<td>75 µl</td>
<td>75 µl</td>
<td>75 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>15 µl</td>
<td>15 µl</td>
<td>15 µl</td>
<td>15 µl</td>
<td>7.5 µl</td>
</tr>
<tr>
<td>10 % (w/v) APS</td>
<td>150 µl</td>
<td>150 µl</td>
<td>150 µl</td>
<td>150 µl</td>
<td>75 µl</td>
</tr>
<tr>
<td>1.5 Tris, pH 6.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>480 µl</td>
</tr>
</tbody>
</table>

Table 2.6: Recipe for Tris-Acetate gel percentage

<table>
<thead>
<tr>
<th>Components</th>
<th>7 %</th>
<th>Staker</th>
</tr>
</thead>
<tbody>
<tr>
<td>3M Tris-Acetate pH7.0</td>
<td>675 µl</td>
<td>470 µl</td>
</tr>
<tr>
<td>30% Acrylamide</td>
<td>2.33 ml</td>
<td>700 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>7 ml</td>
<td>5.83 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>12.5 µl</td>
<td>8.75 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>47.5 µl</td>
<td>33.25 µl</td>
</tr>
</tbody>
</table>

2.9 Recombinant DNA techniques and bacterial protein expression

2.9.1 Transformation of bacteria

Competent *Escherichia coli* (*E. coli*) bacterial cells were thawed gently on ice. 50 µl of cell solution was pipetted into sterile 10 ml culture tubes (Falcon). 1 µl of plasmid was pipetted into each tube. Tubes were flicked to mix and left to incubate on ice for 20 minutes. Cells were heat shocked by holding tubes in a 42 °C water bath for 45 seconds then plunging them into ice for 2 minutes. 0.9 ml of S.O.C. medium (Invitrogen) was added to tubes and they were incubated for 30 minutes at 37 °C with shaking at 250 rpm.
150 µl of each transformation mixture was plated out onto LB agar plates containing ampicillin (0.1 mg/ml) and grown over night.

2.9.2 Purification of plasmid DNA from E. coli

Single colonies of plasmid containing E. coli were inoculated into 5 ml LB media supplemented with appropriated antibiotics. For small scale plasmid purifications inoculates were grown over night at 37 °C with shaking at 250 rpm. Subsequently, 500 µl of small scale bacteria were added to 250 ml of LB supplemented with appropriated antibiotics and incubated at 37 °C over night with agitation.

E. coli cultures were centrifuged at 5000 rpm at 4 °C and pellets subjected to DNA extraction using HiSpeed® Plasmid Maxi Kit (QIAGEN). The kits were used according to the manufacturer’s protocol.

2.10 Statistical analysis

Data was analysed using Student t- test in order to show statistically significance where relevant. Student t test used were two sided, paired tests. Whitney-Mann statistical test was used to analyse non-parametric data. Two-way ANOVA statistical test was used to analyse data in more than three groups. Results were considered statistically significant when \( p \) was less than 0.05 (*), if \( p < 0.01 (** \) and if \( p < 0.001 (*** \). Statistical analyses were performed using Microsoft Excel or GraphPad Prism.

2.11 Materials

2.11.1 Buffers and Solutions used

**Ampicillin**

1 g of Ampicillin dissolved in 20 ml of ultrafiltration (UF) water (50 mg/ml)

**APS (Ammonium Persulfate) stock**

10 % APS (w/v) in UF water
**BCA Protein assay Kit**
50 parts of buffer A missed with 1 part of buffer B (Fisher Scientific)

**Blocking buffer (immunoblotting)**
5 % (w/v) bovine serum albumen (BSA, SIGMA), 0.1 % (v/v) Tween-20 (Sigma) in 1X Tris buffered saline (TBS) solution diluted in UF water

**DMEM media**
Dulbecco’s modified Eagle’s medium with antibiotics (DMEM, ThermoFisher Scientific) supplemented with 4.8 mM glutamine (ThermoFisher Scientific) and 10 % heat-inactivated FBS (ThermoFisher Scientific)

**Cell lysis buffer**
120 mM Tris pH 6.8, 5 % glycerol (Fisher Scientific), 1 % SDS, 225 mM DTT, 1X of Protease Inhibitor buffer, 10 mM NaF (Fluka), 10 mM β-glycerophosphate (Alfa Aesar), 1 mm Na$_3$VO$_4$ and UF water

**Crystal violet**
0.25 % crystal violet (SIGMA) in 50 % methanol (Fisher Scientific)

**Cytoskeletal (CSK) buffer**
0.5 % TritonX-100, 10 mM HEPES, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl$_2$, Protease and phosphatase inhibitors

**Dye extraction (Crystal violet)**
40 % methanol, 10 % acetic acid (VWR), 50 % UF water

**FluoroBrite medium**
FluoroBrite medium (DMEM, ThermoFisher Scientific) supplemented with 4.8 mM glutamine (ThermoFisher Scientific) and 10 % heat-inactivated FBS (ThermoFisher Scientific)

**Freeze-down medium**
10 % DMSO (v/v) (SIGMA) in heat-inactivated FBS
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**Gel-running buffer (immunoblotting)**
1X of Epho buffer, 1 % of SDS (BioRad) in UF water

**LB Broth**
10 tablets diluted in 500 ml of UF water

**PBS solution**
1 tablet (Thermo Fisher) in 500 ml of UF water

**Propidium Iodide (PI)**
1 mg/ml (SIGMA) dissolved in PBS

**Puromycin**
2 µg/ml (SIGMA)

**10X Proteinase inhibitor buffer**
1 Complete EDTA-free Proteinase Inhibitor mini-pill (ThermoFisher scientific) per ml of UF water

**RPMI media**
Roswell Park Memorial Institute (RPMI, ThermoFisher Scientific) 1640 medium supplemented with 4.8 mM glutamine (ThermoFisher Scientific), 10 % heat-inactivated FBS (Thermo Fisher), 4500 mg/L D-glucose (SIGMA), 1500 mg/L sodium bicarbonate (SIGMA) and 1 mM sodium pyruvate (SIGMA).

**10X Running (Epho) buffer (immunoblotting)**
0.25 M Tris (Fisher), 1.92 M of Glycine (SIGMA) in UF water

**Running buffer (Tris-Acetate gel)**
50 mM Tris, 50 mM tricine, 0.1 % SDS, 1.3 mM sodium bisulfite in UF water
6X Laemli loading buffer
120 mM Tris pH 6.8, 30 % glycerol, 6 % SDS, 225 mM DTT, bromophenol blue and UF water

Spreading buffer (DNA fibre assay)
200 mM Tris-HCl pH 7.4, 50 mM EDTA, 0.5 % SDS and UF water

SYTOX® green nucleic acid stain
5 mM solution dissolved in FluoroBrite medium, stocked as 1 mM (Fisher Scientific)

TBST (TBS-Tween) Wash buffer (immunoblotting)
0.1 % Tween-20 (v/v) (SIGMA) in 1X TBS diluted in UF water.

Transfer buffer (immunoblotting)
1x protein electrophoresis buffer, 20 % (v/v) methanol, UF water

Transfer buffer (Tris-Acetate gel)
1 X NuPAGE transfer buffer (ThermoFisher Scientific), 25 mM sodium bisulfite (SIGMA), 10 % methanol, UF water

10X Tris-buffered saline (TBS) (immunoblotting)
1.4 M NaCl, 250 mM Tris, pH 7.5, UF water

Trypsin
Trypsin-EDTA solution 0.25 % (SIGMA)

Waymouth’s medium
Waymouth’s medium (ThermoFisher Scientific) supplemented with 4.8 mM glutamine (ThermoFisher Scientific) and 10 % heat-inactivated FBS (ThermoFisher Scientific)
Table 2.7: Antibodies

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* used for immunofluoresce
CHAPTER THREE

DNA damage repair pathway inhibitors screening in RB1-mutant cancers
3.1 Introduction
Beside its roles in cell cycle control, RB1 has been reported to play an important role in DNA damage checkpoints. Indeed, RB1 deficiency has been associated with multiple forms of CIN, therefore supporting tumorigenesis by removing safeguards that limit oncogenic transformation (Dyson 2016, Manning and Dyson 2012). Furthermore, it has been shown that RB1 can control chromatin condensation by the interaction between the LxCxE amino acid domain of RB1 and Condensin II subunit Cap-D3 (Manning et al. 2014). These publications suggest a role of RB1 inactivation in genome instability, which could be driving tumourigenesis.

Moreover, in human cells, loss of functional RB1 leads to accumulation of DSBs (Pickering and Kowalik 2006). Essentially, RB1 has been reported to initiate both HR as well as NHEJ repair (Cook et al. 2015, Velez-Cruz et al. 2016). These evidences led to the hypothesis that RB1 may be involved in the regulation of DSB repair pathways. Therefore, a better understanding of the consequences of RB1 loss in cancer treatment is crucial to identify new therapeutic approaches for tumours characterized by RB1 loss.

Following diverse lines of evidence for a role of RB1 loss in DNA repair, I set up an in vitro target assessment of candidate inhibitors in order to identify a possible link between RB1 loss and DDR pathway inhibitor sensitivity.

3.2 DDR pathway inhibitor profiling in RB1-mutant cancers
To identify a reliance of RB1-mutant tumours for specific DNA metabolic functions, I performed an inhibitor screen using a cell survival assay.

For this study I used a group of tumour cell lines characterised by RB1 gene mutation/deletion [H2009 (NSCLC), H82, H446 (SCLC) and SAOS2 (Osteosarcoma)] and a histotype matched tumour lines harbouring the RB1 wild type gene [H1975, H2122, H1299 (NSCLC) and U2OS (Osteosarcoma)].

Initially, all cell lines used were tested to confirm their RB1 status via immunoblotting (Fig. 3.1). Subsequently, these cell lines were tested via a cell survival assay, using Resazurin dye (Fig 3.2).
Figure 3.1: Cell lines RB1 status of cell lines used in his study

A) and B) Levels of total RB1 were established by immunoblotting. Equal amounts of total protein were loaded for all samples. GAPDH served as a loading control.
Figure 3.2: DDR pathway inhibitor profiling in RB1-mutant- as well as RB1-wildtype cancer cell lines

A) to D) Assessment of cell survival. A panel of histotype matched cancer cell lines differing in RB1 status were assessed for sensitivity to inhibitors against enzymes required for DDR. Cells were seeded in 96 well plates for 24 hours, then exposed to inhibitors at the indicated concentrations. Cell viability was determined after 5 days of drug exposure using Resazurin dye. Data are presented relative to vehicle and represent the mean of n=3 biological replicates each run in triplicate.

A) Treatment of cells with ATM inhibitor KU60019,
B) treatment of cells with DNA-PKcs inhibitor NU7441,
C) treatment of cells with PARP inhibitor Olaparib,
D) treatment of cells with ATR inhibitor AZD6738.

Data were analysed by a curve fitting process.

E) Cell lines and relative tumour type used for compounds.

F) Table with relative IC\textsubscript{50} values for each cell line and inhibitor. The IC\textsubscript{50} values indicated refer to the mean of n=3 biological replicates.

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Figure 3.3: Assessment of sensitivity using multiple and chemically unrelated ATR inhibitors

Experiments were performed essentially as described in Fig. 3.1, using an extended cell line panel. A) Response of cells to ATR inhibitor AZD6738 with relative cell lines tested. B) Response of cells to ATR inhibitor BAY1895344 with relative cell lines tested. C) ATR inhibitor VE822 and D) VE821 with relative cell lines tested. Data represent the mean of n=3 biological replicates, each run in triplicate. E) Table with relative IC₅₀ values for each cell line and inhibitor. The IC₅₀ values indicated refer to the mean of n=3 biological replicates.
Tumour cells were seeded into 96 well plates and treated with different DDR inhibitors: ATM (KU60019, Fig 3.2A), DNA-PK (NU7441, Fig. 3.2B), PARP (Olaparib, Fig. 3.2C) and ATR (AZD6738, Fig. 3.2D). Cells were treated with decreasing concentrations of inhibitors starting with 100 µM and the cell growth was determined measuring Resazurin reduction after 5 days. Differences in cell proliferation were calculated by normalisation to a vehicle control. KU60019 (ATMi), and NU7441 (DNA-PKi) (Fig. 3.2A and B) inhibitors did not show any significant difference in response between RB1-mutant and RB1 wild type cell lines, as also indicated by the IC$_{50}$ values (in red) shown in the table (Fig. 3.2F). In contrast, Olaparib (PARPi) as well as AZD6738 (ATRi) (Fig. 3.2C and D) showed different responses between RB1-mutant and RB1 wild type cells, indicating an increased sensitivity of the RB1-mutant cancer cells in comparison to RB1 wild type.

Further ATR inhibitors were utilised in order to confirm the observed effects in RB1-mutant as well as RB1 wild type cell lines (Fig. 3.3). The cell lines panel to be tested was also expanded and RB1-mutant and RB1 wild type cell lines responded differently to the treatment with three different ATR inhibitors (BAY1895344, VE821 and VE822), indicating sensitivity towards these inhibitors in RB1-mutant background. The IC$_{50}$ values (in red) shown in the table (Fig. 3.3E) indicate a difference in sensitivity between RB1-mutant- and RB1 wild type cell lines.

The data from these experiments suggest that RB1-mutant cancer cells are more sensitive to ATR inhibition compared to RB1 wild type.

### 3.2.1 Effect of ATR inhibition on cell colony growth

To test if ATR inhibition affects cell proliferation and colony growth in RB1-mutant as well as in RB1 wild type cells, colony formation assays were performed (Fig. 3.4).
Figure 3.4: Effect of ATR inhibition on cell colony growth
Assessment of clonogenic growth. Cancer cell lines differing in RB1 status were seeded at low cell density into 6 well dishes. Cells were subsequently exposed for 48h to the indicated concentrations of ATR inhibitor (AZD6738) or vehicle. A) and B) representative raw plate images showing RB1-mutant B) and RB1 wt cell lines A). C) Data shown following curve fitting. D) IC_{50} values from colony assay deduced. p values were calculated using unpaired student t test, **p<0.01. Data represent the mean of n=3 biological replicates, each run in triplicate.
Chapter Three: DNA damage repair pathway inhibitors screening in *RBI*-mutant cancers

*RBI*-mutant cells were used (Osteosarcoma cell line SaOS2, Gliosarcoma cell line SF539, NSCLC cell line H2009 and Breast cell line BT-549) as well as *RBI* wild type cells (Osteosarcoma cell line U2OS, Gliosarcoma cell line SF259, three NSCLC cell lines H1975, H1993 and H1299, and SCLC cell line DMS 114) were seeded at low cell density in 6 well plates and their ability to form colonies following ATR inhibition was observed. After 24 hours, cells were treated at different concentrations with ATR inhibitor (AZD6738) or a vehicle control. The medium containing the drug was replaced after 48 hours with fresh drug-free media. Afterwards cells were left growing for 10-12 days. Subsequently, plates were fixed with 37% formaldehyde and stained with crystal violet.

A stronger reduction in colony numbers was observed in *RBI*-mutant cell lines following treatment with the ATR inhibitor (Fig. 3.4B), compared to the majority of *RBI* wild type cell lines (Fig. 3.4A). Figure 3.4C shows the quantification of the clonogenic activity using a curve fitting. Relative IC$_{50}$ values deduced are shown in Figure 3.4D. These data again support previous findings shown in Figures 3.2 and 3.3, *RBI*-mutant cell lines are sensitive to ATR inhibition.

### 3.3 Real time cell fate assessment in *RBI*-mutant cancer cells upon ATR inhibition

To understand the effect of ATR inhibition on cell fate and if it may differ in *RBI*-mutant or *RBI* wild type cell lines, live cell imaging was performed in the presence of the death dye SYTOX$^\text{TM}$ green.

The cells were seeded in 96 well plates using FluoroBrite DMEM media and SYTOX$^\text{TM}$ green nucleic acid stain was added. Cells, which undergo apoptosis, will emit a green fluorescent signal. Two different ATR inhibitors were tested, AZD6738 (Fig. 3.5) and VE821 (Fig. 3.6).

Signal curves of green fluorescence intensity levels after AZD6738 treatments are shown in Figure 3.5A, B and C for *RBI*-mutant cells (H446, H2009 and SaOS2) and in Figure 3.5D, E and F for *RBI* wild type cells (H2122, H1975 and H1299). The green fluorescence intensity levels after VE821 inhibitor treatments are shown in Figure 3.6A and B for *RBI*-mutant cells (H446, H2009) and in Figure 3.6C and D for *RBI* wild type cells (H2122, H1975).
Figure 3.5: Real time cell fate assessment in RB1-mutant cancer cells upon ATR inhibition

Cancer cell lines were seeded into 96 well plates in media containing SYTOX™ green nucleic acid stain. After 24 hours ATR inhibitor AZD6738, was added at indicated concentration. Cells were imaged using an Incucyte live cell imager. Images were taken once every two hours for five consecutive days, recording green fluorescence and phase contrast. Curves represent mean values for integrated SYTOX™ fluorescence of n=3 technical replicates, normalised to cell density. A) RB1-mutant cell line H446 (SCLC), B) RB1 wt cell line H2122 (NSCLC), C) RB1-mutant cell line H2009 (NSCLC), D) RB1 wt cell line H1975 (NSCLC), E) RB1-mutant cell line SAOS2 and F) RB1 wt cell line H1299. G) Data for the RB1-mutant and RB1 wt groups at indicated concentrations. H) Interaction statistics using a two-way ANOVA test comparing drug effect for RB1-mutant and RB1 wt cell lines. I) Immunoblot analysis showing PARP cleavage in RB1-mutant and RB1 wt cell lines. Cells were treated with ATR inhibitor and harvested at indicated time points; L) quantification relative to GAPDH. GAPDH served as a loading control. p values were calculated using two-way ANOVA *p<0.05, **p<0.01, ***p<0.001. Data represent the mean of n=3 biological replicates, each run in triplicate.
Chapter Three: DNA damage repair pathway inhibitors screening in RB1-mutant cancers

Figure 3.6: Real time cell fate assessment response in RB1-mutant cancer cells after ATR inhibitor VE821

The experiment was executed as described in Fig. 3.4. After 24 hours ATR inhibitor VE821 was added at the indicated concentration. Curves represent mean values for integrated SYTOX\textsuperscript{TM} fluorescence of n=3 technical replicates, normalised to cell density. **A** RB1-mutant cell line H446 (SCLC), **B** RB1 wt cell line H2122 (NSCLC), **C** RB1-mutant cell line H2009 (NSCLC), **D** RB1 wt cell line H1975 (NSCLC). **E** Interaction statistics using a two-way ANOVA test comparing drug effect for RB1-mutant and RB1 wt cell lines. p values were calculated using two-way ANOVA *p<0.05, **p<0.01, ***p<0.001. Data represent the mean of n=3 biological replicates, each run in triplicate.
The cell death levels in RB1-mutant lines started to be recorded after 30 hours of drug treatments and increased over time, reaching a peak after 5 days. These levels were not recorded in RB1 wild type cells. The death rate was calculated by normalising SYTOX™ green fluorescence intensity to cell confluency.

Representative images of green fluorescence levels show higher fluorescence intensity in RB1-mutant cells compared to RB1 wild type induced by ATR inhibition after five days incubation (Fig. 3.5G).

The difference in fluorescence levels between RB1-mutant and RB1 wild type cells is significant for each single drug concentration of the two ATR inhibitors used in the experiments. Data was analysed by two-way ANOVA and the interaction is significant for both inhibitors tested; for AZD6738 at 10 μM (p=0.0011), 3 μM (n.s.), 1 μM (p=0.0001) and 0.3 μM (p=0.0036) (Fig. 3.5H); for VE821 at 10 μM (p=0.0008), 3 μM (p=0.0028), 1 μM (n.s.) and 0.3 μM (n.s.)) (Fig. 3.6E). This difference signifies that RB1-mutant cell lines underwent cell death in higher levels compared to RB1 wild type cells and this could explain the observed difference in sensitivity shown in Figures 3.2 and 3.3. Furthermore, ATR inhibition leads to PARP cleavage observed selectively in RB1-mutant cell lines but not in RB1 wild type cells, indicating apoptotic cell death in RB1-mutant cells (Fig. 3.5I and L).

These results directly support the hypothesis that loss of RB1 is directly responsible for the increasing levels in cell death upon ATR inhibition and are in agreement with results described in section 3.2 and 3.2.1.

### 3.4 Differences in ATR pathway regulation between RB1-mutant and RB1 wild type cell lines

Having established that RB1-mutant cells are sensitive to ATR inhibition and that ATR inhibition increases cell death in those cells, I sought to investigate the underlying mechanism. In a recent publication by Buisson et al., the authors showed how ATR inhibition induces ssDNA accumulation and replication fork catastrophe in a fraction of early S-phase cells.
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Figure 3.7: Mitigation of ATR inhibition in cells with different RB1 status
A) to D) Immunoblot analysis assessing DNA-PKcs, CHK1 and CHK2 activation in cells with different RB1 status following ATR inhibition. Cells were seeded into 6 well plates. After 24 hours cells were treated with ATR inhibitor (AZD6738) at indicated concentrations alone or in combination with DNA-PKcs inhibitor (NU7441). Lysates were prepared 4 and 8 hours post inhibitor addition and analysed by western blots. A) Data for RB1 wt cell line U2OS, RB1-mutant cell line H446 and RB1 wt cell line H1975, B) data for RB1-mutant cell line SaOS-2, RB1 wt cell line H1299 and RB1-mutant cell line H69, C) data for RB1 wt cell line U2OS, RB1-mutant cell line H2009 and RB1 wt cell line H2122 and D) data for RB1 wt cell lines U2OS, H1993 and DMS 114. GAPDH served as a loading control. Data represent the mean of n=2 biological replicates.
In other S-phase cells, however, ATR inhibition induces moderate ssDNA and triggers a DNA-PK and CHK1-mediated back-up pathway to suppress origin firing. The back-up pathway creates a threshold so that the ATR inhibitor selectively kills cells under high replication stress (Buisson et al., 2015).

My hypothesis was that RB1-mutant cell lines are sensitive to ATR inhibition because they cannot effectively trigger the DNA-PK and CHK1-mediated back-up pathway activation, consequently DSBs and stalled forks are not repaired.

In order to test this hypothesis, RB1-mutant cell lines (SaOS-2, H446, H2009 and H69) as well as RB1 wild type cell lines (U2OS, H1299, H1975, H2122, H1993 and DMS 114) were treated with ATR and DNA-PKcs inhibitors for 4 and 8 hours, keeping the same drugs concentrations used in Buisson et al. (Fig. 3.7). The U2OS cell line was used as control as described by Buisson et al. The data obtained for this cell line is in agreement with the data published by Buisson. An up-regulation of phospho-DNA-PKcs (Ser2056) was observed following ATR inhibition, which was clearly detectable in U2OS and reduced after combined treatment with ATR and DNA-PKcs inhibitors (Fig. 3.7A and C and D). Similar behaviour was observed in other RB1 wild type cell lines: H1975 (Fig. 3.7A) and in H2122 (Fig. 3.7C), H1993 and DMS 114 (Fig. 3.7D). The regulation of phospho-CHK1 (Ser345) in these cells followed the results by Buisson and supports that activation of the DNA-PKcs leads to checkpoint activation involving CHK1.

On the other hand, RB1-mutant cell lines showed a clearly different response upon ATR inhibition. In fact, three out of four RB1-mutant cell lines (H446, H69 and H2009), did not show detectable activation of phospho-DNA-PKcs compared to three RB1 wild type cell lines (U2OS, H1975, H2122, H1993 and DMS 114). An exception was the RB1 wild type cell line H1299 which also did not show phospho-DNA-PKcs activation (Fig. 3.7B).

The decreased activation of phospho-DNA-PKcs in RB1-mutant cells might support the data by Cook et al., documenting a reduced ability of RB1 defective cells to induce DNA damage repair involving this enzyme, and decreased activation of DNA-PKcs strongly correlates with ATR inhibitor sensitivity. Importantly, and in addition to poor activation of DNA-PKcs in RB1-mutant cell lines, these cell lines only had minor and poor
activation of CHK1 compared to the most of \( RB1 \) wild type cell lines and this is evidence of a low level of DNA-PKcs activation, data in disagreement with Buisson et al.

### 3.5 Summary

Considering published evidence on \( RB1 \) inactivation and consequent accumulation of DSBs, the hypothesis that RB1 may regulate indirectly DSB repair pathways has been investigated.

Different inhibitors were tested, and it was found that \( RB1 \)-mutant cells are sensitive to multiple ATR inhibitors compared to \( RB1 \) wild type cancer cells. The data was confirmed by a reduction in colony growth upon treatments in \( RB1 \)-mutant cell lines. Additionally, high levels of cell death were observed in \( RB1 \)-mutant cells compared to \( RB1 \) wild type cells upon ATR inhibition and PARP cleavage was observed in \( RB1 \)-mutant cells indicating an apoptotic pathway activation in a RB1 loss scenario.

Moreover, data published by Buisson et al., showed that high levels of replication stress can be tolerated because of the DNA-PK and CHK1-mediated back-up pathway activation, which induces fork stalling rescue upon ATR inhibition (Buisson et al., 2015). The low levels of DNA-PKcs activation in \( RB1 \)-mutant cell lines could explain their sensitivity towards ATR inhibition.

Taken together, the results presented strongly support a correlation between RB1 loss and ATR inhibitor sensitivity, supporting further investigation of the role of RB1 in DDR applying a siRNA and shRNA approach.
CHAPTER FOUR

*RB1* gene modification and ATR inhibition
Chapter Four: \textit{RB1} gene’s modification and ATR inhibition

4.1 Introduction

In previous published work, it has been shown that RB1 loss drives the accumulation of DSBs compelling to the hypothesis that RB1 may be involved in the regulation of DSB repair pathways. Indeed, RB1 has been reported to initiate both HR and NHEJ repair (Cook et al. 2015, Velez-Cruz et al. 2016). As previously shown in chapter three, \textit{RB1}-mutant cancers are sensitive to ATR inhibition, showing high level of cell death and apoptotic pathway activation. In order to increase confidence that RB1 loss is directly responsible for an increased sensitivity towards ATR inhibition, I carried out additional validation by reducing \textit{RB1} expression in \textit{RB1} wild type cancer cells using RNA interference.

4.2 Assessing the contribution of RB family proteins loss to ATR inhibitor sensitivity

As described in chapter three, RB1 loss is linked with hypersensitivity to ATR inhibition. In order to show that loss of functional \textit{RB1} is directly responsible for an increased sensitivity towards ATR inhibition, siRNA and shRNA approaches were used. Sensitivity to the ATR inhibitor AZD6738 was assessed following siRNA mediated \textit{RB1} knockdown in \textit{RB1} wild type cancer cells. As the three RB family protein show an overlap in their function, siRNAs directed against all three family members were used. Two \textit{RB1} wild type cell lines (Colorectal cancer line HCT-116, Fig. 4.1A, B and NSCLC line H1975 Fig.4.1C-E) were transfected with a mixture of SMARTpool siRNAs against \textit{RB1}, \textit{RBL1} and \textit{RBL2}, or non-targeting siRNA in 96 well plates. These cell lines were chosen because of their high transfection efficiency for siRNA constructs. To allow a sufficient knockdown of RB family members, siRNA mixtures and cells were incubated for 36 hours. Subsequently cells were treated with ATR inhibitor (AZD6738) at decreasing concentrations for 5 days. The cell proliferation rate was calculated as described in chapter three (section 3.2).

As shown in Figure 4.1A and C, there is a three-fold decrease of the IC\textsubscript{50} values in cells treated with pooled siRNAs against RB family members, compared to lipid control, non-targeting siRNA control or maternal cell lines.
Figure 4.1: Assessment of ATR inhibition sensitivity in RB1 knockdown cell lines
Cells with RB1 wt status were seeded in 96 well plates, cells were transfected with siRNA targeting RB proteins and a non-targeting oligonucleotide or lipid control for 36 hrs. Cells were subsequently treated with ATR inhibitor (AZD6738) at indicated concentrations. Cell viability was determined after 5 days of drug exposure using Resazurin dye, data analysis performed as Fig. 3.3A-L. A) Data for RB1 wt HCT116 Colorectal cell line, C) data for RB1 wt H1975 NSCLC cell line. IC50 values for values derived from the different conditions are indicated. Loss of RB1 was confirmed by immunoblot analysis after 36 hours of RB1 knockdown in B) for HCT-116 and in D) for H1975. E) presents data for RB1 knockdown using single oligonucleotides in H1975 cell line. GAPDH served as a loading control. p values were calculated using one-way ANOVA *p<0.05, **p<0.01, ***p<0.001. Data represent the mean of n=3 biological replicates, each run in triplicate.
Chapter Four: RBL1 gene’s modification and ATR inhibition

The RBL1 knockdown efficiency has been validated via immunoblot for HCT-116 (Fig. 4.1B) and for H1975 (Fig. 4.1D). Furthermore, to test the effect of RBL1 knockdown without targeting RBL1 and RBL2, single oligonucleotides targeting RBL1 were tested in H1975 cells (Fig. 4.1E). RBL1 knockdown samples show a four-fold decrease in IC50 values for the ATR inhibitor AZD6738 between siRNA and control treated cells. This data supports the hypothesis that loss of RBL1 is involved in the increased sensitivity to ATR inhibition observed in RBL1-mutant cells (chapter three).

To confirm that RBL1 loss also alters cell fate after ATR inhibition, I performed live cell imaging in presence of the cell death dye SYTOX™ green. Experiments were performed as described in chapter three, section 3.3, (Fig. 3.5).

Treatment with the ATR inhibitor induced increased levels of cell death in cells treated with siRNAs targeting RBL1 compared to lipid control or non-targeting control for both, HCT-116 (Fig. 4.2A) and H1975 (Fig. 4.2B) cell lines. The differences in cell death levels between RBL1 knockdown and controls tested significant as shown in Figure 4.2C for HCT-116 and in Figure 4.2D for H1975 (two-way ANOVA analysis). These results directly support the hypothesis that RBL1 loss is directly responsible for increased levels in cell death upon ATR inhibition, and are in agreement with results shown in Figure 3.4 (chapter three).

4.3 Assessment of ATR inhibitor sensitivity in RBL1 knockout cell lines

Previous results presented in section 4.2, which are based on the siRNA-mediated knockdown of RBL1 (Fig. 4.1), were additionally confirmed using lentivirus transmitted short-hairpin RNAs targeting RBL1.

Two RBL1 wild type cell lines were used to show the effect of a RBL1 knockout, DMS 114 (SCLC) (Fig. 4.3A-C) and H1299 (NSCLC) (Fig. 4.3D-E), and to test their sensitivity towards ATR inhibition. These cell lines were chosen because of their high transfection efficiency for shRNA constructs.
**Figure 4.2: Real time cell fate determination in RB1 knockdown cell lines upon ATR inhibition**

Cells fate was assessed using life cell imaging for 5 days. Cells with RB1 wt status were seeded in 96 well plates in media with SYTOX green nucleic acid stain. Cells were transfected with siRNA targeting RB family members (RB1, p107, p130) and a non-targeting oligonucleotide control or lipid control for 36 hours. Cells were subsequently exposed to ATR inhibitor (AZD6738) at the indicated concentrations. A) and C) show data for RB1 wt cell line HCT-116, B) and D) show data for the RB1 wt cell line H1975. Death rate was calculated by normalising SYTOX™ intensity to cell confluency C) and D) Data analysis performed as in Fig. 3.4H.

p values were calculated using two-way ANOVA **p<0.01, ***p<0.001. Data represent the mean of n=3 biological replicates, each run in triplicate.
**Figure 4.3: Assessment of ATR inhibitor sensitivity in *RB1* knockout cell lines**

Cells with *RB1* wt status were infected with individual lentiviruses delivering shRNA expression vectors targeting *RB1* gene or an empty vector control. A) to E) Cell viability assessment, cells were seeded in 96 well plates, and subsequently treated with two ATR inhibitors (AZD6738 and VE822) at indicated concentrations. Cell viability was determined after 5 days of drug exposure using Resazurin dye. A) and B) *RB1* wt cell line DMS 114 (SCLC) and in D) and E) *RB1* wt cell line H1299 (NSCLC). The knockout was confirmed via immunoblot analysis for DMS 114 in C) and for H1299 in F). Data points represent mean of n=3 technical replicates, normalised to control cell density. GAPDH served as a loading control. Data represent the mean of n=3 biological replicates, each run in triplicate.
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**Figure 4.4: Effect of ATR inhibition on colony growth in *RB1* knockout cell lines**

Cells were seeded at low cell density into 6 well plates and subsequently exposed to indicated concentrations of ATR inhibitor (AZD6738) or vehicle for 48h. Subsequently, media was replaced with fresh media. Cells were fixed after 12 days and stained using crystal violet. **A** and **C** Representative raw plate images showing DMS 114 and H1299 cell lines upon *RB1* knockout. Graphs depicting colony growth was determined by crystal violet dye extraction. **B** Data was analysed by a curve fitting process for DMS 114 line and shRNA-RB(1 and 2) and in **D** for H1299 line and shRNA-RB(2 and 6). Data presented relative to vehicle control and results from one experiment of *n*=3 technical replicates are shown.
Cell lines expressing RB1 targeting constructs: shRB(1), shRB(2) and shRB(6) showed a decrease in IC$_{50}$ values upon ATR inhibitor treatment using AZD6738, (Fig. 4.3A and D) compared to cells infected with the empty vector control or parental cell lines. RB1 knockout was confirmed via immunoblot analysis shown in Figure 4.3C for DMS 114 cell line, and in Figure 4.3F for H1299 cell line. The ATR inhibitor sensitivity of cell lines harbouring a knockout of RB1 was further tested with an additional ATR inhibitor, VE822, (Fig. 4.3B and E).

In line with previous data shown in Figure 4.1, the knockout of RB1 led to a sensitisation of cells towards ATR inhibition (Fig. 4.3). Cell lines treated with shRNA constructs targeting RB1 showed an increased sensitivity towards ATR inhibition leading to two-fold lower IC$_{50}$ values compared to controls (Fig. 4.3B and E).

In order to further validate changes in cell proliferation upon RB1 knockout combined with ATR inhibition, I performed colony formation assays using shRNA cell lines. The experiments were executed as described in chapter three, section 3.2.1, (Fig. 3.4).

A stronger reduction in colony growth was observed in cells treated with RB1 knockout constructs (Fig. 4.4A for DMS 114 and Fig. 4.4C for H1299 lines), compared to cell lines treated with the empty vector control. Figure 4.3B and D show the quantification of clonogenic growth using a curve fitting method and the resulting IC$_{50}$ values.

The data again supports previous findings shown in Figures 3.2 and 3.3. RB1 knockout cell lines are more sensitive to ATR inhibition and less able to grow in colonies under ATR inhibitor treatments compared to parental cell lines.

**4.4 Real time cell fate determination in RB1 knockout cell lines upon ATR inhibition**

To investigate the cell fate upon ATR inhibition in cells treated with RB1 knockout constructs (H1299 and DMS 114 cell lines), I performed live cell imaging in the presence of the death dye SYTOX$^\text{TM}$ green. Experiments were performed as described in chapter three, section 3.3, (Fig. 3.5).
Figure 4.5: Real time cell fate determination in RB1 knockout cell line H1299 upon ATR inhibition

A) to D) Real time cell fate assessment. Cancer cell lines were seeded into 96 well plates in media containing Propidium iodide (PI) nucleic acid stain. After 24 hours ATR inhibitor, AZD6738, was added at indicated concentration. Cells were imaged using an Incucyte live cell imager. Images were taken once every two hours for five consecutive days, recording red fluorescence and phase contrast. Data shown for H1299 cell line in A), empty vector control in B) and shRNA(RB2) and shRNA(RB6) in C) and D). Data points represent mean of n=3 technical triplicates, normalised to cell density, data shown is representative of one experiment. E) Two-way ANOVA test comparing drug effects for shRNA(RB2 and 6) lines compared to treated H1299 cell line for each single drug concentration. F) Immunoblot analysis assessing PARP cleavage in H1299 cell line, empty vector control (Ctrl) and shRNA(RB6) lines. Cells were treated with ATR inhibitor and harvested at indicated time points; G) quantification relative to GAPDH. GAPDH served as a loading control. p values were calculated using two-way ANOVA **p<0.01, ***p<0.001.
Figure 4.6: Real time cell fate analysis in RB1 knockout cell line DMS 114 upon ATR inhibition

A) to D) Real time cell fate assessment. Cancer cell lines were seeded into 96 well plates in media containing Propidium iodide (PI) nucleic acid stain. After 24 hours ATR inhibitor, AZD6738, was added at the indicated concentrations. Cells were imaged using an Incucyte live cell imager. Images were taken once every two hours for five consecutive days, recoding red fluorescence and phase contrast. Data for DMS 114 cell line are shown in A), empty vector control in B) and shRNA(RB1) and shRNA(RB2) in C) and D). Data points represent mean of n=3 technical triplicates, normalised to cell density, data shown is representative of one experiment. E) Two-way ANOVA test comparing drug effect for shRNA(RB1) and 2) lines compared to treated DMS 114 cell line for each single drug concentration. Immunoblot analysis assessing PARP cleavage in DMS 114 cell line, empty vector control (Ctrl) and shRNA(RB2) lines. Cells were treated with ATR inhibitor and harvested at indicated time points; G) quantification relative to GAPDH. GAPDH served as a loading control. p values were calculated using two-way ANOVA **p<0.01, ***p<0.001.
Treatment with AZD6738 showed increased florescence intensity recorded in \textit{RB1}-knockout cells (Fig. 4.5C and D for H1299 and Fig. 4.6C and D for DMS 114) compared to parental cells and empty vector control cells (Fig. 4.5A and B for H1299 and Fig. 4.6A and B for DMS 114).

The difference in fluorescence levels between \textit{RB1}-knockout and treated maternal cell lines is significant for 10µM, 3µM and 1µM of ATR inhibitor, AZD6738, used in the experiments (Fig. 4.5E for H1299 and Fig. 4.6E for DMS 114), (data analysed by two-way ANOVA). Moreover, the effect of ATR inhibition showed PARP cleavage after 72- and 96-hours of treatment indicating apoptotic cell death in \textit{RB1} knockout cells (Fig. 4.5F and G for H1299 and Fig. 4.6F and G for DMS114) compared to parental cell lines and empty vector control treated cells.

Taken together the data presented in this section supports the results of Figure 4.1, indicating that \textit{RB1} deficiency increases ATR inhibitor sensitivity and leads to an increase in apoptosis levels. Therefore, RB1 loss could serve as a potential biomarker for ATR inhibitor sensitivity.

4.5 Differences in ATR pathway regulation in \textit{RB1} knockdown or knockout cell lines

In line with data presented in section 3.4, I sought to test whether cell lines treated with \textit{RB1} knockdown or knockout constructs would show similar ATR pathway regulation as \textit{de novo} \textit{RB1}-mutant cell lines.

To investigate this hypothesis, the U2OS cell line was transfected with a single oligonucleotide targeting \textit{RB1} as well as a mixture of SMARTpool siRNAs targeting \textit{RB1}, \textit{RBL1} and \textit{RBL2}, or non-targeting siRNAs in 6 well plates. The siRNA mixtures were incubated for 48 hours with the target cells and subsequently the cells were treated with an ATR inhibitor alone or in combination with a DNA-PKcs inhibitor for 4 and 8 hours (Fig. 4.7A). As shown in Figure 4.7A, U2OS cells treated with siRNAs targeting \textit{RB1} did not present the same pattern of pathway activation as observed in \textit{de novo} \textit{RB1}-mutant cell lines (section 3.4). For example, \textit{de novo} \textit{RB1}-mutant cell lines did not show detectable activation of phospho-DNA-PKcs compared to \textit{RB1} wild type cell lines (Fig. 3.7).
Figure 4.7: Differences in ATR pathway regulation in RB1 knockdown or knockout cell lines
A) Immunoblot analysis assessing DNA-PKcs activation in the U2OS cell line upon RB1 knockdown and ATR inhibition. B) and C) Immunoblot analysis showing CHK1 activation in the H1299 RB1 knockout cell line following ATR inhibition. Cells were seeded into 6 well plates. After 24 hours cells were treated with ATR inhibitor (AZD6738) at indicated concentrations, alone or in combination with DNA-PKcs inhibitor (NU7441). Lysates were prepared 4 and 8 hours post inhibitor addition and analysed using western blots. A) Data for U2OS cell line after siRNA transfection targeting RB1 alone or RB family (pool). B) Data for U2OS, H1299 maternal, empty vector control (Ctrl) and shRNA(RB2) cell lines. C) Data for U2OS, H1299 empty vector (Ctrl) and shRNA(RB6) cell lines. GAPDH and Vinculin served as loading controls. Data represent the mean of n=2 biological replicates.
Whereas U2OS cells treated with siRNA constructs targeting \textit{RB1} show an up-regulation of phospho-DNA-PKcs upon ATR inhibition and there is no difference in between U2OS cells treated with lipid control, siRNA \textit{RB1} or siRNA \textit{RB1} pool.

In addition to the use of siRNA against \textit{RB1} and other RB family members, I set up a further approach employing short-harpin RNAs targeting \textit{RB1}. Firstly, the \textit{RB1} wild type cell line H1299, was investigated using two different shRNA constructs targeting \textit{RB1} (shRNA-RB(2) and shRNA-RB(6)). The cell line U2OS served as a positive control showing activated CHK1 (Fig. 4.7B and C) like in Fig. 3.7A, C and D. Cells were seeded in 6 well plates and treated with ATR and DNA-PKcs inhibitors at indicated concentrations over 4 and 8 hours. H1299 shRNA-RB(2) and (6) did not show any changes in CHK1 phosphorylation differently from \textit{de novo} \textit{RB1}-mutant cell lines (section 3.4), where these cell lines only had minor and poor activation of CHK1 compared to the most of the \textit{RB1} wild type cell lines.

\section*{4.6 Summary}

In order to confirm that loss of RB1 is directly responsible for an increased sensitivity towards ATR inhibition, I carried out additional validation by suppressing the expression of RB family members in \textit{RB1} wild type cancer cells using RNA interference.

Upon inhibition of \textit{RB1} gene expression via siRNA tools, it was shown that cells acquired sensitivity towards ATR inhibition and presented increased levels of cells death compared to control treated cells.

Subsequently, I tested an additional approach using short-harpin RNAs targeting \textit{RB1}. Cell lines harbouring a knockout of \textit{RB1} showed an increased sensitivity towards ATR inhibition determined by cell proliferation assays. The data was confirmed by the determination of colony formation potential of \textit{RB1} knockout cell lines and controls upon ATR inhibition. Cell lines treated with shRNA constructs targeting \textit{RB1} are less able to form colonies upon ATR inhibition compared to cell lines treated with an empty vector control.

Additionally, high levels of cell death were observed in \textit{RB1} knockout cells compared to parental cells or cells treated with and empty vector control upon ATR inhibition. Together with the data of an increase in PARP cleavage in \textit{RB1} knockout cells upon ATR inhibition.
inhibitor treatment, these findings indicate the activation of apoptotic pathways in a RB1 loss and ATR inhibitor treated setting.

Although RB1 knockdown as well as knockout cell lines do not show the same ATR pathway regulation as *de novo* RB1-mutant cells, the observations presented in this chapter strongly support the data discussed in chapter three and point to a correlation between RB1 loss and ATR inhibitor sensitivity.

Based on the data described in this chapter and in chapter three, it was thought to assess whether RB1 loss has an impact on DNA metabolic functions.
CHAPTER FIVE

RB1 loss and DNA replication progression
5.1 Introduction
As discussed in the introduction (section 1.7), ATR is an essential DDR kinase due to its activities associated with DNA replication. ATR kinase is activated at stalled and collapsed replication forks and it phosphorylates several substrates that function in DNA replication and repair, chromatin remodelling, transcription, protein synthesis, cell cycle progression and cell death. A key ATR function is to phosphorylate and activate the protein kinase CHK1 (Guo et al. 2000). When activated CHK1 promotes proteasomal degradation of CDC25A, a phosphatase that removes inhibitory modifications from CDKs (Bartek et al. 2004). Therefore, CDC25A inactivation by CHK1 in response to genotoxic stress, slows or arrests cell cycle progression by reducing CDK2 activity. This holds S phase progression to allow time to complete replication of the genomic DNA and prevents premature entry into M phase. In case of extended damage, CHK1 activation leads to p53 activation that will induce senescence and apoptotic pathways.

ATR kinase activity is also implicated in the regulation of unperturbed DNA replication, as ATR inhibition is known to induce unscheduled origin firing and reduces replication fork speed.

Various published studies have linked the RB1-E2F pathway with the control of DNA replication. Through its effects on E2F, RB1 loss alters the expression of numerous proteins that are needed for S phase and RB1/E2F proteins have also been shown to physically interact with replication factors (Srinivasan et al., 2007). Changes in the levels and/or activity of replication proteins may influence the process of DNA replication in RB1-deficient cells inducing a possible under-replication scenario due to a short G1 phase. RB1 loss has been shown to affect the spatial organisation of DNA synthesis within the nucleus (Barbie et al., 2004), indeed RB1 was found to control chromatin cohesion through the interaction between the LxCxE amino acid domain of RB1 with the Condensin II subunit Cap-D3 (Coschi et al. 2010, Isaac et al. 2006). In a work published by Bester et al., it has been shown how the disruption of RB1 pathway alters S phase progression and promotes replication fork stalling (Bester et al. 2011). The idea that RB1 is necessary for normal replication, and that its loss causes replication stalling and DSBs, is consistent with multiple studies showing that RB1 loss results in increased DNA damage and results in increased genomic instability (Manning and Dyson 2012).
Considering the important role of ATR in preserving genome stability and that RB1 loss is responsible for increasing levels of DNA damage and genome instability (Marshall et al. 2019), I decided to investigate the role of RB1 loss on DNA replication progression under basal condition and under ATR inhibition.

5.2 Assessing DNA replication progression in \textit{RB1}-mutant and \textit{RB1} wild type cell lines

To assess the impact of RB1 loss on DNA replication fork activation and progression under basal condition, I performed a DNA fibre assay in cell lines previously used. Two \textit{RB1}-mutant (SCLC cell line H446 and NSCLC cell line H2009) and two \textit{RB1} wild type (NSCLC cell lines H1975 and H1993) cell lines were exposed to a first pulse with CldU for 20 minutes (red tracts) to label active replication forks, then exposed to a second pulse with IdU for further 20 minutes (green tracts) to monitor fork extension. An experiment diagram is shown in Figure 5.1A. The analysis is based on measuring the second pulse (IdU).

This analysis revealed a significant increase in the frequency of asymmetric forks in \textit{RB1}-mutant cells (Fig. 5.1F for H446 and G for H2009), when compared to \textit{RB1} wild type cells (Fig. 5.1H for H1975 and I for H1993). The analysis of the tract ratio (IdU/IdU) gave significant differences between \textit{RB1}-mutant and \textit{RB1} wild type cells (Fig. 5.1L), indicating that \textit{RB1}-mutant cell lines have asymmetric forks.

Fork stalling events in response to replication stress are associated with dormant origin activation and a corresponding reduction of both IOD (inter-origin distance) and fork speed, this most likely due to compensation mechanisms based on exhaustion of replication factors and deoxyribonucleoside triphosphate (dNTP) levels (Techer et al., 2017, Bester et al., 2011). Fibre assay analysis revealed that \textit{RB1}-mutant cells showed a reduction in fork speed (Fig. 5.1B) and a significant decrease of the IOD (Fig. 5.1C and D) compared to \textit{RB1} wild type cells (Bellelli et al., 2018, Bellelli et al., 2020, Rodriguez-Acebes et al., 2018). These results seem to be in agreement with published data by Bester et al. 2011, showing that cells with an abnormal activation of the RB1-E2F pathway by viral or cellular oncogenes, leads to perturbed progression of the replication fork indicating a poor processivity of the replication machinery.
Figure 5.1: Assessment of RB1 defective and RB1 wild type cell fibre tract lengths under basal conditions

A) Schematic of experiment design, involving CldU/IdU pulse-labelled with representative image. B) Dot plot graph of raw data displaying IdU tract length for individual replication forks comparing RB1 wt and RB1-mutant cells. C) Representative image of inter-origin distance (IOD). D) Mean IOD values from RB1-mutant and RB1 wt cells. E) Representative image of asymmetric DNA fibre from RB1-mutant cell line H446. Analysis of fork symmetry of RB1-mutant cells F) for H446 and G) for H2009, and RB1 wt cells H) for H1975 and I) for H1993. L) IdU/IdU ratio for each single cell lines are shown. M) Percentage of initiation events for RB1-mutant and RB1 wild type cell lines. Scale bar, 16.1 µm. ORI: replication origin. p values were calculated using Kruskal-Wallis test, *p<0.05, **p<0.01. Data represent for one of n=2 representative experiment, data generated in parallel.
The analysis of initiation events did not show any difference between \textit{RB1}-mutant and \textit{RB1} wild type cell lines (Fig. 5.1M). Together these data show that \textit{RB1}-mutant cell lines show replication stress features characterised by shorter IOD distance, slower replication fork speed and fork asymmetry compared to \textit{RB1} wild type cells.

5.3 Investigating replication fork speed in \textit{RB1}-mutant and \textit{RB1} wild type cells upon ATR inhibition

To test whether or not RB1 loss combined with ATR inhibition has an impact on DNA replication speed, I performed DNA fibre analysis in cell lines previously used. Two \textit{RB1}-mutant and two \textit{RB1} wild type cell lines were exposed to a first pulse with CldU for 20 minutes to label actively replication forks, then exposed to 1 μM ATR inhibitor in normal media for 10 minutes, then exposed to a second pulse with IdU together with 1 μM ATR inhibitor for further 20 minutes to monitor fork extension. An experiment diagram is shown in Figure 5.2A. The analysis is based on the measurement of the second pulse (IdU).

As indicated in Figure 5.2B, there is an evident difference in the length of the IdU incorporated DNA (in green) between \textit{RB1}-mutant (H446 and H2009) and \textit{RB1} wild type cells (H1975 and H1993) after ATR treatments. \textit{RB1}-mutant cells exposed to ATR inhibitor appear to have reduced IdU tracts, (in green, Fig. 5.2B).

The analysis of CldU tracts (in red) did not show any difference in length between \textit{RB1}-mutant- and two \textit{RB1} wild type cell lines for DMSO and ATR treatments (Fig. 5.2C and D).

The IdU incorporated fibres were measured for all samples and the frequency distribution was calculated for each treatment (ATR and vehicle). As shown in Figure 5.2E and G, ATR inhibition caused a reduction in fork speed in both \textit{RB1} defective cancer cell lines (H446 and H2009), while replication speed was unaltered in the two \textit{RB1} wild type cancer cell lines (H1975 and H1993) (Fig. 5.2I and M). Thus \textit{RB1}-mutant cells respond with a significant reduction in fork speed after ATR inhibition treatment (Fig. 5.2F and H), compared to DMSO vehicle.
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Figure 5.2: Fork speed analysis in RB1 defective and RB1 wild type cells upon ATR inhibition

A) Schematic of experiment design, involving CldU/IdU pulse-labelled and treatment with 1µM of ATR inhibitor (AZD6738) for 30 minutes or vehicle as indicated. B) Representative images of CldU and IdU replication tracts in vehicle and ATR treated RB1-mutant and RB1 wt cells. Scale bar, 16.1 μm. C) and D) Dot plot graphs of CldU raw data in DMSO and ATR treatment. Frequency distribution of replication fork speed for vehicle or ATR inhibitor treated cells based on IdU tracts length in RB1-mutant cell line H446 in E) and H2009 cell line in G), and RB1 wt cell lines H1975 in I) and H1993 in M). Dot plot graph of raw data are shown in F), H), L) and N) displaying IdU tract length for individual replication forks comparing RB1 wt and RB1-mutant cells treated with vehicle are shown in O). P) Immunoblot analysis showing CHK1 phosphorylation after ATR inhibition in RB1-mutant and RB1 wt cell lines with relative normalisation in Q), cells were harvested after 1 hour treatment, GAPDH served as a loading control. Statistical analysis was conducted using Mann-Whitney test (***p<0.001). Data represent for one of n = 2 representative experiment, data generated in parallel.
Figure 5.3: Evaluation of fork symmetry in RB1 defective and RB1 wild type cells upon ATR inhibition

Data relative to experiment shown in Fig. 5.2. A) Representative image of fork symmetry. Analysis of fork symmetry in RB1-mutant cells in B), C) and D) for H446 cell line and E), F) and G) for H2009 cell line, and RB1 wt cell lines H), I) and L) for H1975 and M), N) and O) for H1993. D), G), L) and O) IdU/IdU ratio for each single cell lines are shown. Scale bar, 16.1 µm. ORI: replication origin. Statistical analysis was conducted using Mann-Whitney test (**p<0.001). Data represent for one of n = 2 representative experiment, data generated in parallel.
To test whether the data shown in Figure 5.2 are in agreement with Bester’s data, the vehicle values were plotted to compare \( RB1 \)-mutant and \( RB1 \) wild type cell replication speed without treatment. However, in contrast to predictions based on the experiment in Figure 5.1B and data by Bester et al., the data in Figure 5.2O did not reveal that unchallenged \( RB1 \)-mutant cells possess a reduced replication fork speed compared to \( RB1 \) wild type cells.

The downregulation of CHK1 phosphorylation was assessed via immunoblotting as a marker of pathway modulation downstream of ATR (Fig. 5.2P and Q), revealing a reduction of CHK1 phosphorylation following ATR inhibition regardless of \( RB1 \) status. Moreover, the analysis of fork symmetry (Fig. 5.3A) showed an increase in frequency of asymmetric forks in vehicle control samples in \( RB1 \)-mutant cells (Fig. 5.3B for H446 and E for H2009), and this frequency became higher after treatment with an ATR inhibitor (Fig. 5.3C for H446 and F for H2009). The differences in fork symmetry were not observed at the same levels of \( RB1 \)-mutant cells in \( RB1 \) wild type cells treated with vehicle control (Fig. 5.3H for H1975 and M for H1993), or after ATR inhibitor treatments (Fig. 5.3I for H1975 and N for H1993). The analysis of the green tract ratios (IdU/IdU) revealed significant differences between \( RB1 \)-mutant and \( RB1 \) wild type cells (Fig. 5.3 D, G, L and O), showing a ratio different from 1, indicating fork asymmetry.

Taken together, the data described above indicate that ATR inhibition induces a reduction in speed of the replication forks and an increase in fork asymmetry in \( RB1 \)-mutant cells compared to \( RB1 \) wild type cell lines.

### 5.4 Effects of \( RB1 \) knockout and ATR inhibition on DNA replication fork speed

In order to confirm that \( RB1 \) loss is responsible for the reduction in DNA replication speed in response to ATR inhibition, I performed fibre assays using \( RB1 \) knockout cell lines previously used (Fig. 5.4 to Fig. 5.9). The experiments in Figures 5.4 and 5.5, were executed in the same way as experiments shown in Figure 5.2, the experiment diagram is shown in Figure 5.4A for H1299 cell line and Figure 5.5A for DMS 114 cell line. The analysis is based on the measurement of the second pulse (IdU).
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Figure 5.4: Determination of fork speed in RB1 knockout cell line H1299 upon ATR inhibition

Cells were labelled with CldU/IdU and treated with ATR inhibitor (AZD6738) or vehicle. A) Schematic of experiment design, involving CldU/IdU pulse-labelled and treatment with 1µM of ATR inhibitor (AZD6738) for 30 minutes or vehicle as indicated. Frequency distribution of replication speed for H1299 shRNA(RB6) [shRB(6)] in B), cells infected with shRNA expression empty vector control (Ctrl) in D), data for H1299 in F). Relative dot plot of IdU tract length for individual replication forks in vehicle and ATR treated are shown in C), E) and G). H) Immunoblot analysis showing CHK1 phosphorylation after ATR inhibition in RB1-mutant and RB1 wt cell lines with relative normalisation in I), GAPDH served as a loading control.

Statistical analysis was conducted using Mann-Whitney test (***p<0.001). Data represent one of n = 2 representative experiment, data generated in parallel.
Figure 5.5: Evaluation of fork speed in RB1 knockout cell line DMS 114 upon ATR inhibition

Cells were labelled with CldU/IdU and treated with ATR inhibitor (AZD6738) or vehicle. 

A) Schematic of experiment design involving CldU/IdU pulse-labelled and treatment with 1 µM of ATR inhibitor (AZD6738) for 30 minutes or vehicle as indicated. Frequency distribution of replication speed for DMS 114 shRNA(RB2) [shRB(2)] in B), cells infected with shRNA expression empty vector control (Ctrl) in D), data for DMS 114 in F). Relative dot plot of IdU tract length for individual replication forks in vehicle and ATR treated are shown in C), E) and G). H) Immunoblot analysis showing CHK1 phosphorylation after ATR inhibition in RB1-mutant and RB1 wt cell lines with relative normalisation in I), GAPDH served as a loading control.

Statistical analysis was conducted using Mann-Whitney test (***p<0.001). Data represent for one of n = 2 representative experiment, data generated in parallel.
Figure 5.6: Validation of RB1 knockout cell line H1299 replication speed upon ATR inhibition

Cells were treated with ATR inhibitor (AZD6738) or vehicle and labelled with CldU/IdU. A) Schematic of experiment design, involving treatment with 1µM of ATR inhibitor (AZD6738) for 2 hours or vehicle as indicated and CldU/IdU pulse-labelled. Frequency distribution of replication speed for H1299 shRNA(RB6) [shRB(6)] in B), cells infected with shRNA expression empty vector control (Ctrl) in D), data for H1299 in F). Relative dot plot of IdU tract length for individual replication forks in vehicle and ATR treated are shown in C), E) and G). H) Representative image of inter-origin distance (IOD). Mean IOD values from H1299-shRB(6) cells in I) from H1299-Ctrl in L) and H1299 in M).

Statistical analysis was conducted using Mann-Whitney test (**p<0.001). Data represent for one of n = 2 representative experiment, data generated in parallel. Scale bar, 16.1 µm. ORI: replication origin.
Figure 5.7: Analysis of fork symmetry in RB1 knockout cell line H1299 upon ATR inhibition

Data relative to experiment shown in Fig. 5.6. A) Representative image of fork symmetry. Analysis of fork symmetry for H1299 shRNA(RB6) [shRB(6)] cells vehicle treated in B) ATR inhibitor treated in C) cells infected with shRNA expression empty vector control (Ctrl) vehicle treated in E) ATR inhibitor treated F) data for H1299 vehicle treated in H) ATR inhibitor treated in I). D), G) and L) IdU/IdU ratio for each single cell lines are shown. M) and N) IdU/IdU ratio for DMSO and ATR treatments. Statistical analysis was conducted using Mann-Whitney test (***p<0.001). Scale bar, 16.1 μm. ORI: replication origin.
Figure 5.8: Validation of RB1 knockout DMS 114 cell line replication speed upon ATR inhibition

Cells were treated with ATR inhibitor (AZD6738) or vehicle and labelled with CldU/IdU. A) Schematic of experiment design, involving treatment with 1μM of ATR inhibitor (AZD6738) for 2 hours or vehicle as indicated and CldU/IdU pulse-labelled. Frequency distribution of replication speed for DMS 114 shRNA(RB2) [shRB(2)] in B), cells infected with shRNA expression empty vector control (Ctrl) in D), data for DMS 114 in F). Relative dot plot of IdU tract length for individual replication forks in vehicle and ATR treated are shown in C), E) and G). H) Representative image of inter-origin distance (IOD). Mean IOD values from DMS 114-shRB(2) cells in I) from DMS 114-Ctrl in L) and DMS 114 in M).

Statistical analysis was conducted using Mann-Whitney test (**p<0.001). Data represent for one of n = 2 representative experiment, data generated in parallel. Scale bar, 16.1 μm. ORI: replication origin.
**Figure 5.9: Analysis of fork symmetry in RB1 knockout cell line DMS 114 upon ATR inhibition**

Data relative to experiment shown in Fig. 5.6. A) Representative image of fork symmetry. Analysis of fork symmetry for DMS 114 shRNA(RB2) [shRB(2)] cells vehicle treated in B) ATR inhibitor treated in C) cells infected with shRNA expression empty vector control (Ctrl) vehicle treated in E) ATR inhibitor treated F) data for DMS 114 vehicle treated in H) ATR inhibitor treated in I) D), G) and L) IdU/IdU ratio for each single cell lines are shown. M) and N) IdU/IdU ratio for DMSO and ATR treatments. Scale bar, 16.1 µm. ORI: replication origin. Statistical analysis was conducted using Mann-Whitney test (***p<0.001). Data represent for one of n = 2 representative experiment, data generated in parallel.
RB1 knockout cell lines showed a significant reduction in DNA replication speed upon ATR inhibition (Fig. 5.4B and C for H1299 and Fig. 5.5B and C for DMS 114) compared to cells treated with vehicle control. The difference in DNA replication speed observed in RB1 knockout cells was not observed in cells modified using the empty vector control (Ctrl) (Fig. 5.4D and E for H1299 and Fig. 5.5D and E for DMS 114) nor in unmodified cells (Fig. 5.4F and G for H1299 and Fig. 5.5F and G for DMS 114). A biomarker modulation analysis was set up in parallel and the effect of ATR inhibition was validated via immunoblots in Figure 5.4H for H1299 and Figure 5.5H for DMS 114, which showed a reduction in phospho-CHK1 signal after ATR inhibition regardless of RB1 status. The level of CHK1 phosphorylation was quantified and shown in Figure 5.4I for H1299 and Figure 5.5I for DMS 114. To independently confirm the data shown in Figure 5.4 and 5.5, and further within the same experiment to allow a fair assessment of origin distance response, a different pulsing scheme was used. The experiments diagram is shown in Figure 5.6A for H1299 and Figure 5.8A for DMS 114.

Cells were treated with an ATR inhibitor for 2 hours, subsequently pulsed for 20 minutes with CldU followed by 20 minutes with IdU. The data shown in Figures 5.6 and 5.8, which use this second pulsing scheme, are in agreement with data shown in Figures 5.4 and 5.5. The analysis is based on the measurement of the second pulse (IdU). In fact, ATR inhibition reduced replication fork speed in RB1 knockout lines (Fig. 5.6B for H1299 and Fig. 5.8B for DMS 114) compared to maternal lines (Fig. 5.6F for H1299 and Fig. 5.8F for DMS 114) and to the empty vector control (Ctrl) cells (Fig. 5.6D for H1299 and Fig. 5.8D for DMS 114). Moreover, in agreement with the data shown in Figure 5.1 and 5.2, there is a reduced IOD in RB1 knockout lines (Fig. 5.6I for H1299 and Fig. 5.8I for DMS 114) compared to maternal cell lines and to the empty vector control treated (Ctrl) cells, most likely to refer to under-replication phenomena. Additionally, analysis of the fork symmetry showed an increase in the frequency of asymmetric forks in RB1 knockout lines (Fig. 5.7B, C and D for H1299 and Fig. 5.9B, C and D for DMS 114) compared to the control cell lines, indicating the features of replication stress after RB1 knockout amplified by ATR inhibition. The analysis of the green tract ratios (IdU/IdU) resulted in significant differences between RB1 knockout and maternal cell lines (Fig. 5.7D for H1299 and 5.9D for DMS 114). Moreover, the analysis of DMSO vehicle and ATR inhibitor treated cells showed a ratio
different than 1 in RB1 knockout cell lines, indicating fork asymmetry (Fig. 5.7M and N for H1299 and 5.9M and N for DMS 114.
These data show that RB1 status can influence the DNA replication speed upon ATR inhibition and are in agreement with data showed in Figure 5.1 and 5.2.

5.5 Summary
Considering the importance of ATR function during DNA replication, and the role of RB1 loss in increasing genome instability, I wished to test DNA replication progression in RB1-mutant and RB1 wild type cells using DNA fibre analysis.

As previously reported, cells deficient in various cell cycle checkpoint or DNA repair proteins display reduced replication fork speed and under- or over-replication events in absence of exogenous challenge (Techer et al. 2017). Indeed RB1-mutant cells displayed the key features of replication stress with a significant increase in the frequency of asymmetric forks and reduced IOD under basal conditions. Those replication stress factors were enhanced after ATR inhibition, suggesting that cells under replication stress have increased origin initiation inducing DNA damage formation.

Based on the data discussed in this chapter, I sought to assess how RB1 loss may impact the DNA replication progression under the effect of replication fork stalling agent Hydroxyurea (HU).
CHAPTER SIX

RB1 loss and DNA replication fork stability
Chapter Six: RB1 loss and DNA replication fork stability

6.1 Introduction

As previously discussed in the introduction (section 1.8), DNA replication is a very vulnerable process that can lead to replication impairments causing replication stress. To prevent replication stress-induced DNA damage, cells have developed many fork protection mechanisms to reduce such genotoxic effects by stabilising, repairing and restarting stalled forks (McGrail et al., 2018).

It has been reported that functional defects in replication fork protection correlates with ATR inhibitor sensitivity (Hill et al., 2018). Hill et al., developed organoids from high grade serous ovarian cancers samples and used them for functional analysis of DNA repair to predict patients’ clinical response to DNA repair inhibitors. They show that, regardless of the genetic status, a stalled fork protection defect was present in 61 % of the organoid lines tested, and furthermore this defect was associated with VE-822 (ATR inhibitor) sensitivity (Hill et al., 2018).

Considering the observations of Hill et al. and what has been discussed in previous chapters regarding RB1-mutant cells sensitivity towards ATR inhibitors, I sought to test whether there is a lack of fork protection in RB1 defective cancer cells and if RB1 loss may be the cause.

6.2 RB1 status and replication fork stability

In order to determine whether RB1 status may affect replication fork stability, cells were treated with the fork stalling agent Hydroxyurea (HU) and DNA replication progression was analysed via fibre assay (Fig. 6.1). HU is an antimetabolite drug, which acts by disrupting the DNA replication process of dividing cells. In cells, HU induces replication stress and blocks the DNA replication by inhibiting the ribonucleoside diphosphate reductase. Ribonucleotide diphosphate reductase is an enzyme that catalyses the formation of deoxyribonucleotides from ribonucleotides. It catalyses this formation by removing the 2’-hydroxyl group of the ribose ring of nucleoside diphosphates. This reduction produces deoxyribonucleotides which are in turn used for the synthesis of DNA.
Figure 6.1: RB1 status and replication fork stability

A) Schematic of CldU/IdU pulse-labelling samples were harvested either directly (T0) or following treatment for 5 hours with 2 mM HU (5h HU). B), D), F) and H) Dot blot of IdU to CldU tract length ratios for individual replication forks in T0 and 5h HU samples. B) and C) RB1-mutated cells H446, D) and E) RB1-mutated cells H2009, F) and G) RB1 wt cells H1975 and H) and I) RB1 wt cells H1993. C) G), H) and I) Dot plot of IdU and CldU raw data tracts length for individual replication forks in T0 and 5 hours HU treated RB1-mutated cells are. L) Detection by immunoblot of CHK1(pS345) and CHK1 in cells treated with 2 mM HU or vehicle for 5 hours, GAPDH served as a loading controls. Statistical analysis was conducted using Mann-Whitney test (***p<0.001). Data are represented for one independent experiment, data generated in parallel.
Two RB1-mutant and two RB1 wild type cell lines were exposed to two sequential pulses with CldU and IdU. Following this, DNA replication was arrested for 5 hours with 2 mM of HU. The control samples (T0) were harvested at the same time that HU was added whereas the treated cells were processed after 5 hours of HU treatments (Fig. 6.1A).

The degree of fork degradation was then assessed by measuring the ratio of IdU to CldU tract lengths in response to HU. If the ratio of the track lengths is one, then the fork was protected during replication stalling. If the ratio of the track lengths is less than one, then the fibre containing the second analogue was degraded, indicating that the tumour cells are unable to protect their stalled forks.

RB1-mutant cells (H446 and H2009) show a significantly reduced IdU/CldU tracts length (lower than one) after 5 hours of HU-treatment cells compared to vehicle (T0) (Fig. 6.1B for H446 and D for H2009). In contrast, RB1 wild type cells (H1975 and H1993) did not show any difference in IdU/CldU ratios between vehicle- or HU-treated cells (Fig. 6.1F for H1975 an H for H1993).

Figure 6.1 also shows the raw data of the tract lengths as Kb/min, both in T0 and after 5 hours HU treatment in RB1-mutant (Fig. 6.1C for H446 and E for H2009) and RB1 wild type cells (Fig.6.1G for H1975 and I for H1993). These data indicate that the length of the IdU-DNA fibres treated with HU are shorter compared to the rest of the IdU-DNA fibres T0 in RB1-mutant cells (H446 and H2009), whereas in RB1 wild type cells (H1975 and H1993) the tracts length of the IdU-DNA is the same in the vehicle samples (T0) and in the HU-treated samples. Furthermore, there was no difference in track length between T0 and HU in CldU-DNA fibres, indicating that the difference in the ratio is not due to a difference in initial replication speed.

These data together indicate that RB1-mutant cells are not able to protect the replication fork under replication stalling conditions such as that induced by HU treatment, in contrast to RB1 wild type cells, where the IdU/CldU ratio was equal to one.
Figure 6.2: Mitigation of ATR inhibition in cells with different RB1 status

Immunoblot analysis assessing CHK1 engagement in cells with different RB1 status upon ATR and HU treatments. Cells were seeded into 6 well plates. After 24 hours cells were treated with ATR inhibitor (AZD6738) at the indicated concentrations alone or in combination with HU. Lysates were prepared 5 hours post inhibitor addition and analysed using western blots. A) Data for H1975, H1993, H2009 and H446. B) Quantification relative to GAPDH. GAPDH served as a loading control. Data represent for one of n = 2 representative experiment.
To test the activation of replication stress cascade induced by HU treatments, I set up a biomarker modulation test (Fig. 6.1). The ATR pathway modulation was tested via immunoblotting (Fig. 6.1L), which showed an increase in CHK1 phosphorylation, indicating pathway activation. The response seen in RB1-mutant and RB1 wild type cells indicates that cells responded to HU even though the fork stability was only affected in RB1 defective cells. To test whether there is a difference in the replication stress cascade due to the RB1 status, I set up another biomarker modulation test (Fig. 6.2). Again, the ATR pathway activation was tested via immunoblotting (Fig. 6.2A), which showed an increase in CHK1 phosphorylation after treatment with HU alone for all cell lines. However, when HU was combined with the ATR inhibitor the RB1-mutant cells show a lower phospho-CHK1 activation compared to RB1 wild type.

6.3 RB1 gene knockout and replication fork stability

In order to validate whether RB1 loss causes destabilisation of the replication fork under stalling agents such as HU, I performed fibre assays using RB1 knockout cell lines (Fig. 6.3 and 6.4). The RB1 knockout in these cell lines was validated via immunoblot after the shRNA infection and antibiotic selection, a new vial of cells was thawed before every single experiment was carried out (see section 2.3.1).

The experiments were executed in the same way as experiments shown in Figure 6.1, the experiment diagram is shown in Figure 6.3A for H1299 cell line and Figure 6.4A for DMS 114 cell line.

As in the previous experiment, fork degradation was assessed by measuring the ratio of IdU to CldU tract lengths in response to a 5 hours treatment with HU.

As observed in RB1-mutant cells (Figure 6.1), RB1 knockout cells show significantly reduced IdU/CldU tracts length in HU-treated cells (Fig. 6.3B for H1299 and 6.4B for DMS 114) compared to T0. On contrary, empty vector controls and maternal lines do not show any difference in IdU/CldU ratios (Fig. 6.3D and F for H1299 and 6.4D and F for DMS 114). Figure 6.3C and Figure 6.4C show the raw data of the tracts length for individual replication forks at T0 and after 5 hours HU treatment. While RB1 gene knockout induces an IdU-DNA fibres degradation under HU effects; this effect was not observed in the empty vector control (Fig. 6.3E for H1299 and Fig. 6.4E for DMS 114) nor in the maternal line (Fig. 6.3G for H1299 and Fig.6.4G for DMS 114).
Figure 6.3: *RB1* knockout and replication fork stability in H1299 cell line

**A)** Schematic of CldU/IdU pulse-labelling followed by 5 hours HU (2 mM) treatment. **C), E) and G)** Dot blot of IdU to CldU tract length ratios for individual replication forks at T0 or 5h HU. **B), D) and F)** Raw data for H1299 cells infected with individual lentiviral targeting RB1 or vector control (Ctrl). **B) and C)** H1299 cells infected with individual lentiviral shRNA(RB6) [shRB(6)]. **D) and E)** cells infected with shRNA expression empty vector (Ctrl), **F) and G)** RB1 wt cells H1299. **H)** Detection by immunoblot of CHK1(pS345), CHK1 and GAPDH of cells treated with 5 hours 2 mM HU or vehicle. GAPDH served as a loading control.

Statistical analysis was conducted using Mann-Whitney test (**p<0.001). Data are represented for one independent experiment, data generated in parallel.
Figure 6.4: *RB1* knockout and replication fork stability in DMS 114 cell line

A) Schematic of CldU/IdU pulse-labelling followed by 5 hours HU (2 mM) treatment.

C), E) and G) Dot blot of IdU to CldU tract length ratios for individual replication forks at T0 or 5h HU. B), D) and F) Raw data for DMS 114 cells infected with individual lentiviral targeting RB1 or vector control (Ctrl). B) and C) DMS 114 cells infected with individual lentiviral shRNA(RB2) [shRB(2)], D) and E) cells infected with shRNA expression empty vector (Ctrl), F) and G) RB1 wt cells DMS 114. H) Detection by immunoblot of CHK1(pS345), CHK1 and GAPDH of cells treated with 5 hours 2 mM HU or vehicle. GAPDH served as a loading control.

Statistical analysis was conducted using Mann-Whitney test (**p<0.001). Data are represented for one independent experiment, data generated in parallel.
Figure 6.5: Assessment of DNA replication progression in RB1 knockout cells under basal conditions

A) Schematic of inter-origin distance (IOD). B) and C) Dot plot graph of raw data displaying IOD comparing RB1 knockout and RB1 wt cells. Mean IOD values from H1299 in B) and DMS 114 in C). D) Schematic of asymmetric DNA fibres. Analysis of fork symmetry analysis for RB1-knockout cells H1299-shRB(6) in E) and H) for DMS 114-shRB(2). F) Fork symmetry analysis for empty vector control for H1299 and I) for DMS 114. Analysis of fork symmetry in RB1 wt cells H1299 in G) and DMS 114 in L). p values were calculated using Kruskal-Wallis test, *p<0.05. ORI: replication origin.

The ATR pathway modulation analysis after treatment with 2 mM of HU was validated via immunoblots (Fig. 6.3H for H1299 and Figure 6.4H for DMS 114). The increase in
phospho-CHK1 signal under HU treatment, indicates the activation of the pathway under replication stress in both RB1 knockout and RB wild type cells.

Moreover, the analysis of vehicle treated cells, showed a significant decrease of the IOD (Fig. 6.5B and C) observed in RB1 knockout cells compared to RB1 wild type cells for H1299 (Fig. 6.5B) and DMS 114 (Fig. 6.5C).

The analysis of fork symmetry (Fig. 6.5E-L), showed an increase in frequency of asymmetric forks in RB1 knockout cells (Fig. 6.5E for H1299-shRB(6) and H for DMS 114-shRB(2)). The difference in fork symmetry were not observed in RB1 wild type cells (Fig. 6.5G for H1299 and L for DMS 114), nor in empty vector control (Fig. 6.5F for H1299-Ctrl and I for DMS 114-Ctrl).

Taken together these data indicate that RB1 loss causes nascent DNA degradation in cells after replication stress. Furthermore, RB1 knockout cell lines show replication stress features characterised by short IOD distance and fork asymmetry.

6.4 Chromatin-bound proteins enrichment after RB1 knockout

As previously described, RB1 loss induces fork resection after treatment with fork stalling agent HU. To test whether there is a recruitment of DNA nucleases to the chromatin, I set up a chromatin fractionation test and I analysed the soluble, chromatin and total fractions via immunoblot (Fig. 6.6).

As described in the introduction (section 1.8.3), MRE11 performs short-range resection. Decreased levels of MRE11 in the chromatin fraction were detected after HU treatment for both H1299 maternal and H1299 sh(RB6) (Fig. 6.6A and B) compared to loading controls. There was no difference between soluble and total fraction between different treatments.

As previously discussed in section 1.8, under conditions of replication stress, the regulation of origin firing becomes more critical, because excessive or continued origin firing generates an excess of ssDNA, exhausting the RPA pool which eventually causes replication fork collapse.
Chapter Six: *RB1* loss and DNA replication fork stability

Figure 6.6: Chromatin-bound proteins enrichment upon ATR and HU treatments in cells with *RB1* knockout H1299 cell line

Immunoblot analysis assessing MRE11, RPA32 and RB1 engagement in H1299 RB1 knockout cell line following ATR and HU treatments. Cells were seeded into 6 well plates. After 24 hours cells were treated with ATR inhibitor (AZD6738) at the indicated concentrations alone or in combination with HU. Lysates were prepared 2 and 6 hours post inhibitor addition and analysed using western blots. **A**) First repeat for soluble, chromatin and total fractions for H1299 maternal and shRNA(RB6) [shRB(6)]. **B**) Second repeat for chromatin fractions for H1299 maternal and shRNA(RB6). GAPDH and histone H3 served as loading controls.

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**A)** First repeat for soluble, chromatin and total fractions for H1299 maternal and shRNA(RB6) [shRB(6)]. **B**) Second repeat for chromatin fractions for H1299 maternal and shRNA(RB6). GAPDH and histone H3 served as loading controls.
As shown in Figure 6.6A and B, the levels of RPA32 in the chromatin fraction were undetectable, further investigation would be needed. In the soluble part there is also an RPA32 downregulation in the H1299 sh(RB6) samples compared to H1299 maternal line, on the other hand in the total fraction there is no evident difference in RPA32 expression between different samples. Moreover, data in Figure 6.6A show that RB1 was detectable in the soluble and total fraction but not on the chromatin.

6.5 Summary

It has previously been reported that functional defects in replication fork protection is associated with ATR inhibitor sensitivity (Hill et al., 2018). Taking together with the fact that RB1 loss is linked to increased genomic instability; I sought to test whether or not RB1 loss has an impact on fork protection using a DNA fibre analysis.

Data discussed in this section indicate that RBL1-mutant cells are unable to protect the replication fork in the presence of a replication stalling agent such as HU, as the IdU/CldU ratio is different than one in contrast to RBL1 wild type cells. Although RB1 was not detected in the chromatin fraction, further investigation would be needed to better understand its possible function in fork protection.

Moreover, these data were validated in RBL1 knockout cell lines, indicating that RB1 loss could induce defects in replication fork protection under replication stalling agents, data confirmed by the IOD reduction and an increase in fork asymmetry in basal conditions.
CHAPTER SEVEN

Discussion
7 Discussion

The Retinoblastoma tumour suppressor (RB1) is well known as the regulator of S phase entry in the cell cycle. Recent studies have shown a link between RB1 loss and genome instability leading to tumour development (Dyson 2016, Coschi et al. 2010). Additionally, RB1 has been reported to take part in both HR and NHEJ repair (Cook et al. 2015, Velez-Cruz et al. 2016), suggesting the hypothesis that RB1 might indirectly regulate DSB repair pathways which have been investigated in the work presented in this thesis.

7.1 RB1 loss and sensitivity towards PARP and ATR inhibition

RB1 loss is a hallmark of cancer and occurs at different frequencies in multiple types of cancers. Based on published evidence highlighting the importance of RB1 inactivation in the accumulation of DSBs, the aim of this work was to study if the loss of RB1 would lead to an increased sensitivity to DDR pathway inhibition.

Following a DDR pathway inhibitor screen, it has been shown that RB1-mutant cancer cell lines are particularly sensitive to PARP inhibition compared to RB1 wild type cell lines (Fig. 3.2). PARP has an essential and well-documented role in the repair of DNA single strand breaks (SSB). If left unrepaired, SSBs block the DNA replication fork during S phase generating DSBs. PARP also promotes the repair of DSBs via the alternative NHEJ (aNHEJ) repair (Corneo et al., 2007), facilitating the resolution of stalled replication forks and allowing DNA replication restart. Selective hypersensitivity to PARP inhibitors has been shown in BRCA1/2-mutant cancers. Mutations in BRCA1/2 genes are found in breast, ovarian and prostate cancer and lead to defects in HR repair (Bryant et al., 2005). Previously discussed publications indicate that RB1 is required to support NHEJ and HR repair (Cook et al. 2015, Velez-Cruz et al. 2016), supporting the findings of this study that PARP inhibition in RB1-mutant cells leads to cell death. This effect can be described as synthetic lethality. Indeed, it would be interesting to test the HR and NHEJ repair pathway efficiency in the RB1 loss scenario under ATR inhibition.

In this thesis, it has further been shown how RB1-mutant cancer are more sensitive towards multiple ATR inhibitors compared to RB1 wild type cell lines.
Preclinical studies have shown that cancer cells with defects in DNA repair pathways or oncogene-induced replication stress, may be sensitive to ATR inhibition. The development of multiple ATR inhibitors allowed the investigation of these compounds in monotherapy as well as combinatorial trials and their further optimisation. From this, ATR inhibitors have been described to sensitise ATM or TP53-deficient cancer cells to cisplatin (Vendetti et al., 2015, Wengner et al., 2020). Furthermore, the blocking of ATR activity with small molecule inhibitors, either alone or in combination with DNA-damaging agents, can lead to mitotic catastrophe of cancers cells and TP53-independent cell death (Vendetti et al., 2015). Currently, ATR inhibitors in early-phase clinical trials are used in ATM and TP53-deficient cancers (Mei et al., 2019). However, at the moment there is a critical lack of data on whether specific oncogenes or the inactivation of tumour suppressors could serve as predictive biomarkers for ATR inhibitor sensitivity.

Data presented in this thesis shows that ATR inhibition leads to decreased cell viability in RB1-mutant cells using a metabolic activity assay (Fig. 3.2-3). Furthermore, ATR inhibition is shown to elicit a similar effect on colony formation. The clonogenic assay tests the effects of external stress signals on cells survival by measuring the cells ability to retain their reproductive integrity over a prolonged period of time. RB1-mutant cancer cells showed a reduced capacity to form colonies upon ATR inhibition (Fig. 3.4), although the growth rate of the cell lines tested was different, showing RB1 wild type cell lines getting over-confluent compared to RB1-mutant cell lines in the same time frame. This underlines a weakness of these results and further investigations would be needed testing further cell lines and different seeding densities.

Moreover, RB1-mutant cell lines exhibited higher levels of cell death upon treatment with different ATR inhibitors compared to RB1 wild type cells. These results were achieved via SYTOX™ green stain, indicating that upon ATR inhibition the cell membrane integrity becomes damaged (Fig. 3.5-6).

To summarise, RB1-mutant cells are less viable, not able to form colonies and show increased cell death upon ATR inhibition compared to RB1 wild type cells. These conclusions were reinforced by data showing PARP cleavage in RB1-mutant cells which was not observed in RB1 wild type cancer cells. These data confirm the activation of the apoptotic pathway further indicating the RB1-mutant cancer cells sensitivity to ATR
inhibitors. The ATR sensitivity can be linked to the DNA replication phenotype observed in \textit{RB1}-mutant cells discussed in chapter five.

7.2 Differences in ATR pathway regulation between \textit{RB1}-mutant and \textit{RB1} wild type cell lines

In recent published work from Buisson et al., it has been described how ATR inhibition induces ssDNA accumulation and replication catastrophe in a fraction of early S-phase cells. However, in other S-phase cells, ATR inhibition induces moderate ssDNA and triggers a DNA-PK and CHK1-mediated back-up pathway to suppress origin firing. The back-up pathway creates a threshold such that ATR inhibition selectively kills cells under high replication stress (Buisson et al. 2015).

In order to investigate if the findings by Buisson apply to \textit{RB1}-mutant cancers, multiple cancer cell lines with different functional \textit{RB1} status were treated with ATR and DNA-PKcs inhibitors (Fig. 3.7). Interestingly, data obtained for several \textit{RB1} wild type cells is indeed in agreement with Buisson et al., showing an up-regulation of phospho-DNA-PKcs upon ATR inhibition and a down-regulation of phospho-DNA-PKcs after combined treatment with ATR and DNA-PKcs inhibitors. One exception was the \textit{RB1} wild type cell line H1299 which did not show phospho-DNA-PKcs activation, possibly due to its genetic background which could be subject of further investigation. On the other hand, naturally \textit{RB1}-mutant cells do not show detectable activation of phospho-DNA-PKcs or phospho-CHK1 compared to \textit{RB1} wild type cell lines.

The lack of DNA-PKcs and CHK1 activation in \textit{RB1}-mutant cells could explain the sensitivity of these cells towards ATR inhibition, possibly indicating a short G1 phase; a theory which is supported by data published by Cook et al., showing a reduced ability to induce DNA repair, and consequently DSB formation in \textit{RB1}-mutant cell lines and most importantly loss of NHEJ repair function. This observation leaves open the possibility for a different interpretation not related to DNA-PKcs and CHK1 activation but also opens the question as to whether other kinases are relied upon ATR inhibition, such as ATM.

It is very important to consider the weakness of Buisson et al.’s data as well, as only one cell line (U2OS) has been used, which could have a particular genetic background that enabled their conclusions. Indeed, including further cell lines with different genetic backgrounds like in Fig. 3.7 resulted in rather different outcomes compared to Buisson et
al. This would explain the different results presented in chapter 3 in comparison to the data of Buisson et al. and leave room for further studies.

7.3 *RB1* knockdown and knockout in combination with ATR inhibition

To show that ATR inhibitor sensitivity is caused by the loss of functional RB1, *RB1* wild type cells were treated with different siRNA as well as shRNA constructs targeting *RB1*. For the experiments discussed in chapter four, siRNA targeting all three RB pocket proteins were used to remove possible functional redundancy between family members. Indeed, the pocket proteins, share various cellular functions: they all arrest cell cycle progression, principally due to their ability to associate with E2F transcription family members and thus repress E2F gene expression. The pocket proteins regulate gene transcription through their inhibition of E2F and by engaging transcriptional co-regulators that modify histones and chromatin structure.

However, multiple observations suggest important differences between the pocket proteins. It has been shown that the knockout of *RB1* in mice is embryonic lethal, whereas the knockout of p107 and p130 results in a viable mouse (Cobrinik et al., 1996, Lee et al., 1996, Jacks et al., 1992). Notably, RB1 tumour suppressor features are thought to be stronger than p107 and p130 and mutations in p107 and p130 genes, are less common in cancer compared to RB1 (Burkhart and Sage, 2008).

It has been proposed that different E2F transcription factor binding proteins mediate the different pocket protein activities. In humans, RB1 binds E2F1-5, whereas p107 and p130 almost exclusively associate with E2F4 and E2F5. A recently published comparison of the crystal structures of RB1 and p107 identified several residues that contribute to the E2F selectivity of the pocket proteins (Liban et al., 2016).

Experiments of *RB1* knockdown enforced and proved that ATR sensitivity is cause by RB1 loss (Fig. 4.1), as demonstrated by viability assays. Although the *RB1* knockdown showed in Fig. 4.1B and C should have been tested at the completion of the experiment, after 5 days of ATR treatment. Data in Fig. 4.2 showed how RB1 loss increased the levels of cell death upon ATR inhibition, even though RB1 knockdown samples showed initial
cell death levels, maybe due to the cytotoxicity of the siRNA constructs (Fig. 4.2A and B).

The clonogenic assays also showed ATR inhibitor sensitivity upon RB1 loss, although the shRNA construct RB(1) (Fig. 4.4A) seemed to be cytotoxic by itself, affecting DMS 114 cell growth; this will need further investigations and considerations.

Moreover, cells without a functional \textit{RB1} gene undergo higher level of cell death and apoptotic pathway activation compared to \textit{RB1} wild type cells (Fig. 4.5-6).

Interestingly, \textit{RB1} knockdown in \textit{RB1} wild type cell lines did not alter DNA-PKcs phosphorylation (Fig. 4.7), as observed in \textit{RB1}-mutant cell lines (Fig. 3.7). Therefore, the lack of DNA-PKcs activation is unlikely to explain the difference in ATR inhibitor sensitivity between \textit{RB1}-mutant and \textit{RB1} wild type cells.

Furthermore, \textit{RB1} knockdown did not influence CHK1 activation. A possible explanation could be the different genetic backgrounds of \textit{RB1} wild type and \textit{RB1}-mutant cell lines used in this study. Further research into other contributing mutations is necessary to better understand this observation.

\textbf{7.4 RB1 loss and DNA replication fork speed}

As previously discussed in section 5.1, RB1 loss influences the regulation of DNA replication as well as genome integrity.

To ensure that the entire genome is duplicated during every cell cycle in an error-free manner, the S phase is tightly regulated by the correct transcriptional synchronisation of the RB1/E2F pathway (Takeda and Dutta, 2005). RB1 is known to inhibit S phase initiation and several studies have identified different roles of RB1 in regulating DNA replication (Wikman and Kettunen, 2006, Braden et al., 2006). RB1, in fact, is also required for a prompt and proper DNA damage response, while the loss of RB1 results in deregulated replication control (Srinivasan et al., 2007). Considering the different roles of RB1 in DNA replication control, it was important to assess the impact of RB1 loss on DNA replication progression.

Data presented in this thesis shows that \textit{RB1}-mutant cell lines display the main features of replication stress, such as reduced replication speed, asymmetric forks and reduced IOD under basal conditions. Those replication stress indicators were enhanced upon ATR
inhibition (Fig. 5.1-3). Work by Bester et al. in 2011, showed that the slowing of replication fork speed following RB protein loss and p53, appears to result from dNTP precursor deprivation. The data presented in Figure 5.1 seems to be in agreement with Bester et al., showing that RB1-mutant cells under unperturbed conditions replicate their DNA slower compared to RB1 wild type cells. On the contrary, replication speed analysis of unperturbed conditions in Figure 5.2O did not show such a result.

Due to cell replication behaviour or technical issues, leaving open the possibility to exploit the replication speed in RB1-mutant cells in further details. Moreover, Bester et al. suggested an exogenous supply of nucleosides can rescue the replication stress in cells with an abnormal activation of the RB1/E2F pathway. In the work presented here such a scenario could not be reproduced in RB1-mutant cells (data not shown), which could be due to an insufficient sample size within the experiments as well as differences in the genetic background of the cell lines used.

Data obtained from experiments on RB1-mutant as well as RB1 wild type cells has been confirmed by RB1 knock out, resulting in reduced replication fork speed, reduced IOD and increased fork asymmetry upon ATR inhibition (Fig. 5.4-9), again underlining RB1 loss as the cause of replication stress features (Bellelli et al., 2020, Bellelli et al., 2018, Rodriguez-Acebes et al., 2018). In addition, this data has been further supported using a different DNA labelling scheme highlighting the importance of utilising the DNA fibre analysis beyond the conventional protocols.

In order to identify the basis for perturbed DNA replication in RB1-mutant cells and in RB1 knockout cells, it is essential to consider what has been discussed in section 1.7 and in section 5.1. ATR is a critical component of DNA replication stress response and is activated by regions of ssDNA, some of which occur as a result of replication stress. When active, ATR activates CHK1 which limits S phase progression and is essential to the survival of cancer cells that lack other cell cycle checkpoints or possess high replication stress (Massey, 2017, Qiu et al., 2018). In unperturbed conditions, CHK1 loss generates an excess of origin firing and reduces fork elongation (Maya-Mendoza et al., 2007). CHK1 inhibits origin firing by inducing the degradation of CDC25A, with a consequent inhibition of CDK2 (Sorensen and Syljuasen, 2012). According to published work by Gonzalez Besteiro et al. in 2019, loss of CHK1 contributes to the accumulation of
replication fork barriers, which are created by excessive loading of helicase components. CHK1 loss, in fact, activates CDK2 in S phase which subsequently phosphorylates DNA polymerase eta (Polη), inducing the accumulation of replication barriers that can be deleterious to fork protection and fork elongation (Gonzalez Besteiro et al., 2019). In summary, this could offer an explanation for the decrease in replication fork speed observed in RB1-mutant cells and not in RB1 wild type cells described in chapter five. Indeed, it would be interesting to test Polη recruitment upon ATR inhibition in RB1-mutant cell lines.

7.5 RB1 loss and DNA replication fork stability
To maintain genome integrity in dividing cells, complete and accurate duplication of the DNA in S phase is required. This is only possible by complete control of the DNA replication program at the level of both, origin firing and replication fork progression. Many of the components that constitute the replication fork are well defined; recent proteomic analyses have categorised fork components during normal elongation as well as under stress conditions. An increasing amount of data suggests that replication forks recruit repair components even during unstressed replication, revealing that this system is well programmed to respond to any fork impediments.

Cells have developed a multitude of fork protection mechanisms to stabilise, repair and restart stalled forks and consequently reduce genotoxic effects (McGrail et al., 2018). Recently published data revealed that functional defects in replication fork protection correlates with ATR inhibitor sensitivity (Hill et al., 2018).

Using fibre assay as a read out, RB1-mutant cell lines showed fork degradation under replication stress induced by HU, which was not observed in RB1 wild type cells (Fig. 6.1). The findings have been extended and confirmed in RB1 knockout cell lines, which also showed a significant decrease of the IOD and an increase in the frequency of asymmetric forks (Fig. 6.3-5). Taken together, the data produced clearly argues that RB1 loss induces fork resection under replication stress conditions. To further investigate these findings, experiments were performed to test whether increased resection may be explained by increased recruitment of DNA nucleases to the chromatin (Fig. 6.6). However, the results of these experiments show that MRE11, which performs short-range resection, was recruited to the chromatin after HU treatment regardless of RB1 status.
Further experiments would be needed to be able to draw robust conclusions about the DNA resection in RB1-mutant cell lines.

### 7.6 Future work

In addition to further work aiming to confirm the role of RB1 loss as the cause of ATR inhibitor sensitivity, it would be interesting to perform rescue experiments introducing functional RB1 back into RB1-mutant cell lines. To do so, it would be necessary to restore RB1 protein function to endogenous levels and test whether cells gain resistance to ATR inhibition. In the vast majority of cases, mutations in the RB1 gene are accompanied by high levels of cyclin-dependent kinase inhibitor protein (p16-INK4a) expression, precluding activation of CDK4/6 in cancer cells, hence limiting their ability to initiate the cell cycle. In order to enable RB1 activation a p16-INK4a resistant CDK4 or CDK6 variant could be expressed, which could make enforced RB1 expression acceptable in RB1-mutant cancer cell lines.

CDK4/6 would be able to regulate the phosphorylation of RB1 exogenously expressed in the RB1-mutant cell lines. Initial attempts in this direction have been performed (data not shown), however, RB1-mutant cells did not survive the plasmid transfections. Therefore, it could be interesting to explore alternative approaches to restore RB1 function, for example knocking down p16-INK4a.

As discussed previously, it would be necessary to investigate which kinases are being recruited to the chromatin upon ATR inhibition in RB1-mutant cells. This could be studied together with the introduction of replication stress via HU treatments in order to better understand the role of RB1 in DNA replication.

As previously hypothesised, RB1 loss could be considered as a potential new biomarker for patient stratification in clinical trials involving ATR inhibitors. The starting point would be to test RB1 loss and ATR inhibition sensitivity in animal in vivo studies. This would increase the confidence within the scientific community in order to add RB1 status as a predictive biomarker for ATR inhibitors sensitivity in monotherapies which are currently available.


7.7 Final conclusions

Identifying genetic biomarkers for a synthetically lethal drug effect provides one approach in the development of targeted cancer therapies. Mutations in \( RB1 \) gene represent one of the most common alterations in human cancer, but therapeutic approaches that target this defect are not yet clinically available.

Work presented in this thesis shows that defects in the \( RB1 \) gene sensitised tumour cells to clinical inhibitors targeting DNA damage checkpoint kinase ATR, \textit{in vitro}. \( RB1 \)-mutant cells are more sensitive upon ATR inhibition, moreover, \( RB1 \)-mutant cells display numerous features of replication stress in basal condition and even more so upon ATR inhibition. These levels of replication stress could lead to DNA replication catastrophe, explaining ATR inhibition sensitivity and cell death (Fig. 7.1).

\( RB1 \) is an important tumour suppressor and the work described here identifies and functionally validates a previously described role of \( RB1 \) in DNA damage pathways repair, opening the possibilities to explore \( RB1 \) loss as a potential actionable event in cancer therapy.
Levels of replication stress in RB1 loss scenario are enhanced upon ATR inhibition with consequent DNA replication catastrophe and apoptosis.

**Figure 7.1: Tentative network model of action in RB1 loss scenario**

Levels of replication stress in RB1 loss scenario are enhanced upon ATR inhibition with consequent DNA replication catastrophe and apoptosis.
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