

Understanding the Role of B-Myb during DNA Replication in Embryonic Stem Cells

Daniel Blakemore

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Birmingham Centre for Genome Biology
College of Medical and Dental Sciences
University of Birmingham

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Abstract

The acquisition of genetic lesions represents a major threat to embryonic stem cell (ESC) function due to the potential passage of mutations onto the whole embryo. Owing to this, stem cells have a superior capacity to prevent and repair genetic lesions, although, the mechanisms responsible remain elusive. The transcription factor B-Myb, for which deregulation is implicated in tumourigenesis, is found substantially elevated in ESCs. Through the generation of B-*myb* mutant ESCs (B-*myb*^{Δ/Δ}), this study uncovers an important role for B-Myb in control of the replication programme, with its loss resulting in; replication slowdown, elevated origin firing, replication factory activation, cell cycle deregulation and DNA damage accumulation. Further investigation revealed that the activity of CDK1, associated with control replication initiation, was elevated in the B-*myb*^{Δ/Δ}, moreover, inhibition of replication initiation through targeting CDK1 or Cdc7 resulted in a substantial rescue of the replication stress. In addition, inhibition of ATM dependent signalling was epistatic with B-Myb loss for the replication stress phenotype, suggesting B-Myb exerts its role through the DNA damage response. Transcriptome analysis of the B-*myb*^{Δ/Δ} was performed revealing changes in proliferation associated pathways and in the expression of chromatin modifiers. Overall, this study proposes an ATM-CDK-B-Myb axis important for control of cell cycle progression and DNA replication in ESCs.

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Abbreviations

7-AAD	7-Amino-Actinomycin D
A-Myb/MYBL1	MYB Proto-Oncogene Like 1
APC/C	Anaphase promoting complex/cyclosome
AT	Ataxia telangiectasia
ATM	Ataxia-telangiectasia mutated protein
ATR	Ataxia telangiectasia and Rad3-related protein
ATRIP	ATR interacting protein
AURKA	Aurora kinase A
B-Myb/MYBL2	MYB Proto-Oncogene Like 2
BrdU	Bromodeoxyuridine
CDC7	Cell division cycle 7
CDK	Cyclin dependant kinase
CENPF	Centromere Protein F
CKI	Cip/Kip protein family CDK inhibitors
CldU	Chlorodeoxyuridine
C-Myb/MYB	MYB Proto-Oncogene
CPT	Camptothecin
CSC	Cancer stem cell
DDK	Dbf4-dependent kinase
DDR	DNA damage response
DREAM	Dimerization partner (DP), retinoblastoma (RB)-like proteins, E2F and MuvB
DSB	Double strand break
dsDNA	Double stranded DNA
DSE	Double strand end
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EdU	Ethynyldeoxyuridine
EGTA	Ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
EMT	Epithelial to mesenchymal transition
ESC	Embryonic stem cell (mESC=mouse ESC)
FBS	Foetal bovine serum
GSC	Glioma stem cell
GSK3	Glycogen synthase kinase-3
H3-pS10	Phosphorylation of histone H3 at serine 10
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HU	Hydroxyurea
IdU	Iododeoxyuridine
IRES	Internal ribosome entry sequence
KAP1	KRAB-associated protein 1
Kb/min	Kilobases per minute
kDa	Kilodaltons
KDM5D	Lysine demethylase 5D
LIF	Leukaemia inhibitory factor
MAPK	Ras/mitogen-activated protein kinase

MCM	Minichromosome maintenance protein complex
MDC1	Mediator of the DNA damage Checkpoint-1
MEF	Mouse embryonic fibroblast
MET	Mesenchymal to Epithelial transition
MRN	Mre11, Rad50 and Nbs1
ORC	Origin recognition complex
OTM	Olive tail moment
PBS	Phosphate buffered saline
PCA	Principal component analysis
PFA	Paraformaldehyde
PIKK	Phosphoinositide 3-kinase related kinase
PLK1	Polo-like kinase 1
PMSF	Phenylmethylsulfonyl fluoride
PNP2	Purine nucleoside phosphorylase 2
PRC1	Protein Regulator of cytokinesis 1
PSC	Pluripotent stem cell
Q-PCR	Quantitative polymerase chain reaction
RIN	RNA integrity number
ROS	Reactive oxygen species
RPA	Replication protein A
SAC	Spindle assembly checkpoint
SCF	Skp1/CUL1/F-box protein
SDS	Sodium dodecyl sulfate
SSC	Somatic stem cell
ssDNA	Single stranded DNA
STRAP	Serine-threonine kinase receptor-associated protein
TBS	Tris buffered saline
TOP1	Topoisomerase 1
TOP1cc	Topoisomerase I cleavage complex
UFB	Ultra-fine bridge
γ H2AX	Phosphorylated histone variant H2AX

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Chapter 1 Introduction

1.1 Maintenance of genome stability

1.1.1 Genome instability is a prerequisite to carcinogenesis

The genome encodes instructions necessary for directing normal cellular function; hence its maintenance is of utmost importance for viability. Every time a cell divides, the genome must be faithfully duplicated and segregated to provide a copy for each daughter cell. The complexity of DNA processing in combination with the persistent exposure to genotoxic stimuli means that the constancy of the genetic code is constantly under threat. Despite this, the genome is remarkably stable between generations owing to various protective strategies which cells have evolved to prevent unfavourable levels of genomic changes (Aguilera and Gomez-Gonzalez, 2008).

The genome has a natural tendency to undergo minor changes as it passes through cell generations; a trait which is necessary to promote evolution at the molecular level. Particular genetic changes, or ‘driver mutations’, can offer a selective growth advantage to promote proliferation and survival. Genes which are mutated to offer a gain of function are identified as oncogenes, whereas those which promote growth upon their loss are tumour suppressor genes (Alberts B., 2009, Weinstein and Joe, 2006). However, this increased survival and proliferation also encourages physiological changes which increase the propensity for genomic alterations, a process termed ‘genome instability.’ Increased genome instability is detrimental to the health of the host organism and forms the basis for the initiation and the progression of cancer (Cairns, 1975, Stratton et al., 2009, Aguilera and Gomez-Gonzalez, 2008).

Almost 20 years ago, Hanahan and Weinberg were first to describe six alterations in cell physiology coined the hallmarks for cancer generation including; evasion of apoptosis, self-sufficiency in growth signalling, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis and tissue invasion capacity (Hanahan and Weinberg, 2000).

More recently multiple other hallmarks were proposed including evasion of immune surveillance, metabolic stress, replicative stress and several more (Kroemer and Pouyssegur, 2008, Hanahan and Weinberg, 2011, Luo et al., 2009). It is the acquisition of these cellular traits which drive genome instability catalysing the development of cancer.

Although a single genetically unstable founder cell harbouring a selective growth advantage is sufficient to initiate tumour growth, it is also recognised that progression of cancer is a multistep process driven by accumulating genome instability. A progressive increase in spontaneous mutation rate over cell generations prompts the selection of cells with increasing aggressiveness; the result is an expanding heterogeneous population of tumour cells. This model for cancer progression was coined the clonal evolution theory (Loeb and Cheng, 1990, Nowell, 1976).

1.1.2 The eukaryotic cell cycle

The eukaryotic cell cycle refers to the methodical series of cell stages which promote cellular growth (G1 and G2), DNA synthesis (S phase), chromosome segregation and cellular division (M phase) (Figure 1.1). Meticulous extrinsic and intrinsic signalling enables strict control over progression through the cell cycle; this level of regulation is necessary to safeguard the functionality of the genome and to prevent transmission of genome errors onto daughter progeny (Alberts B., 2009, Nurse, 1997). Vitally, changes in cell cycle structure can determine the behaviour of certain cell types. Stem cells during early embryogenesis present a short cell cycle to facilitate their rapid proliferation during this development period (Kapinas et al., 2013). Terminally differentiated cells are permitted to exit the cell cycle and enter a reversible state of inactivity known as quiescence. This cellular state allows cells to be poised for reactivation and is crucial for growth and tissue regeneration in multicellular organisms (Yao, 2014).

1.1.3 Cell cycle progression is governed by cyclin dependent kinases (CDKs)

Responsible for directing the cell cycle is the family of serine/threonine cyclin dependent kinase (CDKs) (Jeffrey et al., 2000, Malumbres et al., 2009) (Figure 1.1). CDKs were first implicated in cell cycle control in yeast where *cdc28* and *cdc2* were shown to influence cell cycle progression (Beach et al., 1982). CDKs alone lack enzymatic activity and must be complexed with their partner regulatory subunits known as cyclins which are also regulated by their timely synthesis and degradation. Moreover, the activity of two ubiquitin ligase complexes, SCF (Skp1/CUL1/F-box protein) and APC/C (anaphase promoting complex/cyclosome), are crucial to controlling the timely degradation of cyclins to aid in control of CDK activity (Nakayama and Nakayama, 2006). Most of the CDKs are also subject to negative and positive phosphorylation which also contributes to their activity (Nurse, 1990, Lim and Kaldis, 2013, Glotzer et al., 1991). In addition, the activity of the Ink4 and Cip/Kip protein family CDK inhibitors (CKIs) serve to silence CDK activity in unfavourable conditions (Sherr and Roberts, 1999). There are over 20 members of the CDK family, however, only the interphase CDKs (CDK2, CDK4 and CDK6), and the mitotic CDK (CDK1) have been identified as important for cell cycle progression (Malumbres et al., 2009).

1.1.4 Cell cycle checkpoints

Several regulatory events termed cell cycle checkpoints function to assess the integrity of the genome to ensure that damaged DNA is not passed through the cell cycle and onto cellular offspring. The cell cycle checkpoints are operated by the fluctuating activity of CDKs, moreover, in the presence of unfavourable conditions, cell cycle progression is blocked, providing an interval for repair (Sherr and Roberts, 1999, Hochegger et al., 2008).

1.1.5 The G1/S checkpoint

The ‘restriction point’ or ‘G1/S checkpoint’ represents one of the major checkpoints identified in somatic cells and is responsible for mediating commitment to cell cycle entry (Pardee, 1974) (Figure 1.1). The transition is governed through the activity of the E2F transcription factors (E2F1-8) and their regulation by the Rb pocket protein family members, pRb, p107 and p130 (Attwooll et al., 2004, Dimova and Dyson, 2005). At the start of G1, Rb pocket proteins are bound to E2F which suppresses the transcription of cell cycle progression associated genes. In the presence of favourable conditions, increased expression of cyclin D and E, which form complexes with cdk4/6 and cdk2 respectively, results in phosphorylation of the pRb p107 and p130 pocket proteins allowing release of E2F. This is followed by the transcription of cell cycle entry associated E2F target genes (Almasan et al., 1995, Qin et al., 1994, Weinberg, 1995). Importantly, recent work using single cell analysis, revealed that cell cycle commitment correlates with APC inactivation which happens much later than E2F activity, challenging the canonical pathway of CDK4/6 dependent cell cycle entry (Cappell et al., 2016, Dong et al., 2018). After cell cycle commitment, Cyclin E bound to CDK2 partakes in a second wave of Rb activation resulting in transcription of proteins associated with the initiation of DNA replication (Harbour et al., 1999, Malumbres and Barbacid, 2005). The G1-S checkpoint is often defective in cancer cells through a variety of different mechanisms. One example is through amplification of Cyclin D or its binding partners CDK4 and CDK6, shown in multiple cancer entities. Disruption of G1-S checkpoint control results in unrestricted transcription of E2F target genes and permits unscheduled entry into the cell cycle (Choi et al., 2012, Costello et al., 1997, Musgrove et al., 2011, Wang et al., 1994).

1.1.6 The G2/M checkpoint

The G2/M checkpoint initiates during the latter stages of DNA replication, governed by the activity of CDK1. The activation of CDK1 not only relies on its binding to one of many cyclin proteins, but also upon its phosphorylation on multiple residues through the activity of post-translational modifiers (Nigg, 2001, Bailly et al., 1992, Takizawa and Morgan, 2000, Santamaria et al., 2007) (Figure 1.1). WEE1 and MYT1 kinases are responsible for inducing inhibitory tyrosine and threonine phosphorylation of CDK1, providing a target for its degradation (Booher et al., 1997, Chow and Poon, 2013, De Witt Hamer et al., 2011, Heald et al., 1993). The activity of these kinases is countered by CDC25 phosphatase through removal of these inhibitory marks (Kumagai and Dunphy, 1991, Strausfeld et al., 1991). An appropriate balance between the activity of WEE1 and CDC25 is controlled by various mechanisms including via a feedback loop with CDK1 itself (Harvey et al., 2005, Hoffmann et al., 1993). In the presence of unfavourable conditions, CDK1 activation is suppressed to arrest the cell cycle providing a window to alleviate the issue before commitment to mitosis (Harbour et al., 1999, Malumbres and Barbacid, 2005). Loss of G2/M checkpoint activation could potentially result in the passage of under replicated/damaged DNA into mitosis (De Souza et al., 1999, Knauf et al., 2006). Moreover, changes in G2/M checkpoint control are reported in cancer, for example, WEE1 is deregulated in multiple malignancies representing a mechanism for G2/M checkpoint deregulation (Ge et al., 2017, Magnussen et al., 2013, Magnussen et al., 2012, Slipicevic et al., 2014). Interestingly, inhibition of WEE1 was shown to sensitize cancer cells to DNA damage resulting in their death, highlighting a potential therapeutic target (Mir et al., 2010).

1.1.7 The spindle assembly checkpoint

There also exists the spindle assembly checkpoint (SAC) which ensures proper segregation of chromatids during mitosis (Figure 1.1). Its activity is governed by APC/C, which marks cyclin B for degradation resulting in a reduction of CDK1 activity. This reaction allows the release of chromatids prior to their separation during anaphase (Nakayama and Nakayama, 2006, Potapova et al., 2006). Deregulation of the SAC has been shown to cause aneuploidy, which is commonly found in cancer (Diaz-Rodriguez et al., 2008, Weaver et al., 2007).

1.1.8 The intra S phase checkpoint

Finally, there is the intra-S phase or DNA replication checkpoint. This checkpoint differs from the typical cell cycle checkpoints as it represents a continuous global regulation of replication progression rather than a fixed cell cycle transition decision (Figure 1.1) (Iyer and Rhind, 2017). Implicated in this response is a network of signalling pathways known collectively as the DNA damage response (DDR). The DDR pathways are vital for assessing the genome during S phase and relaying information back to cell cycle associated signalling pathways. This allows cells to fine-tune their proliferative behaviour in line with the efficiency of DNA replication (Iyer and Rhind, 2017, Jones and Petermann, 2012, Willis and Rhind, 2009). The DDR is also crucial for initiating an appropriate cellular response to stress and DNA damage. Moreover, the activation of the other cell cycle checkpoints relies on stimulation from the DNA damage response, highlighting its importance. Defects in one or more aspects of intra S phase checkpoint signalling is common in cancer cells, allowing cancer cell proliferation and survival in the presence of enhanced DNA damage and mutation rates (Halazonetis et al., 2008, Jackson and Bartek, 2009).

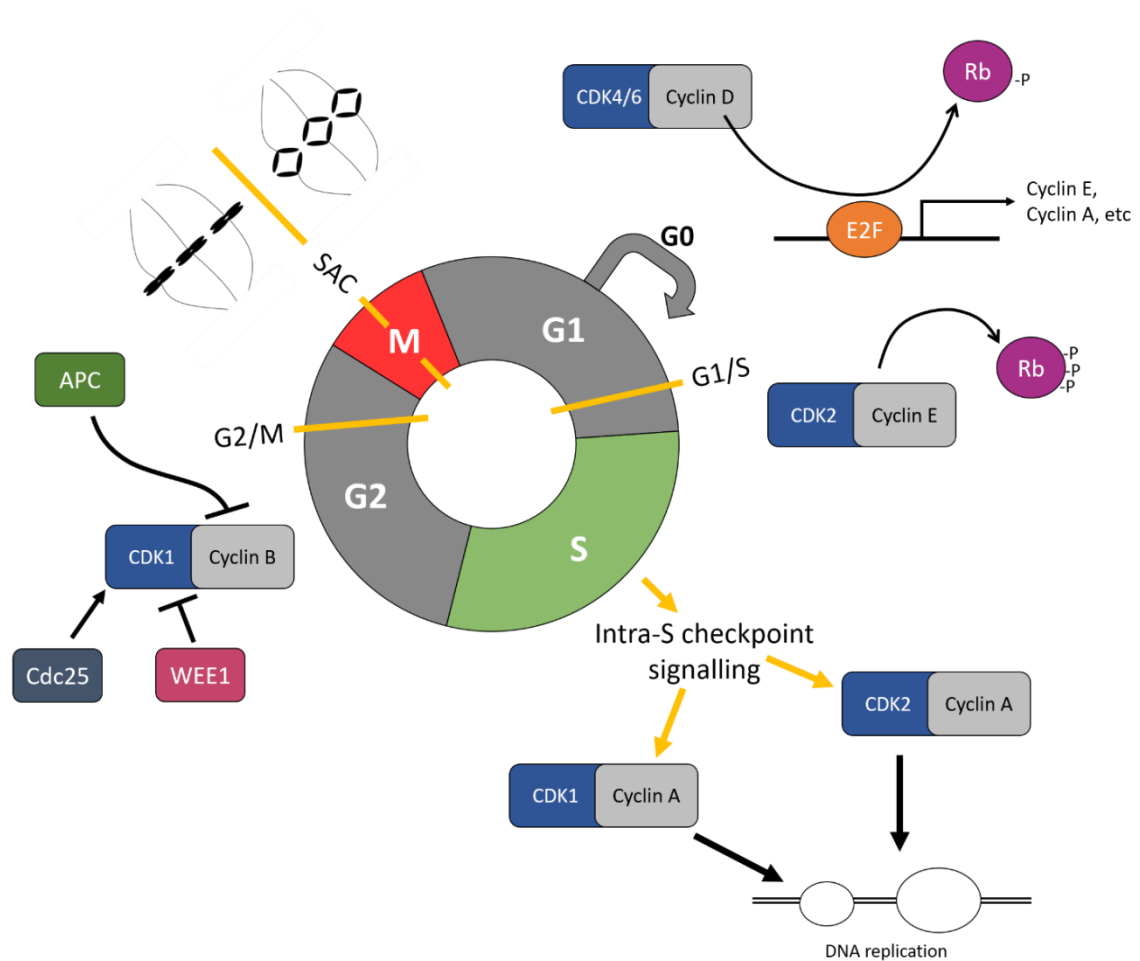


Figure 1.1 Progression through the cell cycle is governed by the activity of CDKs The eukaryotic cell cycle consists of 4 main phases; two gap phases (G1 and G2), S phase in which DNA is replicated and M phase when mitosis takes place. Cells may also exit the cell cycle and enter a cellular state known as quiescence, known as the G0 phase. Progression through the cell cycle is tightly controlled through the activity of CDKs and their partner cyclins. Moreover, their fluctuating activity is vital for the functioning of the cell cycle checkpoints (shown in yellow) which assess the integrity of the genome before progression is permitted (Alberts B., 2009, Sherr and Roberts, 1999). The G1/S checkpoint, or the restriction point, is governed by Cdk4/6 and Cdk2 dependent activation of E2F target genes through phosphorylation of Rb (Malumbres, 2014). Cdk2 and Cdk1 function during replication as a target of intra-s phase signalling to enable fine tuning of the replication programme (Iyer and Rhind, 2017). Cdk1 activity, governed by WEE1 kinase and Cdc25 phosphatase, is also responsible for activation of the G2/M checkpoint ensuring DNA is replicated prior to mitotic entry. Finally, the spindle assembly checkpoint, activated partly through APC ubiquitin ligase dependent degradation of Cdk1, ensures chromosome stability prior to anaphase onset (Santamaria et al., 2007, Malumbres et al., 2009, Nakayama and Nakayama, 2006).

1.2 DNA replication

During every cell cycle, the genome must be duplicated to provide an exact copy to be passed onto daughter progeny. The molecular machinery responsible for DNA replication is highly conserved from prokaryotes to eukaryotes, however, due to the increased size of the genome, timing and organisation of the replication programme in eukaryotes has been demonstrated to be more complex (Alberts B., 2009). Cell cycle related signalling networks permit fine-tuning of the replication programme to promote rapid genome duplication, whilst also balancing the availability of metabolites and the stability of the genome (Recolin et al., 2014). In eukaryotic cells, DNA replication is initiated from thousands of specific sites across the genome known as origins. Initiation of replication is separated into two separate phases; Origin licensing, which takes place during late mitosis and G1, and origin firing, which initiates upon entry into S phase (Fragkos et al., 2015, Huberman and Riggs, 1968).

1.2.1 CDK activity during replication

CDK2 and CDK1 represent the two main CDKs which function during S phase in eukaryotic cells. It was originally thought that their activities were separate, with CDK2 responsible for initiation and progression of replication and CDK1 dedicated to control of mitotic onset (Malumbres and Barbacid, 2005). However, several studies have challenged this dogma. Firstly, CDK1 can substitute for CDK2 for its function during replication in CDK2 deficient cells (Aleem et al., 2005). In addition, knockout experiments demonstrated that interphase CDKs (CDK2, CDK4 and CDK6) are not essential for cell cycle progression in most cell types but are only essential in certain specialised cells (Berthet et al., 2003, Malumbres et al., 2004, Rane et al., 1999, Santamaria et al., 2007). In pre-implanted mouse embryos, in the absence of all interphase CDKs, CDK1 binds to all cyclins and permits normal proliferation of cells until cellular differentiation commences. This work highlighted that, like in yeast, only one CDK (CDK1 in mice) is strictly essential for cell cycle progression (Santamaria et al., 2007).

1.2.2 Origin Licensing and preventing re-replication

Origin licensing commences with the binding of the multi-subunit DNA binding complex, the origin recognition complex (ORC), composed of six polypeptide subunits, ORC 1-6. This forms the foundation for formation of the pre-RC complex through recruitment of Cdc6, followed by loading of Cdt1 and the hexameric Mcm2-7 helicase onto all potential replication origins (Speck et al., 2005, Fernandez-Cid et al., 2013, Sun et al., 2013). Bound MCM2-7 is highly stable with a half-life of several hours to ensure it has the potential to bind for the duration of S phase (Kuipers et al., 2011). Vitally, through various mechanisms, no further MCM2-7 can bind to chromatin upon entry into S phase. This ensures no inappropriate licensing takes place and DNA is replicated only once per cell cycle (Romanowski and Madine, 1997, Diffley, 2011). Moreover, re-replication of parts of the genome has been shown to be highly detrimental to genome stability (Schimke et al., 1986, Davidson et al., 2006, Fragkos et al., 2015). A potential disadvantage to restricting licensing to M/G1 phases, is that cells must anticipate the required number of origins needed to replicate the whole genome. To alleviate this potential handicap, eukaryotic cells license an excess of origins, with only approximately 10% of licensed origins firing during unperturbed replication. The extra 90%, which are termed ‘dormant origins’, provide high plasticity allowing the cell to selectively fire additional origins temporally throughout replication (Donovan et al., 1997, Woodward et al., 2006, Blow and Dutta, 2005) (Figure 1.2).

The timing of origin licensing is in part governed by the fluctuating activity of CDKs. Firstly, licensing is triggered just after APC dependent degradation of cyclin A and cyclin B when CDK levels are low (Acquaviva and Pines, 2006, Peters, 2006). Once licensing has taken place, an increase in the activity of CDKs upon entry into S phase acts as a barrier to prevent further licensing. Evidence for this comes from studies carried out in yeast in which there is only one

CDK (Cdc28). Inhibition of CDK activity in yeast resulted in an abnormal re-licensing of origins highlighting the importance of CDK activity in this process (Itzhaki et al., 1997, Noton and Diffley, 2000). Interestingly, multiple rounds of S phase are induced in yeast after deletion of Cdc13, a mitotic cyclin partner of CDK1 (Hayles et al., 1994), or by overexpression of CDK1 inhibitors (Correa-Bordes and Nurse, 1995). It has also been demonstrated in yeast that CDKs have a direct influence upon replication licensing through targeting specific components of the Pre-RC (ORC, Cdc6, Cdt1 and MCM2-7); disruption of these interactions also resulted in partial re-replication (Nguyen et al., 2001, Tanaka and Diffley, 2002). In xenopus and mammalian cells, there exists more complex mechanisms to prevent re-replication. An example is the role of geminin, a direct inhibitor of Cdt1 licensing activity with its activity prominent during S phase (Tada et al., 2001, Wohlschlegel et al., 2000, McGarry and Kirschner, 1998, Gonzalez et al., 2006). Licensing factors such as Cdc6 and Cdt1 are often deregulated in cancer, moreover, disruption of origin licensing and a susceptibility to re-licensing are common features of cancer cells (Seo et al., 2005, Vaziri et al., 2003).

1.2.3 Origin firing and replication progression

Upon entry into S phase, replication firing ensues through the disassociation of Cdc6 and Cdt1, and the formation of the CMG helicase complex through recruitment of Cdc45, GINS and multiple accessory proteins, to the MCM2-7 double hexamer (Ilves et al., 2010, Gambus et al., 2006). MCM2-7 activation results in its helicase activity promoting the unwinding of the double stranded DNA exposing a template for DNA polymerase. Two replication forks form, the MCM2-7 complex divides into two hexamers and the replication forks travel bi-directionally away from the origin whilst the polymerase copies the DNA template in a 5' to 3' direction (Alberts B., 2009, Deegan and Diffley, 2016) (Figure 1.2).

The firing of replication origins is governed by two main mechanisms; the activity of CDKs, and the activity of the Dbf4-dependent kinase (DDK) complex containing Cdc7 (Cell Division Cycle 7) kinase and its regulatory subunit Dbf4 (Jackson et al., 1993, Yeeles et al., 2015). Evidence for the contribution of CDK activity was demonstrated in yeast; through promoting the complexing and activation of several replication proteins, CDK is important for recruitment of replication factors Cdc45 and GINS to the Mcm2-7 complex (Muramatsu et al., 2010, Masumoto et al., 2002). In addition, there are many components of the replication firing machinery which have been identified as substrates of CDK1 (Ubersax et al., 2003, Holt et al., 2009), highlighting its importance in this process. The DDK complex (Cdc7-Dbf4), also directly interacts with the replication machinery. Cdc7 dependent phosphorylation of MCM4 and MCM6 of the MCM2-7 complex is vital to alleviate their inhibition to enable initiation of replication (Varrin et al., 2005, Sheu and Stillman, 2006, Randell et al., 2010). Cdc7 activity is also required for the initial recruitment of Sld3 and Cdc45 prior to CMG complex formation (Sheu and Stillman, 2010).

Both CDK and DDK activity are important for origin firing, however, which activity comes first and how these complexes interact with each other has been debated in the literature. Firstly, phosphorylation of the MCM2-7 complex by CDK activity was shown to promote DDK dependent MCM modification (Masai et al., 2000). This was supported in yeast where it was suggested that Cdc7 can only function after CDK activation (Nougarede et al., 2000). Conflicting evidence was acquired using xenopus, in which Cdc7 could carry out its role in the absence of CDK2 but not vice versa, suggesting CDK2 could only function after Cdc7 had completed its role (Walter, 2000). More recent work using a reconstituted yeast replication initiation system revealed that the order in which DDK and CDK phosphorylated the MCM2-7 complex did not influence replication efficiency, and both orders of activation were observed (Yeeles et al., 2015). It could be that the conflicting evidence is a result of differences in the

systems used. Overall, these studies provide evidence for potential cooperation between DDK and CDK activity in promoting replication initiation.

1.2.4 The origin firing programme

Although cells license an excess of origins, not all origins fire simultaneously upon entry into S phase. In fact, origins are fired according to a highly organised temporal firing programme. One way this is achieved is through limiting the availability of firing factors. The abundance of Sld2, Sld3, Dpb11 and Dbf4 was demonstrated to be low compared to other firing factors, moreover, overexpression of these proteins resulted in an abnormal increase in early firing origins (Mantiero et al., 2011, McGuffee et al., 2013), highlighting the availability of these factors as important for origin firing control. The origin firing programme also ensures that problems during DNA replication can be dealt with efficiently. Origins are fired in specific clusters throughout the genome known as replication factories. This system places multiple origins in close proximity, therefore, complications such as stalled forks can be dealt with quickly by adjacent origins within the same factory (Dimitrova and Berezney, 2002). Evidence comes from observing an increase in the size of factories in response to fork stalling (Cseresnyes et al., 2009).

An important question is why certain regions of the genome are given priority for origin firing during early replication. Although not completely understood, studies investigating this question have suggested that the process is not completely stochastic, but there is a complex relationship between DNA sequence, chromatin structure and transcription levels which governs which regions fire first. Firstly, in human and mouse cells, replication initiation sites correlate with GC rich regions such as unmethylated CpG islands and regions with actively transcribed genes (Delgado et al., 1998, Sequeira-Mendes et al., 2009). It was also demonstrated

that open chromatin regions with transcriptionally active genes are replicated early but heterochromatin regions or 'hard to replicate regions' are replicated towards late S phase (Ma et al., 1998, Chen et al., 2019). This is supported by an increased level of mutation associated with late replication, when difficult to replicate regions are processed (Stamatoyannopoulos et al., 2009).

Other studies have also highlighted the role of chromatin accessibility in origin selection. It was demonstrated in *Drosophila* that the ORC localises to sequences enriched with H3.3, often at transcription start sites and areas depleted of nucleosomes (MacAlpine et al., 2010). In budding yeast, histone deacetylase activity prevents MCM2-7 complex association with origins by altering nucleosome positioning (Crampton et al., 2008). In human cells, HBO1, a histone acetyl transferase, was shown to interact with and activate Cdt1 to coordinate replication with transcription (Miotto and Struhl, 2008). ORC1 was also shown to bind to chromatin marked with H4 lysine 20 di-methylation which may also affect origin selection (Kuo et al., 2012). Therefore, there is likely a complex relationship between many different factors which determines the organisation of the replication programme encompassing both scheduled changes and stochastic processes. Interestingly, the timing and usage of replication origins changes during development or cellular differentiation (Hyrien et al., 1995, Norio et al., 2005). These studies suggest that changes in origin selection could be dependent upon development associated transcriptional programme modifications and changes in chromatin organisation.

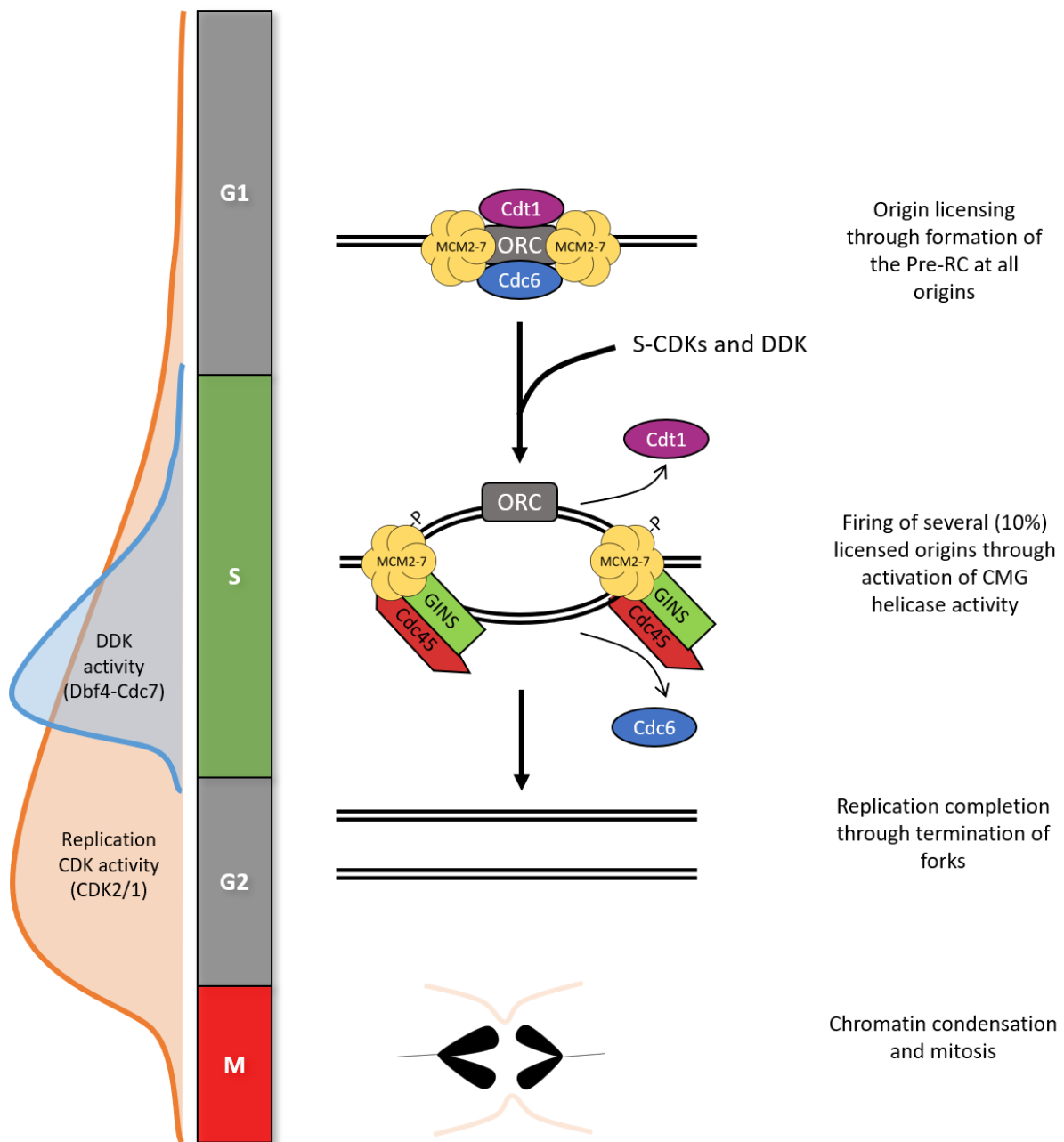


Figure 1.2 Control of the DNA replication programme DNA replication is separated into two phases. Firstly, replication licensing, in which replication initiation sites, known as origins of replication, undergo the binding of the pre-RC, consisting of the ORC, MCM2-7 helicase plus Cdc6 and Cdt1. The second phase, known as origin firing, involves the activation of 10% of origins through the binding of GINS complex and Cdc45, resulting in initiation of helicase activity and unwinding of the double stranded DNA (Fragkos et al., 2015, Huberman and Riggs, 1968). Following this, DNA polymerase activity permits the duplication of the DNA travelling bi-directionally away from the origins until the whole genome is duplicated prior to mitosis. The process of origin initiation is governed by the activity of two main kinase groups, the S phase CDKs (orange curve), and the DDK complex (Dbf4-Cdc7 kinase) (blue curve). Overall, their fluctuating activity permits a high level of control over replication to ensure genome stability is maintained (Jackson et al., 1993, Yeeles et al., 2015).

1.3 The replication stress response

1.3.1 Replication stress, a major hallmark of genome instability

Once replication has commenced, cells must adopt the appropriate balance between replication speed and accuracy to ensure the genome is replicated as efficiently as possible. There are multiple exogenous and endogenous pressures which can negatively influence the replication programme. In the presence of obstacles, communication between the replication programme and cell cycle machinery enables appropriate manipulation of replication progression to ensure genome stability is maintained (Branzei and Foiani, 2010, Zeman and Cimprich, 2014, Hustedt et al., 2013). An inability to adapt to replication pressures can result in replication stress, a phenomenon defined as ‘any influence which hinders the fulfilment of replication.’ Replication stress is characterised by the slowdown and stalling of active replication forks (Zeman and Cimprich, 2014). Replication forks consist of fragile ssDNA intermediates and upon stalling are also prone to collapse resulting in the formation of highly detrimental double-stranded DNA damage. Hence, replication stress is recognised as a major contributor to genome instability; and many diseases including cancer have been linked to the occurrence of replication stress (Zeman and Cimprich, 2014, Mazouzi et al., 2014).

1.3.2 Activity of the DNA damage response during replication

In order to control replication and to respond to the occurrence of replication stress, cells have evolved a network of pathways crucial to safeguarding genome stability known collectively as the DNA damage response (DDR). The DDR has been shown to be specifically important for safeguarding replication progression through multiple mechanisms including its influence upon origin firing through regulating CDK activity (Ciccia and Elledge, 2010, Mazouzi et al., 2014,

Sorensen et al., 2003, Sorensen and Syljuasen, 2012, Bartek et al., 2004). Inhibition of the DDR is shown to perturb replication initiation timing and fork elongation resulting in fork collapse and cell death (Couch et al., 2013, Maya-Mendoza et al., 2007, Petermann et al., 2010, Shechter et al., 2004). Recent work also demonstrated that the activity of the DDR is important to suppress CDK1 activity to stabilise RIF1 and PP1 activity which limits origin firing during unperturbed replication (Moiseeva et al., 2019). These studies highlight the importance of the DDR in preventing the occurrence of replication stress during unperturbed replication. In addition, in response to replication stress, activation of the intra-s phase checkpoint, by signalling through the DDR, induces a paradoxical response comprising both (i) a global decrease in origin firing, to allow time to repair the lesion, plus (ii) activation of origins localised to the region containing the detrimental stimuli, thus promoting replication over the lesion (Mechali, 2010, Yekezare et al., 2013). Overall, it is important to recognise that DDR signalling pathways are not only activated in response to stress but are also important for normal replication progression.

Central to the DDR are two phosphoinositide 3-kinase related kinases (PIKKs), Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR), which serve to monitor chromatin integrity and initiate appropriate signalling (Awasthi et al., 2015, Mazouzi et al., 2014). ATM and ATR are positioned at the top of a hierarchical network of pathways shown to influence hundreds of different proteins participating in a range of cellular processes important for regulation of genome stability (Matsuoka et al., 2007, Pearl et al., 2015). Both ATM and ATR share similar molecular organisation, with kinase domains near the carboxyl terminal and internal regions involved in protein-protein interactions, which defines them as PIKKs; despite this, their activities differ considerably (Cimprich and Cortez, 2008, Perry and Kleckner, 2003).

1.3.3 ATR kinase activity

A hallmark of replication stress is the accumulation of stretches of single stranded DNA (ssDNA). This may be driven by multiple different sources including uncoupling of helicase from the polymerase or due to the degradation of nascent DNA at stalled replication forks (Cimprich and Cortez, 2008, Hashimoto et al., 2010, Symington and Gautier, 2011). ssDNA is quickly coated with replication protein A (RPA) to prevent its degradation (Fanning et al., 2006). Moreover, this forms the basis for the recruitment of ATR kinase supported by studies in which depletion of RPA reduces ATR association with chromatin (Costanzo et al., 2003). This interaction also depends upon the activity of ATR interacting protein (ATRIP) and the RAD9–RAD1–HUS1 (9-1-1) complex which interacts with TOPBP1 resulting in ATR dependent phosphorylation of target proteins (Ball and Cortez, 2005, Cimprich and Cortez, 2008, Zou and Elledge, 2003). The importance for ATR in genome stability maintenance is highlighted in patients with the autosomal recessive disorder, Seckel syndrome, in which ATR is mutated. Seckel syndrome manifests in dwarfism, microcephaly and severe mental retardation (O'Driscoll et al., 2003). Recently, mutations in DONSON, found in patients with microcephaly, has been demonstrated to result from dysfunctional ATR signalling (Reynolds et al., 2017). In addition, deletion of ATR in multiple systems results in genome instability and cell death (Cortez et al., 2001, Brown and Baltimore, 2000).

The role of ATR in maintaining genome stability is in part governed by its local roles at replication forks. Through targeting helicases such as SMARCAL1, ATR prevents replication fork collapse and promotes replication fork restart (Couch et al., 2013, Ragland et al., 2013). ATR was also shown to influence replication fork progression and repair through a role in the Fanconi anaemia pathway important for repair of inter-strand crosslinks (Mutreja et al., 2018, Ceccaldi et al., 2016). As well as local roles, ATR partakes in the activation of a vast phosphorylation cascade which has many indirect influences upon cell cycle dynamics,

replication control, transcription, metabolism and DNA repair (Cimprich and Cortez, 2008, Blackford and Jackson, 2017). The Serine threonine kinase Chk1 is characterised as the primary downstream target of ATR signalling activation (Liu et al., 2000b). The importance of the ATR-Chk1 pathway is highlighted in studies where Chk1 levels are manipulated. Mice generated with an extra copy of Chk1 are more resistant to replication stress inducing agents; whereas, mice with depleted Chk1 display a substantial increase in double strand break (DSB) formation (Lopez-Contreras et al., 2012, Syljuasen et al., 2005). Key Chk1 targets are the CDC25 phosphatases which are crucial for regulating cell cycle progression and DNA replication initiation through manipulating CDK activity (Boutros et al., 2006, Sanchez et al., 1997, Maya-Mendoza et al., 2007, Petermann et al., 2010).

1.3.4 ATM kinase activity

The role of ATM kinase overlaps with ATR dependent function however, ATM has been primarily associated with the response to DNA damage in the form of DSBs (Lee and Paull, 2005). Under stable conditions ATM exists as homodimers or multimers but is converted to monomeric form in response to stress through PP2A and WIP-1 dependent phosphorylation of serine 1981 (Bakkenist and Kastan, 2003, Goodarzi and Lees-Miller, 2004, Shreeram et al., 2006). The requirement for ATM in maintaining genome stability is demonstrated in patients with Ataxia telangiectasia (AT) who present a deficiency of ATM. AT patients present cerebellar degeneration, immunodeficiency and increased risk of cancer. AT patient derived cells fail to activate a checkpoint response and slowdown replication in response to IR, a characteristic termed 'radio-resistant DNA synthesis (Houldsworth and Lavin, 1980, Painter and Young, 1980, Stewart et al., 2001, McKinnon, 2004).

Central to ATM activity is the MRE11-RAD50-NBS1 (MRN) complex which functions upstream of ATM (Stracker et al., 2002). MRN has been shown to assemble at DSBs where it forms a physical bridge spanning the break and provides the signal for recruitment and

activation of ATM (Lee and Paull, 2005, Lavin, 2008, Kudoh et al., 2005). Nbs1, a component of the MRN complex is required for ATM autophosphorylation (Berkovich et al., 2007). The role of the MRN complex is not exclusively ATM recruitment. MRE11, a DNA nuclease and a component of the MRN complex, is vital for several processes during DNA replication and DNA repair, including determining DSB repair pathway choice (Chapman et al., 2012), however, the presence of MRE11 is not required for ATM activity (Buis et al., 2008).

Another major player in the activation of ATM is MDC1 (Mediator of the DNA damage Checkpoint-1), recruited to sites of DNA damage by ATM dependent phosphorylation of H2AX (γ H2AX). This promotes ATM dimerization and in turn results in further recruitment of MDC1 and other associated DNA damage response proteins which serve to activate and amplify the response (Lou et al., 2006, Stewart et al., 2003). Activation of ATM, like ATR, is pivotal for the response to replication stress. Moreover, ATM activation results in initiation of a vast phosphorylation cascade influencing hundreds of different substrates, including BRCA1, Chk2 and p53, involved in processes such as; DNA repair, cell cycle control, DNA replication, apoptosis, chromatin remodeling and transcription (Shiloh and Ziv, 2013, Matsuoka et al., 2007, Lavin, 2008) (Figure 1.3).

The canonical theory for the activation of ATM is solely through its recruitment to sites of DSBs. However, this theory is challenged by the fact that even in response to low dose irradiation, resulting in just a few DSBs, ATM is activated abundantly throughout the cell (Bakkenist and Kastan, 2003). In addition, ATM is only required for the repair of approximately 10% of lesions in response to irradiation (Riballo et al., 2004, Deckbar et al., 2007). Recent work has linked ATM to the repair of DNA damage specifically in heterochromatin regions. Knockout of KAP1, a key ATM substrate important for heterochromatin repair (Ziv et al., 2006), or HP1 and HDAC1/2, which interact with KAP1 to alter chromatin conformation, diminishes the requirement for ATM dependent DNA repair (Goodarzi et al., 2008). It was also

demonstrated that ATM activation is dependent upon H3 lysine 9 trimethylation associated with heterochromatin, further supporting that chromatin context influences the activity of ATM (Olcina et al., 2013). Several studies have identified roles for ATM associated with a much wider and general influence on cellular homeostasis in the absence of DNA damage; notably in response to hypoxic conditions (Olcina et al., 2013, Bencokova et al., 2009, Cam et al., 2010). ATM activity has also been linked to other DNA damage independent roles, such as its role in mitotic spindle checkpoint activation (Yang et al., 2011), its role in preventing re-replication through CDT1 degradation (Iwahori et al., 2014), and its influence upon resolving DSB independent replication fork stalling (Ewald et al., 2008). Overall, these studies suggest that the activity of ATM is potentially more complex and extensive than previously thought.

1.3.5 Crosstalk between ATR and ATM dependent signalling

Crosstalk exists between ATR and ATM in activation of their downstream cascades. ATM dependent end resection promotes recruitment of ATR through ssDNA formation vital for checkpoint activation (Jazayeri et al., 2006, Myers and Cortez, 2006). In contrast, ATR phosphorylates H2AX and ATM directly to promote ATM activity at stressed replication forks (Ward and Chen, 2001, Stiff et al., 2006), highlighting the cooperation between the two pathways. The downstream substrates of ATM and ATR also overlap considerably, moreover, Chk1 was shown to be also activated by ATM (Gatei et al., 2003). Several studies have also highlighted the overlap between ATM and ATR in the activation of a wide range of downstream targets (Matsuoka et al., 2007, Brown and Baltimore, 2003, Chanoux et al., 2009).

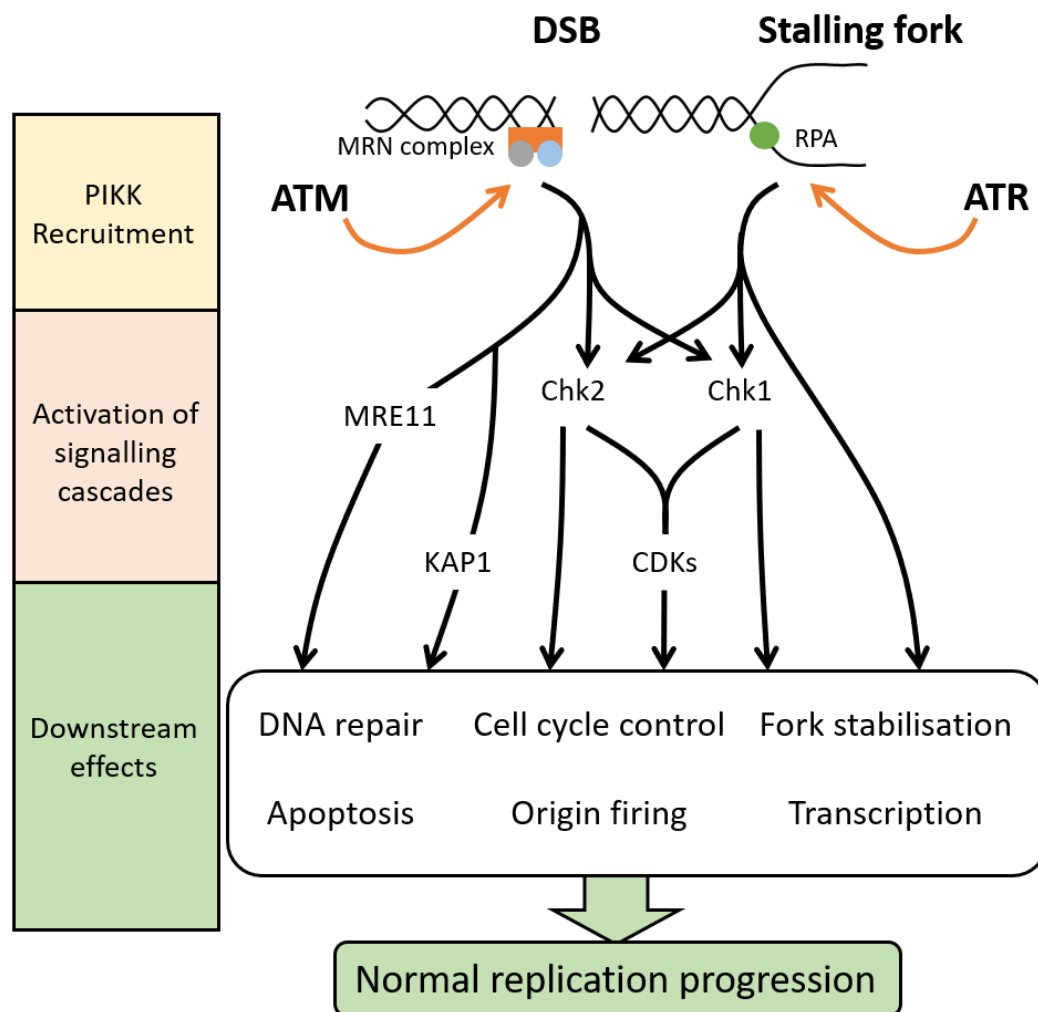


Figure 1.3 The DNA damage response during S phase The DNA damage response encompasses two main pathways which are vital to ensure normal replication progression in unperturbed and DNA damaging conditions. The pathways are initiated by the recruitment and activation of two main kinases; ATR, which plays important role during normal replication, recruited to RPA coated single stranded DNA, and ATM, which is mainly associated with the response to DSBs, recruited to sites of double-strand ends by the MRN complex. Both proteins result in activation of a vast phosphorylation cascade, including the activation of Chk1 and Chk2, identified as the main substrates of ATR and ATM respectively (Matsuoka et al., 2007, Jackson and Bartek, 2009, Zeman and Cimprich, 2014).

1.3.6 The DNA damage response in cancer

Inherited or acquired mutations in the DDR pathways are highly beneficial for cancer cells as they permit cancer cell survival in the presence of replication stress and enhanced mutation rates (Jackson and Bartek, 2009). In early tumours, activation of the DDR is apparent and likely limits their growth potential (Bartkova et al., 2005, Gorgoulis et al., 2005). In contrast, advanced and malignant tumours show deregulation of proteins involved in the DDR (Kandoth et al., 2013, Bartkova et al., 2008, Negrini et al., 2010). Therefore, mutations in DDR pathway associated genes likely act as a crucial hurdle in acquisition of unrestricted and aggressive growth behaviour in developing tumours. Despite providing cancer cells with a growth advantage, the differences in DDR signalling compared to normal cells provides potentially exploitable targets for drug therapy. Changes in the dynamics of DDR signalling pathways in cancer can result in an addiction to an alternative pathway. Moreover, inhibition of such pathways can result in selective toxicity, an approach which has been termed synthetic lethality (Curtin, 2012, Jackson and Bartek, 2009). This tactic has already been successful, one example being the FDA-approved PARP1 inhibitor, Olaparib, which selectively targets cells with a deficiency in DSB repair (Bryant et al., 2005, Golan et al., 2019).

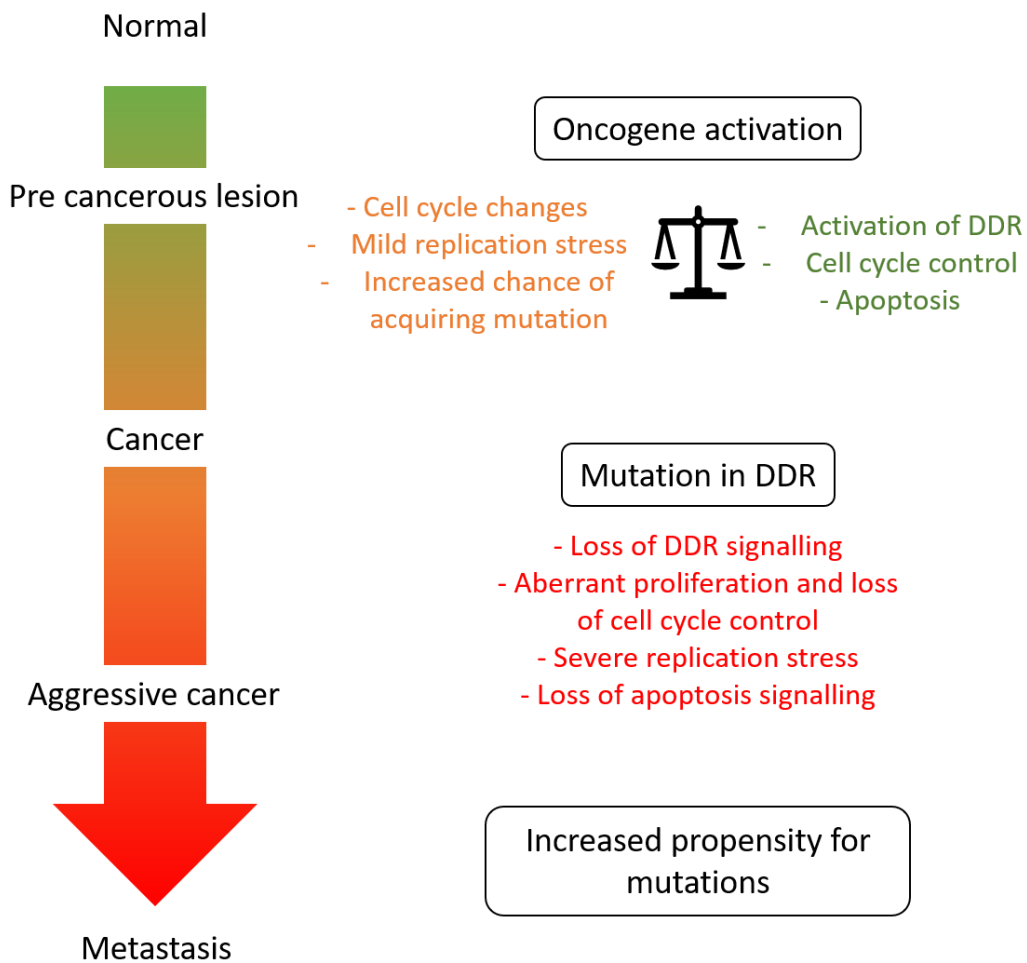


Figure 1.4 Carcinogenesis – the snowball effect Cancer is preceded by a clinically invisible stage in which a single oncogene/driver mutation may increase the chance of acquiring further cancer favouring mutations. Despite this, the cell cycle and DNA damage response are functional and counteracts this increased mutation rate (Bartkova et al., 2005, Gorgoulis et al., 2005). Upon the chance of acquiring further mutations, clonal selection of those cells with increased aggressiveness takes place (Nowell, 1976). Mutations which result in loss of key signalling pathways such as the DDR will increase the mutation rate tremendously as cancer cells are able to ignore anti-growth and apoptosis signals. Eventually, the cancer will evolve to full metastatic status allowing the cancer cells to migrate away from the source (Jackson and Bartek, 2009).

1.3.7 The role of oncogenic mutations in replication stress

Loss of tumour suppressor activity or expression of oncogenes has been related to the development of replication stress (Dereli-Oz et al., 2011, Burrell et al., 2013). MYC, RAS, CDC25A and CCNE are some examples of characterised replication stress inducing oncogenes, moreover, all of them are involved in regulating cell cycle progression and cellular growth (Bartkova et al., 2006, Dominguez-Sola et al., 2007, Bester et al., 2011, Zimmermann et al., 2013, Jones et al., 2013, Spruck et al., 1999). Interestingly, studies looking at specific oncogenes present various different mechanisms for replication stress, with each oncogene inducing a unique fragility landscape on chromatin, therefore, it is difficult to pinpoint any single causes of oncogene induced replication stress (Kotsantis et al., 2018, Miron et al., 2015). Cells in which replication stress was provoked, presented a strong increase in the occurrence of mutations common to tumours, indicating that replication stress contributes to the progression of tumourigenesis (Durkin et al., 2008, Bilousova et al., 2005). It is likely that expression of a single replication stress causing oncogene is enough to increase mutation rate sufficiently to promote cancer cell progression. There has been a multitude of different mechanisms for replication stress described in the literature, some of which will be discussed.

1.3.8 Deregulation of replication initiation as a mechanism for replication stress

Normal cells are set up to deal with harmful stimuli during replication due to the ability of the replication programme to adapt to such pressures, for example, through the firing of dormant origins (Ge and Blow, 2010). However, changes in the dynamics of replication licensing or under-licensing can have devastating effects upon genome stability. Through downregulation of the MCM2-7 complex, cells were shown to proliferate normally, however, were highly sensitive to replication stress and DNA damage occurrence, demonstrating the importance of excess origins in maintaining genomic integrity (Ibarra et al., 2008). Interestingly, deregulation of cyclin E was demonstrated to result in similar impairment of MCM2-7 complex loading and

subsequent replication stress and DNA damage (Ekholm-Reed et al., 2004). Thus, the accelerated cell cycle brought through oncogene activation, results in insufficient preparation prior to replication onset (under-licensing) resulting in an inability to deal with replication pressures (Figure 1.5).

Disruption of control of origin activity during S phase also results in replication stress. An unscheduled increase in origin firing has been reported after overexpression of oncogenes; CCNE (cyclin E), RAS and MYC (Jones et al., 2013, Kotsantis et al., 2018). The mechanism for increased origin firing has been studied extensively. In 2004 it was first reported that inhibition of the DDR through caffeine treatment resulted in global changes in origin firing (Shechter et al., 2004). More specific inhibition of Chk1, a downstream component of the DDR, resulted in deregulation of origin firing and decreased replication fork progression speeds. Interestingly, this reduced fork speed could be rescued by inhibition of CDK or CDC7 activity (Petermann et al., 2010). Therefore, these studies highlight the relationship between the expression of oncogenes and the DDR in deregulation of replication initiation control.

How increased origin firing causes replication stress has also been investigated. Deregulation of CDK1 through inhibition of WEE1 kinase results in an excess of origin firing causing replication stress and DNA damage. Interestingly, supplementation with nucleosides rescued the decreased fork speeds and DNA damage, identifying depletion of nucleotides as the mechanism for the replication stress produced (Beck et al., 2012). Moreover, aberrant activation of cyclin E was also shown to result in depletion of nucleotides, and the resulting stress could also be rescued by nucleotide supplementation, demonstrating a link to oncogene activation (Bester et al., 2011). The pathways directly involved in nucleotide synthesis are also often deregulated in cancer, moreover, the DDR has been shown to be important for appropriate regulation of nucleotide metabolism (Aird et al., 2013, Xie et al., 2014, Pfister et al., 2015).

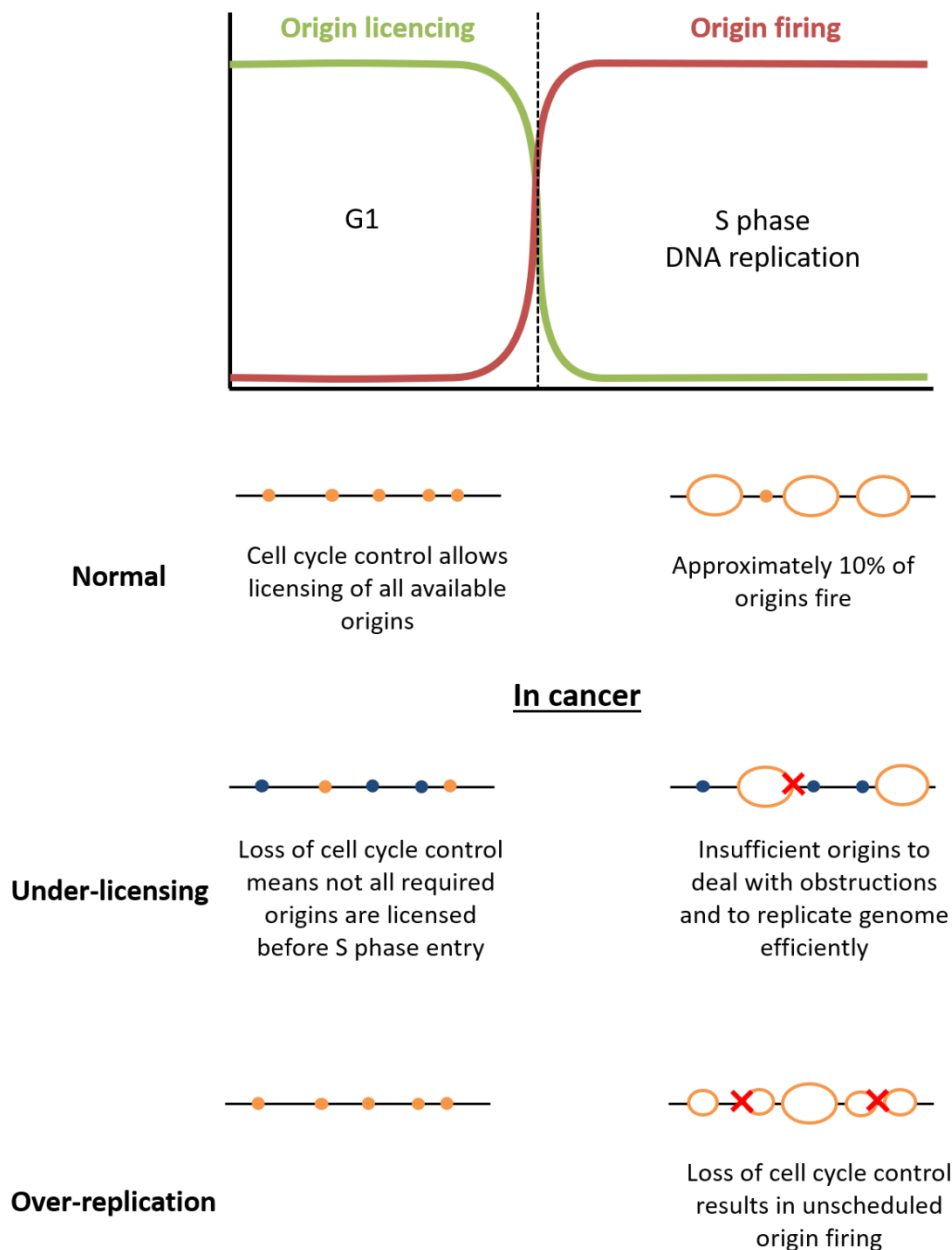


Figure 1.5 Deregulation of replication initiation in cancer Replication initiation is separated into origin licensing during G1, and origin firing during S phase. In normal cells, various mechanisms are in place to ensure that sufficient origins are licensed, as no further origins may be licensed after S phase entry. During S phase, only approximately 10% of licensed origins undergo firing and the dynamics of their activation is carefully controlled (Fragkos et al., 2015, Huberman and Riggs, 1968, Diffley, 2011, Yeeles et al., 2015). Oncogene activation can accelerate replication initiation resulting in under-licensing. Moreover, under-licensed chromatin is less likely to be able to alleviate replication fork problems through the firing of dormant origins (Ge and Blow, 2010). Over-replication is another process associated with cancer cells. This encompasses an unscheduled increase in origin firing which can result in deregulation of the replication programme resulting in replication conflicts and nucleotide depletion (Jones and Petermann, 2012, Kotsantis et al., 2018, Beck et al., 2012).

1.3.9 Replication fork barriers as a mechanism for replication stress

During DNA replication, there are various barriers which can impede replication fork progression. One such barrier is transcription intermediates, moreover, the replication and transcription machinery utilise the same template, so it is not surprising that the two processes have the potential to interfere with each other. Collisions between the replication and transcription machinery has been identified as a causes of replication stress in multiple studies (French, 1992, Helmrigh et al., 2013, Bermejo et al., 2012, Kotsantis et al., 2016). Abnormal transcription intermediates known as R-loops can pose a threat to replication progression, however, replisome activity is fine-tuned to deal with R-loops through regulating a balance between head-on and co-directional conflicts enabling recruitment and activation of the appropriate RNA processing pathways (Hamperl et al., 2017). Oncogene activation, such as increased RAS activity, promotes proliferation through upregulation of certain transcription factors such as TATA box binding protein. This abnormal increase in transcription results in a surge in head-on collisions, accumulation of R-loops and increase replication conflicts ultimately leading to replication stress (Hamperl et al., 2017, Kotsantis et al., 2018, Kotsantis et al., 2016). Interestingly, activity of the DNA damage response through Mec1 and INO80 activity has also been implicated in removal of RNA polymerase from chromatin to prevent its collision with replication (Poli et al., 2016, Lafon et al., 2015).

Deregulation of transcription and replication can also result in a build-up of topological stress upon chromatin. Topological entanglements are another major cause of replication stress due to their ability to prevent progression of the replication machinery (Bermejo et al., 2007, Postow et al., 2001, Keszthelyi et al., 2016). The activity of topoisomerases ensures that topological entanglements are alleviated during replication. Mus81 nuclease is vital for generating topoisomerase dependent DSBs which enables unwinding of the DNA entanglements followed by their subsequent repair (Regairaz et al., 2011, Bermejo et al., 2007, Bermejo et al., 2012).

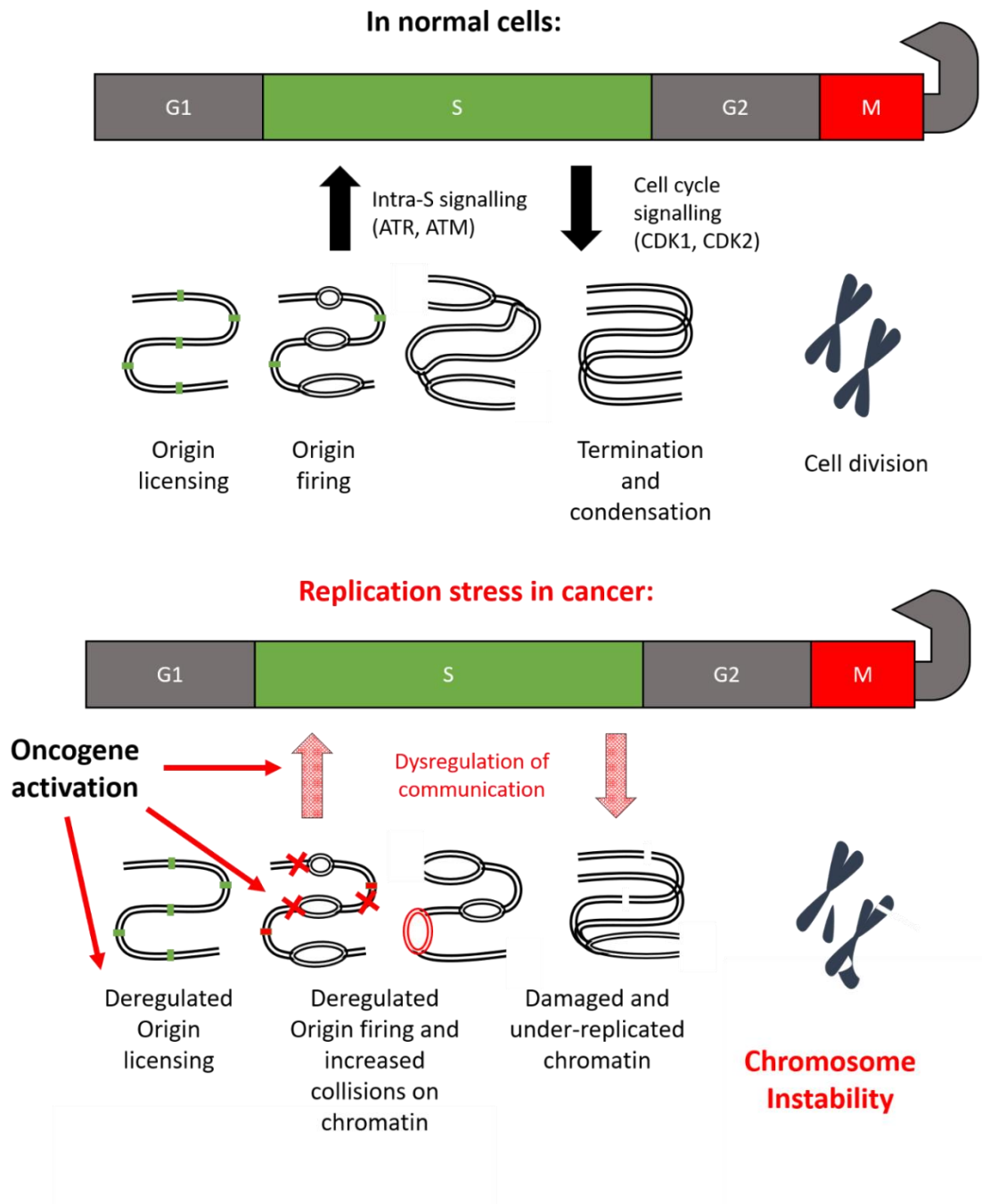


Figure 1.6 The replication stress response in cancer In normal cells, there is constant communication with the replication programme to ensure an appropriate balance between replication progression and genome stability (Iyer and Rhind, 2017). In cancer cells, this communication is often malfunctioned, resulting in disruption of this balance to promote genome duplication at the expense of genome stability. For this reason, replication stress is commonly observed in cancer. Disruption of replication signalling can result in replication stress through; inappropriate replication initiation and progression, abnormally elevated transcription, DNA damage accumulation, increased topological constraints and many more. Loss of checkpoint control, commonly seen in cancer, may allow these issues to accumulate and permit the passage of under-replicated DNA into mitosis resulting in chromosome instability (Kotsantis et al., 2018, Mazouzi et al., 2014, Zeman and Cimprich, 2014).

1.4 Stem cells

1.4.1 Pluripotent stem cells

The creation and survival of multicellular organisms requires the harmonized function of hundreds of diverse cell types. Every cell which constitutes a living organism can be traced back through a cascade of coordinated cell divisions originally instigated by a stem cell. Stem cells possess unique behavioural properties which are tailored to their vital roles for successful development of the mammalian embryo and also their roles throughout adult life. Firstly, their capacity to self-renew indefinitely provides limitless proliferative potential. They may duplicate both symmetrically, generating two identical daughter cells with equivalent developmental potential, or asymmetrically, producing a daughter cell committed to differentiate. This facilitates a balance between expansion and specialisation of cell populations (Young, 2011, Weissman, 2000)

Stem cells also possess high cell potency describing their potential to differentiate into a wide variety of different cell types. Stem cell potency is at its highest upon formation of the zygote, a single stem cell created through encounter of the male and female gametes; an event which marks the beginning of embryogenesis. The zygote is totipotent due to its potential to yield every mature cell of a particular organism (Weissman, 2000). At approximately 5-6 days post coitum, extrinsic signalling guides formation of the blastocyst consisting of an expanding population of stem cells known as the inner cell mass surrounded by a layer of trophectoderm cell specialised to form placental tissue. Stem cells of the inner cell mass are labelled pluripotent due to loss of their capacity to form extra-embryonic tissue (Nichols et al., 1998, Weissman, 2000). As embryogenesis progresses post-implantation, further rounds of cell commitment results in production of multipotent, oligopotent and eventually omnipotent stem cells with the capacity to produce multiple, a few or only one type of cell respectively (Alberts B., 2009, Boroviak et al., 2014, Solter, 2006, Fleming, 1987, Burdon et al., 2002).

In addition to their roles during embryogenesis, stem cells also exist throughout post-natal organisms as small populations in potentially every organ system. Adult or Somatic stem cells (SSCs) function to replenish lost cells in the particular microenvironment or ‘stem cell niche’ they reside in. The stem cell niche provides specific signalling cues guiding behaviour appropriate for that particular tissue (Clevers et al., 2014). The importance of the stem cell niche was first highlighted upon the formation of teratomas after injection of embryo derived stem cells into nude mice, whereas, injection into recipient mouse embryos resulted in normal development (Evans and Kaufman, 1981).

1.4.2 Embryonic stem cells

Pluripotent stem cells first appear during embryogenesis as the inner cell mass of the pre-implantation blastocyst. It was Evan and Kaufman in 1981 who first demonstrated that it is possible to mechanically extract the cells of the inner cell mass and culture them *in-vitro* as embryonic stem cells (ESCs) (Evans and Kaufman, 1981). Under particular culture conditions, ESCs may be maintained in a naive self-renewing state for extended periods of time. A complex interplay exists between extrinsic factors associated with the stem cell niche and intrinsic transcriptional influences dedicated to controlling stem cell behaviour. The molecular mechanisms which enable stem cell populations to remain in a pluripotent, self-renewing state have been under speculation for many years. Moreover, homogenous pluripotent stem cell populations in an identical culture environment will differentiate into a wide variety of different cell types. There is likely a balanced relationship between in-built fate determination, environmental cues and stochastic processes (Li and Belmonte, 2017, Niwa, 2007, Burdon et al., 2002) (Figure 1.7).

1.4.3 Maintaining the pluripotent ground state ‘LIF +2i’

Various soluble factors have been identified which are important to maintaining self-renewal in ESCs. LIF (Leukaemia inhibitory factor) and its activation of the JAK-STAT pathway is perhaps the best characterised, and the most exploited for in-vitro culture of ESCs. LIF belongs to the interleukin 6 cytokine family and is expressed in the trophectoderm during embryogenesis. Through binding to the heterodimeric LIF receptor, LIF induces the phosphorylation of several downstream targets including the signal transducing protein gp130 and gp130-associated JAK kinases and their associated STAT target proteins (Suda et al., 1987, Hirai et al., 2011, Niwa et al., 1998, Burdon et al., 2002). Signalling downstream of gp130 also includes the activation of the Ras/mitogen-activated protein kinase (MAPK) pathway. MAPK targets have known roles in differentiation of trophectodermal lineage (Hirai et al., 2011, Leunda-Casi et al., 2001). Moreover, in 2008 it was reported that supplementation of media with LIF plus combined inhibition of MAPK and glycogen synthase kinase-3 (GSK3), described by the authors as ‘LIF + 2i,’ was sufficient to maintain a pluripotent state in ESCs. (Silva et al., 2008) (Figure 1.7).

1.4.4 The core pluripotency transcription factors

It is well characterised that both mouse and human ESCs rely on the expression of three main transcription factors for the maintenance of pluripotency. Possibly the most distinguished is Oct4 (POU5F1), a member of the POU family of transcription factors, expressed at high levels during early embryonic development. Its sheer importance in self-renewal is highlighted by its knockdown in ESCs resulting in differentiation into trophectoderm like cells and loss of pluripotency, or its overexpression resulting in differentiation into primitive mesoderm and endoderm (Nichols et al., 1998, Niwa et al., 2000). SOX2 and NANOG, expressed in the ICM during embryogenesis, are also important for maintaining pluripotency (Chambers et al., 2003, Masui et al., 2007).

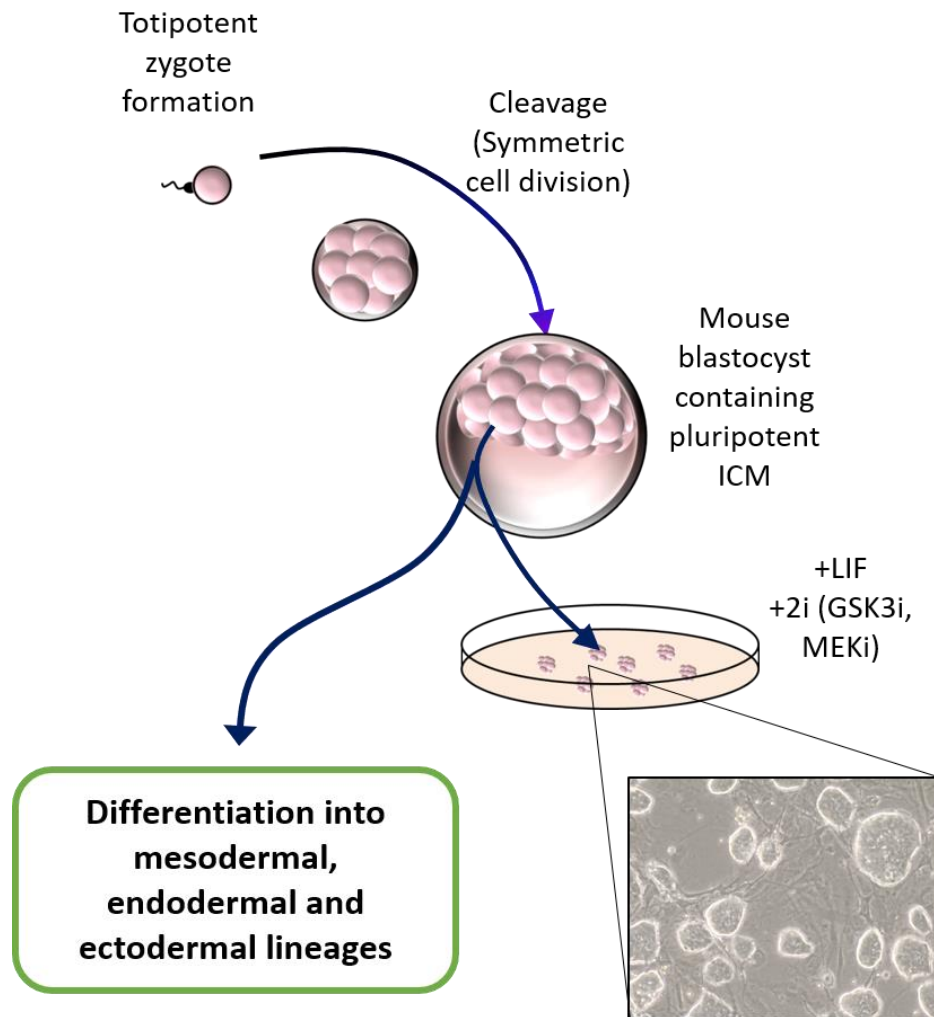


Figure 1.7 Pluripotent stem cells of the inner cell mass Upon fertilisation of the ovum with a sperm cell, the totipotent zygote is produced, with the capacity to produce all cells of that particular organism. The zygote is then signalled to undergo several mitotic divisions with no significant growth forming a morula in a process known as cleavage. Further specialisation promotes formation of the blastocyst consisting of pluripotent stem cells (PSCs) of the inner cell mass surrounded by a layer of trophectodermal cells (Nichols and Smith, 2012, Niwa, 2007). It is at this stage in which the PSCs may be extracted and placed in culture as embryonic stem cells (ESCs). In the presence of 'LIF and 2i', cells will remain in a state of limitless self-renewal (Silva et al., 2008). Upon stimulation, ESCs may be induced to differentiate into any cells derived from the three germ layers; endoderm, ectoderm and mesoderm (Thomson et al., 1998).

1.4.5 Cell cycle control in embryonic stem cells

Embryonic stem cells (ESCs) proliferate very rapidly undergoing a full cell cycle in approximately 10-12 hours. Their behaviour can be attributed to an atypical cell cycle characterised by truncated growth phases and altered cell cycle checkpoint control, namely loss of the G1/S checkpoint. ESCs spend most of their time in S phase, although, the time taken to replicate DNA is the same (Burdon et al., 2002, Coronado et al., 2013, Ahuja et al., 2016, Ballabeni et al., 2011, Kapinas et al., 2013). Analysis of cell cycle regulatory factors in ESCs revealed elevated expression of cyclins and their partner CDKs resulting in their constitutive activation throughout the cell cycle, which likely contributes to their rapid and unhindered cell cycle progression (Ballabeni et al., 2011, Fujii-Yamamoto et al., 2005, Stead et al., 2002) (Figure 1.8).

The mechanisms behind the truncated growth phases and lack of G1-S checkpoint control has been studied extensively. Rb protein, which is important for control of the G1-S checkpoint, is constitutively active in ESCs (Savatier et al., 1994). Intracellular localisation of Chk2 and p53 has also been shown to contribute to the absence of a G1/S checkpoint. In ESCs, p53 and Chk2 are localised to different cellular compartments compared to somatic cells and cannot carry out their typical cell cycle regulatory function; moreover, a G1 arrest can be restored through induced expression of Chk2 (Hong and Stambrook, 2004). Interestingly, the unusual cell cycle is also dependent upon the expression of key pluripotency factors (van der Laan et al., 2013, Zhang et al., 2009, Becker et al., 2006). Upon commitment to differentiate, ESCs override the machinery stimulating pluripotency and upregulate lineage specific transcription factors (Gonzales et al., 2015). Isolated G1 arrested ESCs have a significantly higher differentiation propensity compared to S and G2 cells, demonstrating that the decision to differentiate is during G1 (Sela et al., 2012). It has been suggested that the short G1 serves to avoid differentiation;

this is supported by the finding that cells with a short cell cycle are easier to reprogram to pluripotency (Guo et al., 2014, Ruiz et al., 2011).

1.4.6 Genome stability maintenance in embryonic stem cells

Maintenance of genome stability is exceptionally important in stem cells. Their capacity to instigate the creation of a large proportion of functional cells means that acquisition of a detrimental mutation early in this process could result in catastrophic consequences to the whole embryo or organ in which it resides (Blanpain et al., 2011). ESCs proliferate rapidly, lack normal checkpoint control and utilise the expression of oncogenes, yet, stem cells have adopted mechanisms to compensate for their increased propensity to acquire lesions, in fact, they present a lower (100-fold) mutational rate compared to somatic cells (Cervantes et al., 2002, Fujii-Yamamoto et al., 2005, Kapinas et al., 2013).

One way that ESCs maintain genome stability is through increased DNA damage repair capacity. ESCs were shown to present increased expression of DNA repair components and possess an increased DNA repair efficiency (Maynard et al., 2008). They also favour the more faithful homologous recombination DNA damage repair pathway compared to somatic cells which tend to favour the more error prone non-homologous end joining pathway; this may be attributed to an increase (15-fold) in Rad51 levels in ESCs (Tichy et al., 2010, Tichy et al., 2012). Another mechanism is their robust anti-oxidant responses and a reliance upon anaerobic glycolysis for energy metabolism in ESCs; although less efficient, this strategy reduces the accumulation of reactive oxygen species (ROS) which have the potential to damage the genome (Xu et al., 2013, Dannenmann et al., 2015, Panopoulos et al., 2012).

It has been suggested that stem cells possess enhanced replication coupled mechanisms for genome maintenance, including a highly robust DDR, to compensate for the lack of cell cycle control (Kapinas et al., 2013, Ahuja et al., 2016) (Figure 1.8). Several proteins have been identified which are shown to play specific roles in the DDR in ESCs. The expression of Filia, an ESC specific protein, plays an important role in cell cycle checkpoint control, DNA damage repair and apoptosis through promoting increased PARP1 activity in ESCs (Zhao et al., 2015). Another protein, DICER, was shown to play a role in resolving replication associated DNA damage in rapidly dividing cells such as ESCs (Swahari et al., 2016). Recently, pluripotency factor, Sall4, was demonstrated to be vital for activation of ATM in mouse ESCs through its interaction with Rad50 (Xiong et al., 2015).

Embryonic stem cells also present significant differences in chromatin architecture compared to somatic cells. Moreover, chromatin in ESCs is generally in a more relaxed configuration and is mostly devoid of heterochromatin (O'Neill et al., 2003, Zhou et al., 2011, Gaspar-Maia et al., 2011). This was supported by a decrease in heterochromatin associated epigenetic marks such as histone 3 lysine 9 tri-methylation and an increase in global acetylation associated with euchromatin (Hawkins et al., 2010). It is also found that γ H2AX, a marker usually associated with ATM dependent recruitment to DSBs, is at high levels in ESCs in the absence of DNA damage, with more than 100 γ H2AX foci per cell, which in somatic cells is known to represent the response to approximately 4Gy irradiation (Chuykin et al., 2008, Banath et al., 2009). This phenomenon was attributed to changes in chromatin structure associated with pluripotency, moreover, differentiation resulted in loss of γ H2AX accompanied by loss of histone acetylation (Banath et al., 2009). It is known that chromatin conformation is important for the recruitment and activation of the DDR (see section 1.3.4) (Ziv et al., 2006, Goodarzi et al., 2008, Olcina et al., 2013). Interestingly, induced relaxation of chromatin in ESCs resulted in a hyper-resistance to DNA damage induction, which was shown to be due to an even stronger DDR at lesion sites

(Murga et al., 2007). In addition, Sall4, which has been demonstrated to influence DDR activity in ESCs, is shown to interact with chromatin modifiers such as DNA methyltransferases (Xiong et al., 2015, Yang et al., 2012). This work highlights the potential relationship between pluripotency, chromatin conformation and the strength of the DDR.

A difference in DDR signalling is also supported by observed changes in the replication dynamics in ESCs. Firstly, ESCs have been shown to licence a high level of dormant origins to protect the genome and prevent replication stress (Ge et al., 2015). ESCs were also shown to be proficient in promoting high levels of fork reversal, a mechanism to prevent collapse of stalled replication forks (Ahuja et al., 2016). Another mechanism by which ESCs are able to maintain a highly stable genome is their high sensitivity to apoptosis which allows efficient eradication of mutated cells from the self-renewing pool (Hong and Stambrook, 2004, Desmarais et al., 2016). BAX, an apoptosis associated signalling molecule, is in a constitutively active state in ESCs to enable rapid activation of apoptosis (Dumitru et al., 2012). Overall, these studies strengthen the theory that the recruitment and activity of the DDR in ESCs is potentially unique compared to somatic cells, and that ESCs adopt several strategies to compensate for their increased propensity for mutations, to maintain a highly stable genome.

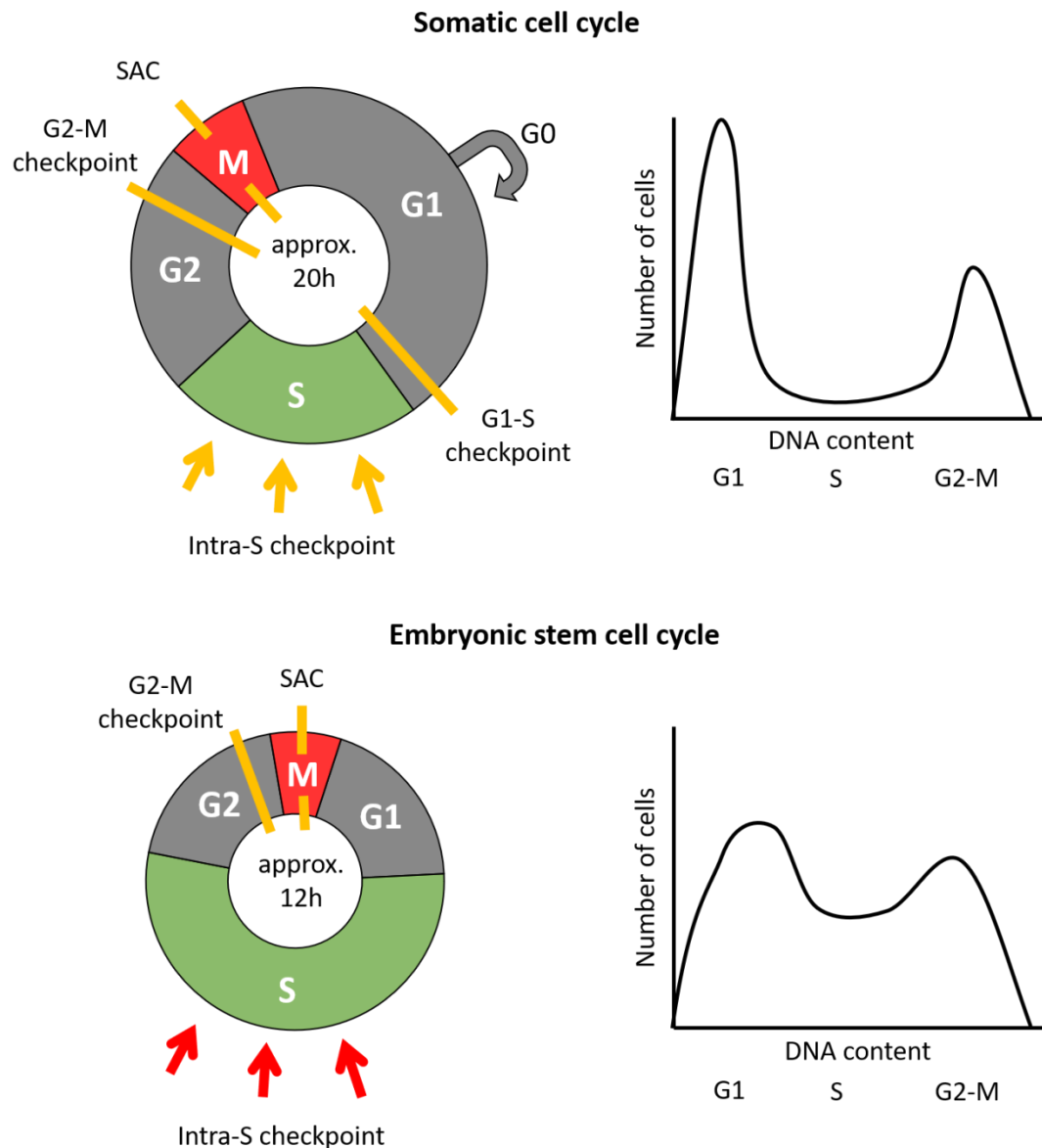


Figure 1.8 The embryonic stem cell cycle Embryonic stem cells proliferate quickly undergoing a full cell cycle in approximately 12 hours. This may be attributed to an atypical cell cycle characterised by highly truncated growth phases, moreover, ESCs spend most of their time in S phase. ESCs lack the typical checkpoint control observed in somatic cells with complete loss of a G1-S checkpoint/restriction point. Therefore, ESCs have been shown to rely on replication coupled mechanisms to maintain genome stability (Ahuja et al., 2016, Burdon et al., 2002).

1.4.7 The stem cell theory of cancer

According to the model proposed by John Dick, there exists populations of naïve cells, known as cancer stem cells (CSCs), within all tumours which are important for tumour growth and maintenance (Kreso and Dick, 2014). CSCs, despite presenting increased deregulation of cellular pathways, share many features with normal stem cells including a capacity for unlimited self-renewal (Nguyen et al., 2012). Experimental evidence for CSCs was first provided in leukaemia through the isolation of naïve haematopoietic stem cell-like cells (Lapidot et al., 1994), but also, CSCs were later uncovered in solid tumours (Al-Hajj et al., 2003). It is also demonstrated that CSCs are likely responsible for resistance to anti-cancer therapies, therefore, CSCs represent a good target for anti-cancer therapeutics (Bao et al., 2006, Zhang et al., 2010).

Like ESCs, CSCs present a highly efficient DDR. Glioma stem cells (GSCs) were shown to present enhanced DNA repair and cell cycle checkpoint contributing to radio-resistance, moreover, this resistance was diminished upon treatment with an inhibitor of Chk1 and Chk2 (Bao et al., 2006). It was revealed that upregulation of NBS1 of the MRN complex contributes to the observed GSC resistance (Cheng et al., 2011). Differences in DDR signalling such as this, represent a potential avenue for targeting CSCs in anti-cancer therapeutics. Therefore, studying the atypical DDR in ESCs also represents a strategy to uncover potential mechanisms to target CSCs.

1.5 The role of B-Myb

1.5.1 Myb family transcription factors

The *Myb* gene family encodes three related nuclear transcription factors in mammals which display similar homology in their molecular structure. Through a highly conserved N-terminal DNA binding domain, all three transcription factors bind Myb binding regions (Biedenkapp et al., 1988, Nomura et al., 1988, Peters et al., 1987). C-Myb (MYB Proto-Oncogene) was the first *Myb* gene product to be established when it was identified as the mammalian homologue to v-Myb, an oncogene carried by the avian myeloblastosis virus and a proto-oncogene highly expressed in immature haematopoietic cells (Graf, 1992). A-Myb (MYBL1/ MYB Proto-Oncogene Like 1) and B-Myb (MYBL2/MYB Proto-Oncogene Like 2) were later uncovered from human cDNA libraries using a C-Myb probe in 1988 (Joaquin and Watson, 2003, Nomura et al., 1988). Although the Myb transcription factors are almost structurally identical and bind to the same promoter regions, their individual expression levels and biological roles are very different. In addition, ectopic expression of each of the Myb transcription factors results in activation of a different set of genes, suggesting that various context specific mechanisms are responsible for their transcriptional activity (Ness, 2003). The expression of A-Myb and C-Myb is cell type specific, with A-Myb restricted to B-lymphocytes and the reproductive system and C-Myb mostly restricted to cells of the haematopoietic system (Toscani et al., 1997). B-Myb however, is expressed in virtually all proliferating cells pointing towards a general function in cell homeostasis (Lam et al., 1992, Kamano et al., 1995). At the protein level, B-Myb consists of a highly conserved amino N terminus responsible for binding to Myb binding sites on DNA, an acidic central portion thought to be responsible for transcriptional activity, a conserved region responsible for protein-protein interaction and a Carboxyl C-terminal negative regulatory domain for inhibiting transactivation activity (Sala, 2005, Henrich et al., 2017).

1.5.2 *B-myb* expression

B-myb is first expressed during G1, a time which correlates with the expression of E2F target genes. The dimerization partner (DP), retinoblastoma (RB)-like proteins, E2F and MuvB core (DREAM) complex is a chief regulator of *B-myb* expression and activity. During late G1, through increased activity of CDK4/6, phosphorylation of Rb-like proteins, p107 and p130, promotes their dissociation away from the MuvB core allowing E2F to activate numerous cell cycle associated genes, including *B-myb* (Georlette et al., 2007, Sala, 2005, Sala et al., 1997, Sadasivam and DeCaprio, 2013). The presence of E2F binding sites upon the promoter region of *B-myb* confirms its identity as a bone fide E2F target gene (Lam and Watson, 1993). Moreover, mutation of these sites results in constitutive expression of *B-myb* throughout the cell cycle (Hurford et al., 1997, Lam and La Thangue, 1994, Lam et al., 1994, Lam and Watson, 1993) (Figure 1.9).

Full length B-Myb protein displays little to no transactivation activity compared to its phosphorylated form (Foos et al., 1992, Ziebold et al., 1997). Upon degradation of cyclin D during late G1, B-Myb is phosphorylated by the Cyclin A-Cdk2 complex. Moreover, this releases B-Myb from its repression mediated through the activity of its repressors N-CoR and SMRT (Joaquin et al., 2002, Sala et al., 1997, Ziebold et al., 1997, Li and McDonnell, 2002). A transcriptional co-activating protein, p300, whose activity is also dependent upon cyclin A, promotes acetylation of B-Myb, increasing its activity further (Bartusel et al., 2005). Contrary to this, cyclin D has been shown to inhibit B-Myb activity (Horstmann et al., 2000). A whole network of additional co-factors have been identified which aid in control of B-Myb transcription, often at the epigenetic level, adding to the complexity of its activation (Seong et al., 2003, Bartusel et al., 2005, Joaquin and Watson, 2003, De Falco et al., 2000). Increased Cyclin A dependent phosphorylation of B-Myb also provides a target for its degradation through the ubiquitin protease pathway. Moreover, B-Myb degradation was significantly

increased upon transfection of cells with *skp2*, involved in ubiquitin ligase activity during S phase (Charrasse et al., 1999, Saville and Watson, 1998, Charrasse et al., 2000) (Figure 1.9).

1.5.3 The role of B-Myb in maintaining normal cellular proliferation

The importance of B-Myb for cellular proliferation was described in an early study in which down-regulation of B-Myb resulted in significant inhibition of growth (Arsura et al., 1992). From the expression profile of B-Myb, it could be assumed that B-Myb exerts its function during the G1 or early S phase, moreover, initial studies described a transcriptional role for B-Myb in governing the transition into S phase (Iwai et al., 2001, Lin et al., 1994). However, more recent studies revealed the major roles of B-Myb in trans-activating the promoters of genes responsible for the G2/M transition (Okada et al., 2002, Osterloh et al., 2007, Katzen et al., 1998, Henrich et al., 2017, Lorvellec et al., 2010, Knight et al., 2009, Wolter et al., 2017). More specifically, the MuvB core cooperates with B-Myb to recruit FOXM1, providing activation of genes such as Cyclin B, survivin and CDC25 phosphatase, responsible for mediating G2–M checkpoint control (Down et al., 2012, Lefebvre et al., 2010, Martinez and Dimaio, 2011, Sadasivam and DeCaprio, 2013, Sadasivam et al., 2012). Supporting this interaction, knockdown of either B-Myb or MuvB inhibited their shared transcriptional activity at target promoters and reduced FOXM1 gene promotor binding (Sadasivam et al., 2012). A recent study also described an ATR-CDK1-FOXM1 axis which interacts with B-Myb and is responsible for governing proper exit from replication into G2 (Saldivar et al., 2018). It was also demonstrated that YAP, a component of the HIPPO signalling pathway, interacts with the B-Myb-MuvB complex to influence expression of mitotic genes (Pattschull et al., 2019). These novel findings highlight that the roles of B-Myb in cell cycle control are complicated and may be influenced through multiple mechanisms.

Much of the findings related to B-Myb function have come from studying other organisms; for example, in *Drosophila*, the Dm-Myb gene is very closely related the vertebrate B-Myb gene (Okada et al., 2002). Furthermore, Dm-Myb mutants present aberrant transition through the cell cycle with chromosomes undergoing premature histone phosphorylation and condensation subsequently resulting in increased polyploidy and aneuploidy (Manak et al., 2002, Fung et al., 2002). Studies using zebra fish containing the *crash&burn* (crb) mutation, which culminates in loss of B-Myb function, have exposed non-transcriptional roles of B-Myb in regulating the mitosis machinery. These fish exhibit defects in mitotic spindle formation, display genome instability and therefore have an increased susceptibility to cancer formation. It is thought that termination of cyclin B regulation by B-Myb is partly responsible for this effect (Shepard et al., 2005). B-Myb also forms a complex with filamin and clathrin; the Myb-clafi complex, a necessary component for mitotic spindle formation (Yamauchi et al., 2008). Further non-transcriptional interactions of B-Myb have been revealed; it was shown that B-Myb promotes cell cycle transitions independently of its DNA binding activity (Werwein et al., 2012). B-Myb also interacts and positively regulates the serine-threonine kinase receptor-associated protein (STRAP) involved in inhibition of TGF- β dependent apoptosis, cellular growth signalling and p53 localisation (Seong et al., 2011).

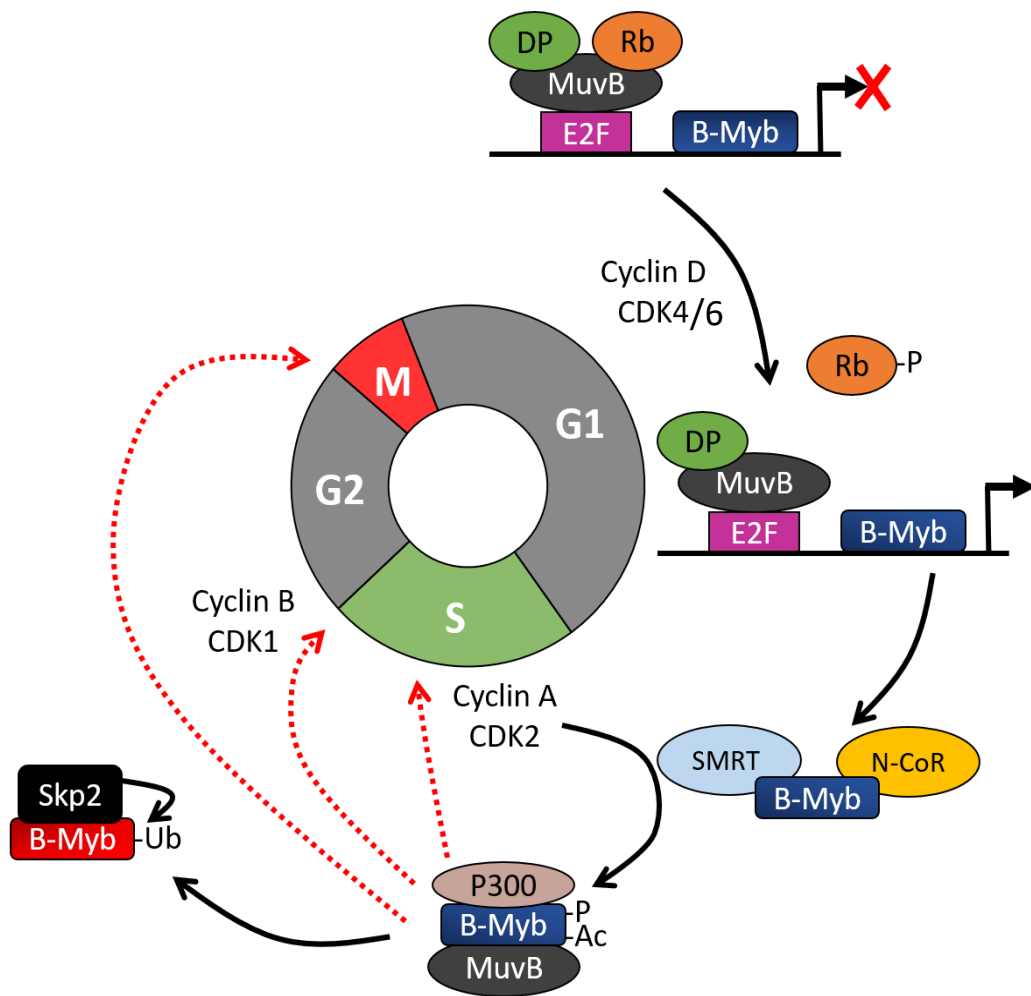


Figure 1.9 The expression of B-Myb is cell cycle regulated B-Myb expression is cell cycle regulated with levels at their highest during S phase. Its expression is dependent upon the activity of the DREAM complex. During G1, increased CDK4/6 activity promotes dissociation of Rb away from the complex allowing E2F dependent transcription of B-Myb. B-Myb is further activated upon entry into S phase via various mechanisms including, release from inhibitory SMRT and N-CoR binding, phosphorylation by CDK2 plus further post-translational modification by multiple proteins including P300 and Cyclin D (Sala, 2005, Sadasivam and DeCaprio, 2013, Joaquin et al., 2002, Sala et al., 1997, Ziebold et al., 1997, Li and McDonnell, 2002). Increased B-Myb activity also signals its degradation by skp2 dependent ubiquitin mediated proteolytic degradation (Charrasse et al., 2000). B-Myb, although maximally expressed during S phase, has been shown to play roles throughout the cell cycle; during replication, during the S/G2 and G2/M transitions, and directly in mitosis (Musa et al., 2017).

1.5.4 The requirement for B-Myb during embryogenesis

B-Myb is ubiquitously expressed in all cells; however, its expression is 1000-10000 times more in ESCs and other highly proliferative cell types of the developing embryo, indicating a potentially important role during this time (Tarasov et al., 2008, Sitzmann et al., 1996). Knockout of B-Myb in the developing embryo results in embryonic death owing to improper maturation of the epiblast. Moreover, this phenotype has made it difficult to study the role of B-Myb during this time when cells of the inner cell mass undergo their initial maturation (Tanaka et al., 1999). These studies led to the belief that B-Myb must be an important regulator of pluripotency, moreover, a decrease in Oct4 expression and protein levels upon B-Myb knockdown supported this theory (Tarasov et al., 2008, Singh and Dalton, 2009). However, through the generation of mouse ESCs with both alleles of B-Myb knocked-out, more definitive deletion of B-Myb was accomplished. Expression analysis of the B-Myb mutant ESCs revealed no change in the expression or protein levels of the core transcription factors, challenging the requirement for B-Myb for pluripotency (Lorvellec et al., 2010, Pauklin and Vallier, 2013). Recently, B-Myb was shown to be a chromatin binding partner for Oct4, Sox2 and Nanog in ESCs, suggesting potential interaction of B-Myb with pluripotency associated transcription factors (Rafiee et al., 2016). B-Myb levels are also crucial for efficient somatic cell reprogramming. Overexpression of B-Myb during reprogramming has a detrimental influence upon the chromatin landscape resulting in deregulation of genes important for the mesenchymal to epithelial transition (Ward et al., 2018). Overall, there is conflicting evidence for the role of B-Myb in regulating pluripotency, which could be related to variations in the models used in these studies.

1.5.5 The role of B-Myb in maintenance of genome stability

An essential role for B-Myb in the maintenance of genome stability has been demonstrated in multiple systems, including; drosophila (Manak et al., 2002), chicken DT40 cells (Ahlbory et al., 2005), zebra fish (Shepard et al., 2005), human megakaryocytes (Garcia and Frampton, 2006) and embryonic stem cells (Lorvellec et al., 2010). Although, the mechanism by which B-Myb exerts an influence upon genome stability is not well characterised. Recent work has revealed potential roles for B-Myb in the DDR. Knockout of B-Myb decreases survival after treatment with UV irradiation (Ahlbory et al., 2005) and B-Myb is required for recovery from DNA damaged induced G2 cell cycle arrest (Mannefeld et al., 2009). Recently, a central region (amino acid residues 446–559) of B-Myb was shown to interact with NBS1, a component of the MRN complex required for activation of the ATM dependent DDR (Henrich et al., 2017). B-Myb also localises to UV induced DNA damage sites and undergoes phosphorylation by protein kinase Gsk3 β . Moreover, this causes its dissociation from the MRN complex to play roles in transcription of G2/M checkpoint associated genes. Interestingly, ATM activity was necessary for the phosphorylation of B-Myb in this system, further implicating B-Myb in the DDR (Henrich et al., 2017).

B-Myb levels are also important in progression of myelodysplastic syndromes through a role in the DDR in haematopoietic stem cells. Reduced levels of B-Myb resulted in sensitivity to ionizing irradiation due to unsustained ATM dependent phosphorylation of KAP1 and telomere fragility (Bayley et al., 2018a). Another recent study described an ATR dependent transcriptional network responsible for governing correct timing for entry into G2 after replication completion. Moreover, this study described an ATR-CDK1-FOXM1 transcriptional axis which encompasses B-Myb activity and governs the correct timing for activation of genes responsible for this transition (Saldivar et al., 2018). Overall, these studies reveal potential roles

for B-Myb implicated in the DDR incorporating both direct protein-protein interactions as well as influences upon transcription.

1.5.6 B-Myb as an oncogene

Considering the widespread roles of B-Myb, it is not surprising that its deregulation is associated with tumourigenesis. B-Myb is found overexpressed in multiple cancers and is associated with poor patient outcome (Inoue and Fry, 2016, Ren et al., 2015, Bayley et al., 2018b, Calvisi et al., 2011, Nord et al., 2010, Sakai et al., 1993). A study looking at primary tumour samples from 108 breast cancer patients showed increased expression of B-Myb in some breast cancer types. Subsequent RNA silencing of B-*myb* in these tumour cells resulted in a cell cycle arrest plus reduced migration and invasion capacity (Deyou Tao., 2014). In addition to its role as an oncogene, a contrasting loss of B-Myb results in detrimental effects to cell proliferation and genome stability, therefore, B-Myb has also been described as a tumour suppressor protein (Martinez and Dimaio, 2011, Lorvellec et al., 2010). One mechanism for B-Myb deregulation is amplification of the chromosome location in which it resides (ch20q13); a genetic alteration which has been described in multiple cancer types (Shi et al., 2011, Tanner et al., 2000). Alternatively, deregulation of associated signalling pathways such as DREAM complex or p53 signalling, has also been proposed to influence B-Myb activity (Martinez and Dimaio, 2011, Quaas et al., 2012, Fischer et al., 2016, Parikh et al., 2014), highlighting indirect mechanisms for its deregulation in cancer.

A role for B-Myb in the epithelial to mesenchymal transition, a process necessary to initiate tumour metastasis, has also been proposed. Knockout of B-Myb in breast cancer cells restores the expression of E-cadherin and suppresses cell invasiveness. In contrast, its overexpression resulted in loss of E-cadherin and gain of mesenchymal associated markers (Tao et al., 2015).

Moreover, this is supported by the recently identified role of B-Myb in regulating mesenchymal to epithelial transition (MET) associated genes during somatic cell reprogramming. Moreover, this was attributed to an influence of B-Myb deregulation upon chromatin accessibility (Ward et al., 2018). In support of this theory, B-Myb is found substantially upregulated in metastasised cancer tissue compared to primary tumour cells (Sakai et al., 1993, Deyou Tao., 2014).

1.5.7 The role of B-Myb in DNA replication

A dedicated role for B-Myb in regulating the DNA replication programme has also been described. Moreover, Pdp1, which binds and interacts with the DNA replication and repair machinery, was shown to bind and interact with B-Myb and PCNA simultaneously (Werwein et al., 2012). A more in-depth insight into the role of B-Myb during replication was carried out in B-Myb mutant ESCs. Loss of B-Myb activity in this system resulted in a severe slowdown of replication progression and an increase in replication stress associated behaviour. This was accompanied by an increased recruitment of γ H2Ax indicating DNA damage accumulation (Lorvellec et al., 2010). Interestingly, overexpression of C-MYC and FOXM1 somewhat rescued the B-Myb knockout phenotype, highlighting a potential link with its transcriptional roles (Lorvellec et al., 2010). These studies suggest that B-Myb plays an important role in DNA replication important for maintaining genome stability.

The role of B-Myb as a regulator of cell proliferation	Activation of its own promotor	(Sala et al., 1999)
	Influence upon transcription of cyclins and CDKs.	(Bartusel et al., 2005, Osterloh et al., 2007, Papetti and Augenlicht, 2011, Sadasivam et al., 2012, Zhu et al., 2004).
	Interaction with FOXM1 to promote transcription of S/G2 and G2/M transition associated genes.	(Knight et al., 2009, Lefebvre et al., 2010, Okada et al., 2002, Osterloh et al., 2007, Sadasivam et al., 2012, Tarasov et al., 2008, Pattschull et al., 2019).
	Regulation of genes associated with mitosis - spindle assembly and cytokinesis. e.g. AURKA, PRC1, CENPF and PLK1.	(Iltzsche et al., 2017, Osterloh et al., 2007, Sadasivam et al., 2012, Wolter et al., 2017).
	Regulation of Topoisomerase 2 activity.	(Brandt et al., 1997).
	Regulation of cell survival genes (e.g. BCL, clusterin).	(Grassilli et al., 1999, Cervellera et al., 2000).
	Regulation of pluripotency regulators (e.g. OCT4, NANOG).	(Rafiee et al., 2016, Tarasov et al., 2008, Singh and Dalton, 2009, Zhan et al., 2012, Lorvellec et al., 2010).
	Regulation of genes associated with epithelial to mesenchymal transition (EMT).	(Tao et al., 2015, Ward et al., 2018, Deyou Tao., 2014).
	Regulation of the S-G2 transition.	(Saldivar et al., 2018).
	Regulation of the serine-threonine kinase receptor-associated protein (STRAP).	(Seong et al., 2011).
The role of B-Myb in maintaining genome stability	Important for genome stability maintenance in multiple systems (zebra fish, drosophila, human megakaryocytes, etc).	(Fung et al., 2002, Manak et al., 2002) (Shepard et al., 2005, Ahlbory et al., 2005, Garcia and Frampton, 2006, Lorvellec et al., 2010).
	Roles in DNA replication.	(Lorvellec et al., 2010, Werwein et al., 2012).
	Roles in the DNA damage response.	(Saldivar et al., 2018, Henrich et al., 2017, Bayley et al., 2018a, Mannefeld et al., 2009, Ahlbory et al., 2005).
	Formation of the Myb clafi complex important for spindle assembly.	(Yamauchi et al., 2008, Shepard et al., 2005).

Table 1.1 Key studies identifying the roles of B-Myb in cell proliferation and genome stability maintenance

1.6 Aims and hypothesis

B-Myb is vitally important for normal cellular growth through its pleiotropic influences throughout the cell cycle, furthermore, deregulation of B-Myb has been associated with carcinogenesis, acting as both an oncogene and a tumour suppressor. B-Myb is massively overexpressed in stem cells and evidence suggests it plays a vital role in normal DNA replication progression in ESCs, although, the molecular mechanisms are not characterised. Due to the role of B-Myb in cell cycle signalling and the recent implication of B-Myb in the DDR, it was hypothesised that the replication stress upon loss of B-Myb in ESCs may be associated with deregulation of these pathways.

Therefore, the main aims of this study were:

- To recapitulate previous findings concerning the influence of B-Myb loss upon replication progression in ESCs through DNA fibre analysis and immunofluorescence staining for DNA damage markers.
- To further characterise the replication stress phenotype after B-Myb loss in ESCs through analysing cell cycle checkpoints, ultra-fine bridge formation and through measuring cell viability after treatment with various chemical inhibitors.
- To determine the potential role of cell cycle signalling deregulation in the replication stress phenotype in the B-Myb mutant ESCs; through western blot analysis, and by examining the effect of inhibition of cell cycle associated signalling upon replication progression and in combination with B-Myb loss.
- To determine whether the potential identified roles of B-Myb in the DDR are also implicated in the replication stress phenotype observed in ESCs, through examining the effect of various DDR inhibitors upon DNA replication in combination with B-Myb loss.

- To determine whether the replication stress phenotype can be attributed to changes in transcription, through performing global transcriptome analysis of the B-Myb mutant ESCs.
- To investigate the activity of the DNA damage response and its influence upon DNA replication in stem cells compared to somatic cells through performing DNA fibre analysis in ESCs and mouse embryonic fibroblasts (MEFs) after treatment with DDR inhibitors.

Chapter 2 Materials and Methods

2.1 Generation and maintenance of cells

2.1.1 Generation of mouse embryonic fibroblast feeder cells

Mouse embryonic fibroblasts (MEFs) were used as a feeder layer to provide structural and nutritional support for ESC culture. 12.5-day-old mouse embryos were taken, and the heart, liver and cerebellum were removed. The remaining tissue was dissociated using trypsin-EDTA (0.25%, Gibco) for 10 minutes at 37°C before inactivating trypsin in foetal bovine serum (FBS) (Sigma). An 18-gauge needle was used to further disrupt tissue structure and the cell suspension was added to a 15ml falcon tube containing 9ml of MEF media (Table 2.1). After 5 minutes, the remaining large tissue masses had settled to the bottom allowing aspiration of the single cell suspension, which was added to culture dishes pre-treated with 0.2% (w/v) gelatin (Sigma) in MEF media.

Upon reaching passage 3, cells were mitotically inactivated for use as feeder cells. Fresh media was added, and cells incubated at 37°C for 2 hours. Cells were treated with media containing 8µg/ml Mitomycin C (10mg/ml stock) (Sigma, M4287), an alkylating drug with DNA crosslinking activity, for 2 hours at 37°C. Treated cells were washed three times in phosphate buffered saline (PBS) (Sigma) before harvesting cells using Trypsin-EDTA. Cells were collected and trypsin inactivated in FBS before centrifugation at 150g for 10 minutes. Cells were counted and re-suspended in freezing media (70% (v/v) media, 20% (v/v) FBS, 10% (v/v) DMSO (Sigma) at 2×10^6 cells/ml before aliquoting and freezing at -1°C/minute in a Mr. Frosty freezing container (Thermo Fisher). Prior to use, inactivated MEFs were defrosted and resuspended in MEF media before centrifugation at 250g for 5 minutes. Plates/dishes were pre-treated with 0.1% (w/v) gelatin for 20 minutes. The resulting pellet was resuspended in MEF media before culturing at the required density (approximately 4×10^4 cells/cm²) in gelatinised plates/dishes.

2.1.2 Generation of B-myb ablated mouse embryonic stem cells (mESCs)

Prior to commencing this study, ESCs containing a B-myb floxed allele were generated (B-myb^F) through introduction of LoxP sites, 5' of exon 2, and 3' of exon 5, encompassing the DNA binding domain of the B-myb gene. In this system, upon introduction of Cre recombinase, the LoxP flanked site is excised, producing a knockout allele (B-myb^Δ) (Garcia et al., 2005, Lorvellec et al., 2010) (Figure 2.1).

To produce new double knockout B-myb^{Δ/Δ} clones, 1x10⁷ previously generated B-myb^{F/Δ} mESCs were grown in culture before harvesting and resuspending in 700μl PBS. Cells were transfected with a pLv-Cre-eGFP plasmid containing a cre recombinase gene followed by an internal ribosome entry sequence (IRES) linked eGFP reporter gene (Ahmed et al., 2004). The procedure was performed through adding 50μg of plasmid to the cells in PBS before transfection through electroporation at 500μf, 260V using a Gene Pulser II (BioRad). Resulting cells were added in culture on fresh MEF feeder cells. After two days, cells were harvested and GFP positive cells (B-myb^{Δ/Δ}) were isolated through high speed XDP (Beckman Coulter) cell sorting with untransfected cells as a negative control. Positive sorted cells were then cultured at a density of 300 cells per 10cm dish in ESC media (Table 2.1). After 10-15 days, colonies were washed with PBS before being manually picked using a pipette under a microscope at 4x objective. 25μl of trypsin-EDTA in 20μl of PBS was added to the colonies for 4 minutes before inactivating with 20μg/ml trypsin inhibitor (Thermo Fisher, 17075029). Cells were resuspended in 150μl ESC media and grown in culture. After reaching sizeable colonies, clones were resuspended in ESC freezing media (50% (v/v) ESC media, 10% (v/v) DMSO, 40% (v/v) KO-SR) before aliquoting into cryovials and freezing at -1°C/minute in a Mr. Frosty freezing container (Thermo Fisher). For long-term storage, clones were transferred to liquid nitrogen. To determine successful ablation of B-myb in individual clones, firstly, RNA was extracted, and qPCR performed to analyse B-myb expression (section 2.2). Secondly, protein extraction

and SDS-PAGE was performed to analyse B-Myb protein levels by western blotting (section 2.3).

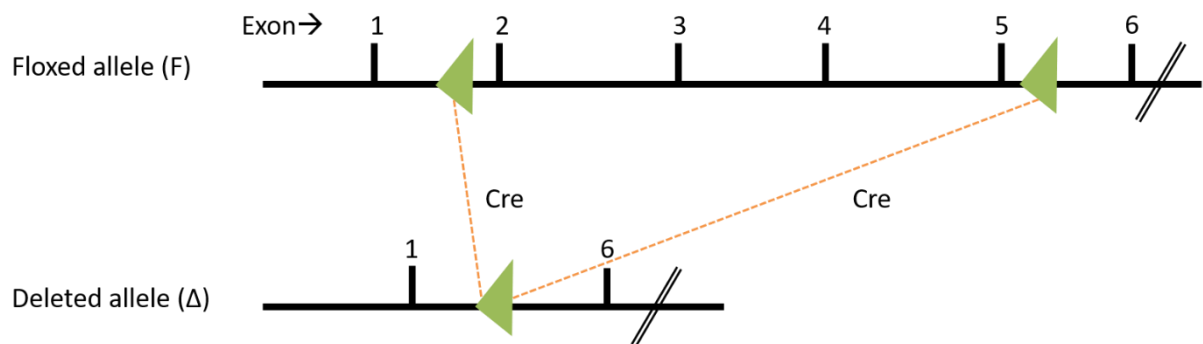


Figure 2.1 Generation of a B-myb conditional knockout using Cre-Lox recombination mESCs containing a floxed (F) allele of B-myb were previously generated. LoxP sites (Green arrows) were inserted through homologous recombination using a vector designed and published in 2005 targeting sites 5' of exon 2, and 3' of exon 5, encompassing the DNA binding domain (Garcia et al., 2005). Deletion of this region was achieved through introducing Cre recombinase producing a deleted allele (Δ).

MEF media	ESC media
- 88% DMEM (Gibco) - 10% FBS - 1% (2mM) L-glutamine (Gibco) - 1% (50U/ml) Penicillin/Streptomycin (Gibco) - 0.0006% (0.1M) β-mercaptoethanol (Sigma)	- 82% Knockout DMEM (Gibco) - 15% Knockout serum replacement (Gibco) - 1% (2mM) L-glutamine (Gibco) - 1% (1X) MEM non-essential amino acids (Gibco) - 1% (50U/ml) Penicillin/Streptomycin (Gibco) - 0.0006% (0.1M) β-mercaptoethanol (Sigma) Added fresh: - 0.01% (1000U/ml) Leukaemia inhibitory factor (Millipore, ESG1107) - 0.03% (3μM) GSK3 inhibitor (Millipore, CHIR99021) (10mM stock made) - 0.01% (1μM) MEK1 inhibitor (Millipore, PD0325901) (10mM stock made)

Table 2.1 The constituents of media used for cell culture

2.1.3 mESC culture conditions and treatments

When required, successful mESC clones were defrosted and resuspended in pre-warmed ESC media. Cells were centrifuged at 250g for 5 minutes and the pellet was resuspended in ESC media before seeding on a pre-prepared MEF feeder layer. Media was replenished daily and upon formation of large colonies (after 2-3 days), cells were passaged onto a fresh feeder layer. In many of the experiments described, the cell media was supplemented with various chemical inhibitors. A summary of the inhibitors used and their concentration can be found in Table 2.2. Importantly, despite ESCs displaying unlimited self-renewal, due to the potential accumulation of genetic abnormalities, ESCs used in the experiments presented here were not grown past twenty passages.

Chemical agent	MOA/ Product code/ Working concentration
Colcemid	Microtubule depolymerising agent. Gibco, 15212012. Used at 0.27µM.
Camptothecin (CPT)	Topoisomerase 1 inhibitor. C9911, Sigma. Used at 2.5-10µM.
Caffeine	Inhibitor of protein kinase involved in DDR (eg. ATM and ATR). C0750, Sigma. Used at 0.0625 – 2mM.
Hydroxyurea (HU)	Inhibitor of nucleotide synthesis. H8627, Sigma. Used at 0.25mM.
Olaparib	Inhibitor of PARP1. AZD2281. Used at 10µM.
MK-1775	Inhibitor of WEE1. M15123, Sigma. Used at 10µM.
Saponin	A detergent. Sigma. Used at 0.1% (w/v)
RO3306	Inhibitor of CDK1. 4181, Tocris. Used at 10µM.
PHA-767491	Cdc7 inhibitor. 3140, Tocris. Used at 10µM.
KU-55933	Inhibitor of ATM kinase. 3544, Tocris. Used at 10µM.
KU60019	More selective inhibitor of ATM kinase. CAY17502-1, Cambridge bioscience. Used at 10µM.
AZ20	Inhibitor of ATR kinase. 5198, Tocris. Used at 10µM.
Mirin	Inhibitor of MRE11 (MRN complex). M9948. Sigma. Used at 20µM.

Table 2.2 List of chemical agents used and their working concentration

2.2 RNA extraction and qPCR

2.2.1 Extraction of RNA

Cells were washed in PBS before pipetting up and down in 200µl Trizol reagent (Invitrogen) to promote cell lysis; lysates were stored at -20°C. Prior to extraction, samples were defrosted before adding 40µl of chloroform (VWR chemicals). The samples were shaken vigorously by hand for 15 seconds before leaving at room temperature for 3 minutes. The mixture was then centrifuged at 12000g for 15 minutes at 4°C before isolating the aqueous phase by carefully pipetting it into a new Eppendorf tube. 100µl of 100% isopropanol (VWR chemicals) was added and the sample was incubated at room temperature for 10 minutes before centrifugation at 12000g for 15 minutes. The supernatant was removed, and the pellet washed in 75% (v/v) ethanol (VWR chemicals) before further centrifugation at 7500g for 5 minutes at 4°C. The resulting pellet was air dried before resuspending in DEPC treated H₂O (Thermo fisher - 750024). RNA concentration in ng/µl was determined using a Nanodrop 2000c spectrophotometer (Thermo scientific) before storing samples at -20°C.

2.2.2 cDNA synthesis

500ng of RNA was added to 500ng of oligo (dT)₁₂₋₁₈ primers (Invitrogen) supplemented with 1µl of 10mM dNTPs mix (Invitrogen) made up to 13µl with RNase-free H₂O (Qiagen). The mix was incubated at 65°C for 5 minutes before placing on ice for 5 minutes. The sample was then mixed with 4µl of 5x first strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂), 1µl 0.1M dithiothreitol (DTT) (Thermo fisher) plus 1µl reverse transcriptase (200U/µl) taken from the SuperScript™ III Reverse Transcriptase kit (Invitrogen, 18080085). The mixture was incubated firstly at 25°C for 5 minutes before transferring to 50°C for 50 minutes and finally at 70°C for 15 minutes to inactivate the enzyme mixture. cDNA concentration was determined using a Nanodrop 2000c spectrophotometer (Thermo scientific).

2.2.3 TaqMan qPCR

To analyse the cDNA, realtime qPCR was performed. For the reaction, a mastermix was produced containing 1µl of the cDNA, 10µl TaqMan assay mix containing forward and reverse primers (Table 2.3), 10µl PCR reaction buffer (Applied biosystems) and 8µl DEPC treated H₂O. The qPCR was carried out in triplicate in a Stratagene Mx3005P (Agilent Technologies). The PCR conditions were 50°C for 2 minutes, 90°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The Ct values generated were used to calculate expression of *B-myb* relative to β_2 microglobulin (Livak and Schmittgen, 2001).

Gene	Applied Biosystems Gene ID
<i>B-myb</i>	Mm00485340_m1 (Chromosome 2, Exon boundary - 6-7, Amplicon length -108)
β_2 microglobulin	Mm00437762_m1 (Chromosome 2, Exon boundary - 1-2, Amplicon length -77)

Table 2.3 Oligo and primer sets for *B-myb* and β_2 microglobulin used in qPCR

2.3 Determining protein levels using western blotting

2.3.1 Protein extraction

Protein was extracted from ESCs using a series of purification steps. Preceding this process, experiment specific treatment was carried out and the cells were harvested using trypsin-EDTA (0.25%, GIBCO) before centrifugation at 250g, 4°C for 5 minutes. Pellets were resuspended in 1ml ice-cold PBS before placing on ice. Cells were counted using a haemocytometer and centrifuged once more. The pellet was resuspended in 50µl total protein lysis buffer per 10⁶ cells on ice (Table 2.4). The cell suspension for each sample was set aside on ice for 20 minutes before centrifugation at 600g for 10 minutes at 4°C. The supernatant from each sample was transferred to an Eppendorf and 2µl of the sample was set aside for determining protein

concentration. Samples were ‘snap frozen’ through introducing 100% ethanol to dry ice containing the samples, before storage at -80°C. To determine protein concentration of extracted samples, a Bradford absorption assay was carried out. A 1µg/µl stock solution of BSA (Sigma, 1076192) was prepared and a range of BSA concentrations (0, 1, 2, 4, 6, 8 and 10 µg/µl) were generated. The BSA samples were diluted in 800ml H₂O plus 200ml Bradford reagent (Sigma, B6916). 1µl of each protein sample was also diluted in the same way. Absorbance was measured at 595nm and a graph of absorbance vs concentration was plotted. The absorbance measured for each sample was used to determine the protein concentration in µg/µl.

2.3.2 Fractionation of soluble and non-soluble (chromatin bound) protein

B-*myb*^{+/+} ESCs were isolated and counted as described in section 2.3.1. To isolate soluble protein, cells were first resuspended in fractionation buffer A (Table 2.4). After 8 minutes, the lysate was centrifuged at 1300g for 5 minutes at 4°C before transferring the supernatant to a separate tube (soluble protein). The pellet (containing nuclei) was then resuspended in fractionation buffer B (Table 2.4) before centrifugation at 1700g for 5 minutes at 4°C, the supernatant was added to the soluble protein fraction. The soluble fraction was snapfrozen and stored at -80°C. The pellet, now containing chromatin with weakly and strongly bound protein, was then incubated once more with buffer B supplemented with 0.1% Triton X (Sigma) and 200mM NaCl, to remove weakly bound protein, before centrifugation at 10000g for 1 minute at 4°C. The supernatant was discarded, and the pellet resuspended in total protein lysis buffer (Table 2.4). The buffer containing the pellet was sonicated three times for 5 minutes using a (Bioruptor Pico, Diagenode) before being snapfrozen and stored at -80°C. To determine protein concentration of extracted samples, a Bradford absorption assay was performed as described previously in section 2.3.1.

Total protein lysis buffer	20mM Tris pH 7.4, 10mM Ethylenediaminetetraacetic acid (EDTA), 100mM Sodium Chloride, 1% (v/v) Triton x 100, 1mM Sodium Fluoride, 1mM β -glycerophosphate, 1mM Ethylene glycol tetra-acetic acid (EGTA), 5mM Polypropiolate sodium, Phenylmethylsulfonyl fluoride (PMSF) (Sigma) (1 in 100), sodium orthovanadate (Na_3VO_4) (Sigma) (1 in 1000), phosphatase inhibitors (PhosSTOP, Sigma) (1 in 10), protease inhibitor (cocktail set III, Calbiochem) (1 in 500).
Fractionation Buffer A	10mM HEPES pH 7.9, 10mM KCL, 1.5mM MgCl_2 , 0.34M sucrose, 10% (v/v) glycerol (Sigma), 1mM Dithiothreitol (DTT), 0.1% (v/v) triton X, protease inhibitor (cocktail set III, Calbiochem) (1 in 500).
Fractionation Buffer B	3mM EDTA, 0.2mM EGTA, 1mM DTT protease inhibitors (cocktail set III, Calbiochem) (1 in 500).
*Chromatin wash buffer	*Supplemented with 0.1% (v/v) Triton X and 200mM NaCl.

Table 2.4 Buffers used for cell lysis and protein extraction

2.3.3 SDS-PAGE and immunoblotting

The purified proteins were prepared for gel electrophoresis by resuspending in 4x sample buffer (40% (v/v) Glycerol, 240 mM Tris/HCl pH 6.8, 8% (w/v) sodium dodecyl sulfate (SDS), 0.04% (w/v) bromophenol blue, 5% (v/v) beta-mercaptoethanol) and heating at 95°C for 8 minutes. 6–10% acrylamide gels (depending on protein size) were prepared and 40 - 60µg of protein was loaded as well as a protein ladder (Thermo fisher, 26634). The protein was subjected to SDS-PAGE at 85V for approximately 2 hours or until the gel front had run to the bottom. Proteins were transferred onto PDVF membrane (Millipore) using a semi-dry transfer system (Biorad transblot turbo) at 25V for 30 minutes. Upon completion, successful protein transfer was checked through Ponceau S staining (Sigma P7170). Membranes were blocked in 5 – 10% BSA in TBST (50 mM Tris-Cl, pH 7.6; 150 mM NaCl plus 0.1% (v/v) Tween20) for 2–12 hours depending on antibody efficacy. After blocking, the membrane was subjected to incubation with a primary antibody for 1 – 12 hours (Table 2.5). The membrane was washed three times in TBST for 10 minutes before applying a HRP conjugated secondary antibody specific to the primary antibody (mouse rabbit or goat) for 1-2 hours (Table 2.5). The membrane was re-washed three times in TBST and developed onto photographic film using Pierce ECL substrate (ThermoFisher, 32106). On occasion, blotting for loading controls was performed after membrane stripping. To strip membranes, they were incubated in 6% (v/v) 1M Tris-HCl pH 6.8, 0.2% (w/v) SDS plus 0.7% (v/v) β-mercaptoethanol in de-ionised H₂O for 30 minutes. The membrane was then washed three times 10 minutes in TBST before re-performing blocking and immunoblotting.

Antibody target	Predicted size and	Immunoglobulin	Cat# and supplier	Dilution
B-Myb (N-terminal)	80kDa	Rabbit	N19, sc724 (SCBT)	1:500
P-CDK1 (Cdc2) (Tyr15)	30kDa	Rabbit	10A11 (Cell signalling)	1:500
CDK1	30kDa	Mouse	A17 (Boster)	1:500
Chk1	56kDa	Mouse	Sc8408 (SCBT)	1:500
P-Chk1 (Ser345)	56kDa	Rabbit	133D3 (CST)	1:500
GAPDH	37kDa	Mouse	Ab8245 (Abcam)	1:5000
Beta-actin	40kDa	Goat	Sc1616 (SCBT)	1:1000
Tubulin	50kDa	Mouse	ab7291 (Abcam)	1:1000
Histone H3	17kDa	Rabbit	ab1791 (Abcam)	1:5000

Secondary antibody	Cat# and supplier	Dilution
Anti-mouse HRP	NXA931 (Amersham)	1:5000
Anti-rabbit HRP	NA9340 (Amersham)	1:5000
Anti-goat HRP	sc-2020 (Santa Cruz)	1:1000

Table 2.5 Primary and secondary antibodies used in western blotting experiments

2.4 Visualising replication through DNA fibre analysis

2.4.1 Labelling and spreading

Exponentially growing cells were subject to various chemical inhibitors and stress inducing agents before labelling replicating DNA through incorporation of thymidine analogues in culture. During labelling, all media was pre-heated to 37°C before adding to cells. Firstly, warm ESC media containing 30µM iododeoxyuridine (IdU) (I7125, Sigma) was added for 20 minutes. Cells were washed gently with warm media before addition of media containing 300µM chlorodeoxyuridine (CldU) (C6891, Sigma) for a further 20 minutes. Cells were washed two times in ice-cold PBS, harvested through treatment with trypsin-EDTA before centrifugation at 250g for 5 minutes. Cells were then washed two times in PBS before counting and resuspending at 10⁶ cells/ml. Labelled cells were mixed 1:1 in untreated cells and were kept on ice up until cell lysis.

In preparation for cell lysis and spreading, a humidified chamber was created through addition of damp paper towels into a light protected slide box. 2.5µl of cell suspension was added to the top of a microscope slide and left exposed to the air for approximately 4 minutes. 7.5µl lysis/spreading buffer (200 mM Tris pH 7.4, 50 mM EDTA, 0.5% (w/v) SDS) was swirled into the cells and incubated with the lid closed for 5 minutes. The slides were then angled at approximately 30° to allow the lysate to slowly spread down the length of the slide. Slides were allowed to air dry before fixing in ice-cold 3:1 methanol: acetic acid for 10 minutes. Slides were dried and stored for at least 24 hours at 4°C.

2.4.2 Immunofluorescence labelling of DNA

For DNA labelling, slides were added to a humidified chamber and rehydrated with deionised water two times for 5 minutes. DNA was denatured through incubating in 2.5M HCl for 1.5 hours before washing twice in PBS. Slides were blocked in blocking buffer (1% (w/v) BSA in PBST) for 1 hour before adding primary antibodies (Table 2.6) diluted in 100µl blocking buffer under a glass coverslip overnight at 4°C. Slides were washed in PBS before fixing in 4% paraformaldehyde (PFA, 16% stock, Perkin Elmer) for 10 minutes. PFA was removed and slides washed before blocking for 30 minutes in blocking buffer. A respective secondary antibody (Table 2.6) conjugated with a fluorescent marker was added, diluted in 100µl blocking buffer under a glass coverslip for 1 hour. Slides were washed in PBS, blocked for 30 minutes and mounted under a glass coverslip in prolong anti-fade mountant (P36961, Thermofisher). They were left overnight in the dark at room temperature to allow curing, before storing at -20°C. Imaging was carried out on a Leica DM6000 fluorescence microscope and images were taken at X60 magnification. Analysis of fibres was performed using the LasX software from Leica.

Primary antibodies	
Rat anti BrdU/CldU	ab6326 – Abcam, 1:200.
Mouse anti BrdU/IdU	ab181664 – Abcam, 1:200.
Secondary antibodies	
Goat anti-Mouse Alexa Fluor 488	A11029 – Thermo fisher
Goat anti-Rat Alexa Fluor 633	A21094 – Thermo fisher

Table 2.6 Antibodies used in DNA fibre experiments

2.4.3 Analysis of DNA fibres

Using LasX, a measuring tool was used to manually measure the length of single replication tracks in micrometres (μm). Replication rate (kb/min) is equal to length of the replication track (μm) multiplied by 2.59 and divided by the incubation time (minutes) (Jackson and Pombo, 1998). For replication rate analysis, only the first incorporated label (IdU) was measured (Figure 2.2). To calculate the percentage of specific replication structures, the LasX counting tool was used to manually count the number of each structure per image. The percentage of each structure was calculated from all of the images analysed (Figure 2.2). The lengths of fibre tracks were also used to calculate fork ratios for a measure of fork stability. This was performed primarily through analysis of elongating forks, in which the fork ratio represents the longest track divided by the smallest. In some cases, the ratio between the lengths of the tracks around new origins was also calculated in the same way (Figure 2.2).

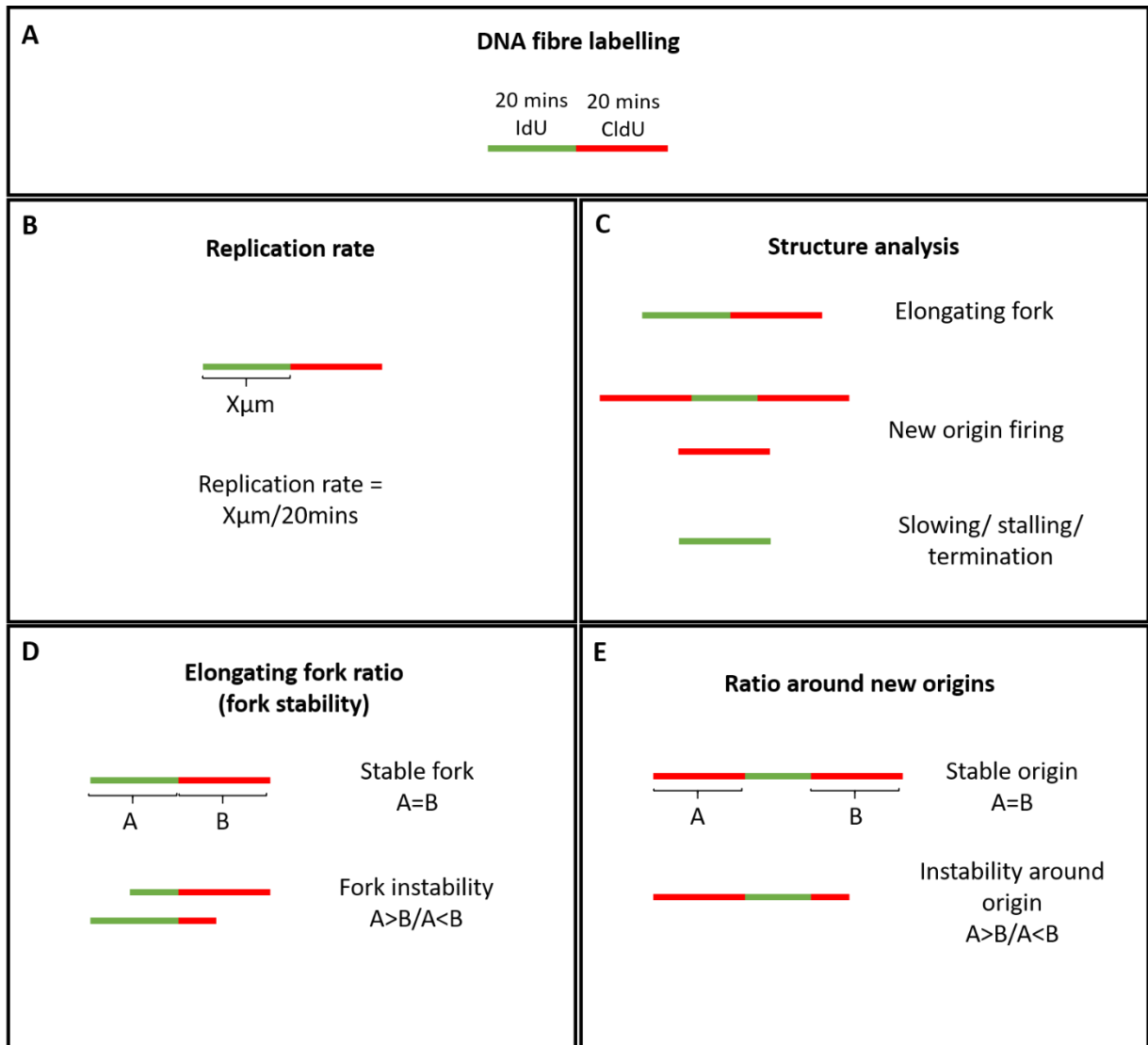


Figure 2.2 Techniques for analysing DNA fibre tracks A) DNA fibre analysis was performed through incorporation of deoxynucleoside analogues, IdU and CldU, for 20 minutes each. B) Replication rate was determined through measuring the length of the IdU label and calculating length relative to time. C) Specific replication structures were identified and quantified to calculate the percentage of each structure. D) For a measure of fork stability, the ratio between the lengths of the first and second incorporated label was calculated. E) To measure replication stability around new origins, the ratio between the lengths of the two labels around new origins was calculated.

2.5 Cell cycle analysis by flow cytometry

2.5.1 Incorporation and staining

50µM BrdU (Sigma, B5002) was added to the ESCs in culture for 1 hour. Cells were harvested and washed in PBS before resuspending in 250µl PBS. 750µl of ice-cold 100% ethanol was added drop-wise whilst vortexing the sample on low speed, before storing the samples at -20°C overnight. Before starting the staining protocol, the ethanol was removed through centrifugation at 300g for 5 minutes at 4°C and washing two times with PBS. The pellet was resuspended carefully in 200µl 2M HCl before incubating for 20 minutes. The HCl was then thoroughly washed off through several PBS washes. The pH was checked using a pH indicator stick (Hydriion 9800, 0.0 to 14.0) before commencing the next step. The sample was incubated two times in blocking buffer (5% (w/v) BSA, 0.1% (v/v) Triton X and 0.1% (v/v) NaN₃) for 5 minutes, removing the supernatant after each wash through centrifugation at 300g for 5 minutes at 4°C. Next, the cells were resuspended in 40µl FITC-conjugated anti-BrdU antibody or 40µl mouse IgG1 κ isotype control antibody from the FITC Mouse Anti- BrdU Set (BD Biosciences, 556028) for 1 hour. Samples were washed two times with PBS before adding 10µg/ml 7-Amino-Actinomycin D (7-AAD) (BD biosciences, 559925) in PBS.

2.5.2 Flow cytometry and analysis

The samples were acquired using a CyAn ADP flow cytometer (Beckman Coulter). Analysis was carried out using the 'Flow Jo' software package. ESCs were gated from MEFs and dead cells through analysing cell size and cell granularity. This was followed by the removal of doublets from the analysis. The number of events for each sample was then made constant before measuring BrdU intensity through the 'log FITC' channel plus DNA content through the 'Lin PE-Cy5' channel, presented as a histogram. Using the staining intensities, the cells were gated further to isolate the different cell cycle stage populations; the number of cells in each gate was calculated as a percentage of total cells.

2.6 Visualisation of replication factories through incorporation and detection of IdU

ESC colonies were exposed to experiment specific treatments before addition of 20 μ M IdU (I7125, Sigma) to the media for 30 minutes. A sample without IdU treatment was used as a negative control. The cells were washed in PBS at 4°C before addition of fixing solution (95% (v/v) ethanol, 5% (v/v) acetic acid) for 30 minutes at room temperature. Next, the samples were rinsed and left in 70% (v/v) ethanol overnight at 4°C. The following day, the ethanol was removed through two washes with deionised water, before treating with 1.5M HCl supplemented with 0.5% tween 20 for 1 hour at room temperature. The HCl was thoroughly washed off through two washes with PBS followed by two further washes in blocking solution (5% (w/v) FBS, 0.1% (v/v) sodium azide plus 0.1% (v/v) tween 20 in PBS). Next, cells were treated with 100 μ g/ml RNase A (Thermo Fisher, EN0531) in PBS for 3 minutes at 37°C. This was followed by incubation with blocking solution for 1 hour at room temperature. The cells were then incubated with a mouse anti-IdU antibody (Invitrogen, MD5000) at a concentration of 1:200 in blocking solution overnight at 4°C. The following day, the primary antibody was washed off through three washes with blocking solution, before adding goat anti-mouse Alexa 488 conjugated secondary antibody (Invitrogen, A11029) at 1:500 for 1 hour at room temperature. The secondary antibody was washed off through two PBS washes before mounting stained ESC colonies in Prolong supplemented with DAPI (Invitrogen, P36931) under a cover slip. Mounted samples were left overnight at room temperature to cure before storing at -20°C. Image acquisition was performed using a Zeiss LSM 780 confocal immunofluorescence microscope.

During the early stages of S phase, the nucleus presents numerous small foci throughout the nucleoplasm, whereas, in mid/late S phase cells, replication factories aggregate at the nuclear periphery before adopting a more clumped appearance (Dimitrova and Berezney, 2002, Ferreira

and Carmo-Fonseca, 1997). Colonies containing early replicating cells were selected and z-stack images were acquired at 100X magnification with a distance of 0.4 μ m between each z slice. The images were then converted to 3D rendered projections using Imaris (x64.3.1) (Bitplane, Zurich, Switzerland). An automatic foci counting tool within the Imaris software was then used to automatically quantify the number of foci per cell. This was through the spots function in the surpass tool. Spots were defined as ‘growing regions’ with an estimated diameter of 0.3 μ m.

2.7 Measuring cell viability using resazurin

Cell viability was measured after treatment with specific stress inducing agents which are summarized in Table 2.2. ESCs were cultured for 24 hours before treatment with a series of concentrations of specific drug treatments in triplicate. Cells were also treated with 0.1% (v/v) Saponin as a positive control for cell death. After 24 hours, the media was replaced with 200 μ l 3.5% (v/v) resazurin (Cell quanti blue, Invivogen) in ESC media and incubated for 1 hour at 37°C. 100 μ l of each sample was transferred to a black 96-well plate before measuring fluorescence on a synergy 2 microplate reader (Fisher scientific, BioTek) using the GEN5 software with a 540 – 620 excitation and emission filter. An average of three fluorescent readings was calculated per treatment and viability was calculated relative to background fluorescence (3.5% resazurin incubated without cells), and to a no drug treatment negative control. Dose response curves were generated, and non-linear regression analysis was carried out using the Graphpad prism software.

2.8 Cell cycle checkpoint analysis through detection of phosphorylated histone H3 at Ser10

Phosphorylation of histone H3 at serine 10 (H3-pS10) is strongly associated with chromatin condensation thus can be used as a marker for cells in mitosis (Li et al., 2005). ESCs were treated with 2.5 μ M CPT for 4 hours; 0.1 μ g/ml (0.27 μ M) colcemid, a microtubule depolymerising agent, was added for the final 3.5 hours to arrest cells which pass into mitosis. Cells were immediately harvested on ice before washing in PBS and centrifugation at 250g for 5 minutes at 4°C. 5x10⁴ cells were cytopun at 300g for 5 minutes (Cytospin 4, Thermo scientific) onto microscope slides and air dried for 15 minutes. Cells were fixed in 4% paraformaldehyde for 20 minutes before washing twice in PBS. Permeabilization and blocking was carried out through treatment with blocking buffer containing 1% (w/v) BSA and 0.3% (v/v) triton X in PBS for 1 hour at room temperature. Cells were then incubated with a mouse anti-H3-pS10 (9701S) primary antibody at a concentration of 1 in 200 in blocking buffer at 4°C overnight. A separate slide was also incubated with an anti-mouse IgG antibody (Santa-cruz, sc2025) for an IgG control. Slides were washed twice in PBS before incubating with goat anti-mouse Alexa 488 secondary antibody (Invitrogen, A11029) at 1 in 500 for 1 hour at room temperature. The secondary antibody was removed using PBS washes before mounting samples in Prolong plus DAPI (P36931) under cover slips. Imaging was carried out on a Leica DM6000 fluorescence microscope and images were taken at x20 magnification. Counting was carried out manually using Image J cell counting plugin; the percentage of mitotic cells was calculated relative to total number of cells.

2.9 Immunofluorescence staining for 53BP1 recruitment

Experiment specific treatments were performed upon the ESCs in culture; for most experiments, cells were also treated with CPT as a positive control for damage. The labelling of replicating cells was also incorporated into most of the 53BP1 staining experiments. For this, 10 μ M EdU (C10337, Thermo fisher) was added to cells in culture for 1 hour before harvesting on ice. Next, cells were counted using a haemocytometer before cytopinning 3x10⁴ cells at 300g for 5 minutes (Cytospin 4, Thermo scientific) onto microscope slides and air drying for 15 minutes. Slides were treated with cold CSK1 buffer (10mM PIPES (Sigma), 300mM sucrose, 100mM NaCl plus 3mM MgCl₂ in H₂O) for 4 minutes, before, adding cold CSK2 buffer (CSK1 buffer plus 0.5% triton X, 100X protease inhibitors (Calbiochem III), 1mM PMSF and 1mM Na₃VO₄) for 1 minute at room temperature. Slides were then washed in PBS before fixing cells in 4% PFA (v/v) for 20 minutes. PFA was removed through several PBS washes before quenching the formaldehyde groups with 50mM ammonium chloride for 5 minutes. Cells were blocked and permeabilised in a blocking solution (3% (w/v) BSA, 10% (v/v) FBS plus 0.3% (v/v) triton X) for 1 hour.

2.9.1 Detection of EdU through a Click reaction

Staining of incorporated EdU was performed through a ‘click’ reaction representing the reaction between an alkyne (conjugated to EdU) and an azide (fluorescently labelled). Using this technique eliminates the need for a DNA denaturation step which could negatively influence 53BP1 detection. A reaction cocktail was made containing 86% Tris buffered saline (TBS) (50 mM Tris-Cl, pH 7.5, 150 mM NaCl), 4% (v/v) 100mM CuSO₄, 0.125% (v/v) Alexa-fluor azide 594 (C10330, Thermo Fisher) and 10% (v/v) 1M sodium ascorbate added in the order presented. 200 μ l of the reaction cocktail was added to each slide under a cover slip and incubated for 30 minutes in the dark. Slides were washed several times in PBS before re-blocking for 30 minutes in blocking solution.

2.9.2 53BP1 detection through immunostaining

For staining of 53BP1, blocking buffer was removed before incubating slides in a rabbit anti-53BP1 primary antibody (Novus Biologicals NB100-304) at 1 in 500 in blocking buffer at 4°C overnight. For the IgG control, cells were incubated with a rabbit IgG (sc-2027, Santa Cruz). The following day, slides were washed two times in PBST (0.1% (v/v) Tween 20 in PBS) before incubating for 1 hour in an anti-rabbit Alexa 488 secondary antibody (A31565, Thermo Fisher) at 1 in 500 at room temperature. After two more washes with PBST, slides were mounted in prolong supplemented with DAPI (P36931) and left to cure at room temperature overnight. Slides were stored at -20°C until ready for imaging. Imaging was performed using a Leica DM6000 fluorescence microscope and images were taken at x40 magnification. The number of 53BP1 foci per nuclei was counted manually using the counting plugin for Image J.

2.10 Immunofluorescence staining for phosphorylated KAP1 (S824)

ESCs were untreated or treated with 10µM CPT for 30 minutes or 4 hours before washing cells with PBS and placing on ice. 4% (v/v) PFA was added to fix the cells for 10 minutes before its removal through three washes with PBS. Next, ice-cold 100% methanol was added for 10 minutes before re-washing. Cells were then incubated in a blocking solution (1% (w/v) BSA, 5% (v/v) FBS plus 0.3% (v/v) Triton X in PBS) for 1 hour at room temperature. A rabbit anti-P-KAP1 (S824) (A300-767A, Bethyl Laboratories Inc) was added at 1:100 in blocking buffer overnight at 4°C. For IgG control, cells were incubated with an anti-rabbit IgG antibody (sc2027, Santa Cruz). Slides were washed in PBST three times before incubating for 1 hour at room temperature in a goat anti-rabbit Alexa 488 secondary antibody (A31565, Thermo Fisher) at 1 in 500 in blocking solution. Finally, cells were washed three times in PBST before

mounting samples in prolong supplemented with DAPI (P36931). Microscopy was performed using a Leica DM6000 fluorescence microscope and images were taken at x40 magnification.

2.11 Staining for formation of ultra-fine bridges

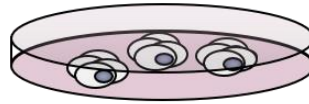
Experiment specific treatments were performed before washing cells with ice-cold PBS and fixation through treatment with 4% (v/v) PFA for 10 minutes. PFA was removed through PBS washes and formaldehyde groups were quenched through treatment with 50mM ammonium chloride for 10 minutes. Cells were washed three times more with PBS before adding ice-cold 100% methanol for 10 minutes. After repeating washes, cells were blocked in blocking buffer, 10% (v/v) FBS, 1% BSA (w/v) and 0.3% (v/v) Triton X in PBS, for 1 hour at room temperature. Antibody staining was performed using an anti-ERCC6 primary antibody (H00054821, Abnova), 1 in 100 in blocking buffer at 4°C overnight, and an anti-rabbit Alexa 488 secondary antibody (A31565, Thermo Fisher) at 1 in 500 in blocking for 1 hour at room temperature. Slides were mounted in prolong plus DAPI before storing at -20°C. Microscopy was performed using a Leica DM6000 fluorescence microscope; anaphase cells were visualised by DAPI staining and the percentage of anaphases with ultra-fine bridges was determined for each sample.

2.12 Alkaline comet assay

In preparation for the assay, 600µl 0.6% (w/v) agarose cooled to 50°C was added to microscope slides overnight; to provide a guide and to promote adherence of the agarose, rectangles were etched into the slides with a diamond pen. The following day, the ESCs were incubated with experiment specific treatments before harvesting on ice. 5×10^4 cells were washed and resuspended in 600µl PBS before adding 600µl 1.2% (w/v) low melting point agarose (50081, Lonza) cooled to 45°C. 600µl of the cell suspension was quickly pipetted onto the slides prepared earlier, covering the previously prepared agarose. Slides were placed at 4°C for 30 minutes before carefully incubating the slides in lysis buffer (2.5M NaCl, 0.1M EDTA pH8, 0.01M Tris pH 10, 0.067M NaOH plus 1% (v/v) DMSO and 1% (v/v) Triton X added fresh) overnight at 4°C in the dark. The following day, slides were washed twice in H₂O before placing in a comet tank (blackened-out 2D gel electrophoresis tank). Next, slides were incubated for 40 minutes in ice-cold running buffer (0.003M EDTA pH8, 0.05M NaOH plus 1% DMSO) before performing electrophoresis at 15V for 30 minutes. After this, slides were quickly washed twice in H₂O before treating with 1M Tris pH7 for 1 hour. This was followed by two additional washes before incubating at 50°C in an oven overnight (Figure 2.3).

Staining of comets was performed through incubating slides directly with DAPI (1µg/ml) before rinsing off the excess and adding a cover slip. Comets were imaged using a Zeiss LSM 780 confocal immunofluorescence microscope. Images were taken at x60 magnification and resulting images were analysed using the CASP analysis software. Moreover, this tool determined the olive tail moment (OTM) for each comet (Figure 2.3).

1. Cells exposed to DNA damage treatments in culture

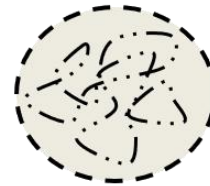


2. Cells embedded in low melting point agarose before cell lysis

Intact genome



Damaged genome



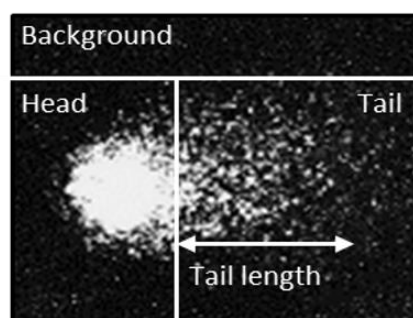
3. Electrophoresis in Alkaline conditions

Smaller DNA fragments travel further through agarose



4. DNA stained by DAPI

Analysis by CASP



$$\text{Olive Tail Moment} = (\text{Tail size} - \text{Head size}) \times \% \text{ of DNA in tail} / 100.$$

Figure 2.3 Measuring DNA damage through single cell gel electrophoresis The alkaline comet assay permits detection of DNA damage in single cells through encompassing single cell gel electrophoresis. Moreover, the assay detects alkali labile sites including DNA double-strand breaks, single-strand breaks, DNA-protein cross-linking, and incomplete excision repair sites. Illustration describes the steps involved in performing the assay plus the equation used by the CASP analysis to quantify the level of DNA damage in each cell.

2.13 Transcriptome analysis through RNA sequencing

2.13.1 RNA extraction and sequencing of the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs

RNA was extracted from the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs using Trizol extraction described in section 2.2.1. RNA clean-up was performed using the RNeasy micro+ kit (Qiagen) as follows. Firstly, RNA was resuspended in 300μl RLT buffer before transferring to a gDNA eliminator column and centrifuging at 8000g for 30 seconds. 350μl 70% ethanol was added to the flow through before transferring to an RNeasy minelute column and spinning at 8000g for 15 seconds. The flow-through was discarded before passing 500μl buffer RPE and 500μl 80% ethanol through the column. After these stages, the column was spun at full speed for 5 minutes inside a 1.5ml Eppendorf. The resulting material containing cleaned RNA was resuspending in RNase free H₂O before freezing at -80°C. RNA extraction from the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs was performed three times. The quality of the RNA was checked through the University of Birmingham genomics service using a Tapestation system to determine sample quality. Moreover, the RNA integrity number (RIN) in all samples of both the B-*myb*^{+/+} and the B-*myb*^{Δ/Δ} ESCs was high (approximately 10) suggesting the RNA extracted was of high quality (TABLE).

	RNA concentration (ng/μl)	RNA Integrity number (RIN)
B- <i>myb</i> ^{+/+} 1	82.7	10
B- <i>myb</i> ^{+/+} 2	156	10
B- <i>myb</i> ^{+/+} 3	163	10
B- <i>myb</i> ^{Δ/Δ} 1	280	10
B- <i>myb</i> ^{Δ/Δ} 2	139	9.7
B- <i>myb</i> ^{Δ/Δ} 3	191	9.9

Table 2.7 Quality control checking of RNA from the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESC samples

10µl of each RNA sample was supplemented with 1µl 3M Sodium acetate and 22 µl 100% ethanol; this stabilisation method ensured the RNA samples were suitable for transportation. The samples were then sent to the transcriptome analysis service provided by ‘Theragenetex’ (<http://bio.theragenetex.com/en/>). cDNA libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit (Illumina) and RNA sequencing was carried out using a HiSeq2500 system (Illumina).

2.13.2 Bioinformatics and RNA sequencing analysis

Bioinformatic analysis of the raw output data and generation of plots was performed by Dr Carl Ward. Firstly, the reads were trimmed of adapters and low-quality reads using Trim Galore v 0.5.0. The reads were then aligned to mm10 assembly indexed with version 18 gencode GTF. Next, gene counts were calculated using RSEM v1.3.1 using ‘rsem-calculate-expression with -star --estimate-rspd --star-output-genome-bam and -- paired-end’ options (STAR version 2.5.4b). Pairwise differential gene expression analysis was performed using the DESeq2 method, representing a highly robust method to accurately quantify bone fide alterations in gene expression (Love et al., 2014). Principal component analysis (PCA) was also performed through this method and a plot was generated using DESeq2 v1.24 in R v3.6.0. An MA plot with a significance threshold of p-value adjusted <0.1, plus a heatmap of the top 50 most altered genes was generated using DESeq2 v1.24 in R v3.6.0. UCSC bigwig tracks were created from RSEM outputted bam files with deeptools version 3.3.0 using bamCoverage with the ‘--normalizeUsing CPM’ option. Gene ontology analysis was performed upon the upregulated and downregulated gene sets using ClueGo, using the KEGG pathway ontology source. Only pathways with a p-value of less than 0.05 were selected. P-values corrected with Bonferroni step down were calculated in ClueGo for each term.

Bioinformatic analysis of the RNA sequencing data was also performed separately by Dr Boris Noyvert from the CRUK Birmingham Centre. The PCA analysis, differential gene expression

analysis, and the top 50 most significantly changed genes, generated through this analysis is presented in the appendix.

2.14 Methods of statistical analysis

For statistical analysis, GraphPad prism v8 was used. Gaussian distribution (normality) of populations was determined using the Shapiro-Wilk normality test with an alpha threshold of 0.05. Normally distributed data was analysed using two-tailed unpaired t-tests; for those data sets with considerable differences in variance, Welches correction was also applied. Non-normally distributed data sets were analysed using non-parametric analysis through the unpaired two-tailed Mann Whitney U test. Statistical analysis of drug response curves was performed using non-linear regression analysis.

Chapter 3 B-Myb is required for normal replication progression and genome stability maintenance in ESCs

3.1 Introduction

B-Myb has been recognised as a key player in regulation of the cell cycle in somatic cells for many years and its deregulation is associated with multiple types of cancer (Musa et al., 2017, Sala, 2005, Bar-Shira et al., 2002, Ren et al., 2015, Thorner et al., 2009). Recent work has revealed its vital roles in the maintenance of genome stability in multiple systems (Ahlbory et al., 2005, Fung et al., 2002, Garcia and Frampton, 2006, Lorvellec et al., 2010, Manak et al., 2007, Shepard et al., 2005), although, the molecular mechanisms behind its roles are not fully understood. *B-myb* is expressed in all cells; however, its expression is exceptionally high in pluripotent stem cells (Tarasov et al., 2008). Knockout of *B-myb* in mouse embryos results in collapse of the inner cell mass at E4.5-6, highlighting a vital role during this developmental period (Tanaka et al., 1999). Work carried out in the Garcia lab, demonstrated that loss of B-Myb in ESCs results in severe replication dysfunction accompanied by the accumulation of DNA damage through a measure of phosphorylated γ H2AX (Lorvellec et al., 2010), suggesting B-Myb plays a specific role during replication in ESCs. In this chapter, I describe the generation of stable *B-myb* mutant ESCs. Moreover, using various techniques, the dynamics of replication and the cell cycle were examined to validate and expand on previous findings regarding the influence of B-Myb loss on ESC replication and genome stability.

3.2 Results

3.2.1 Production of B-Myb mutant mouse ESC

To generate B-Myb mutant ESCs (*B-myb*^{Δ/Δ}), I made use of existing ESCs containing one deleted *B-myb* allele and one *B-myb* floxed allele (*B-myb*^{F/Δ}) (Garcia et al., 2005). This model allows for the conditional deletion of the *B-myb* DNA binding domain *in-vitro* through the expression of cre recombinase protein. *B-myb*^{F/Δ} were transfected with a plasmid that contained the cre recombinase gene followed by an internal ribosome entry sequence (IRES) linked *eGFP* reporter gene (Lenti-CreEGFP plasmid) (Ahmed et al., 2004). After transfection, analysis of the expression of GFP in *B-myb*^{F/Δ} transfected with Lenti-CreEGFP plasmid compared to untransfected cells revealed a transfection efficiency of 26% (Figure 3.1B). GFP positive ESCs were isolated by cell sorting, grown in culture and single colonies were isolated as separate clones (Figure 3.1A).

To determine the successful ablation of *B-myb* in individual clones, the expression of *B-myb* was measured by quantitative PCR (Q-PCR). This analysis revealed complete absence of *B-myb* expression in the majority of clones when compared to wildtype ESC, at exception of clone 6 (Figure 3.2A). Further confirmation was carried out through protein extraction and analysis of B-Myb protein abundance by western blotting. Levels of B-Myb protein were reduced in the floxed heterozygous (*B-myb*^{F/Δ}) clones compared to wildtype ESC (*B-myb*^{+/+}) and completely absent in *B-myb*^{Δ/Δ} ESCs (Figure 3.2B). In culture, the *B-myb*^{Δ/Δ} ESC colonies displayed subtle morphological differences compared to wildtype ESC including increased disorganisation and an increase in cells detaching from colonies. Despite this, the *B-myb*^{Δ/Δ} ESC displayed no defect in propagation suggesting they retain stem cell self-renewal properties similar to normal ESC behaviour (Figure 3.2C).

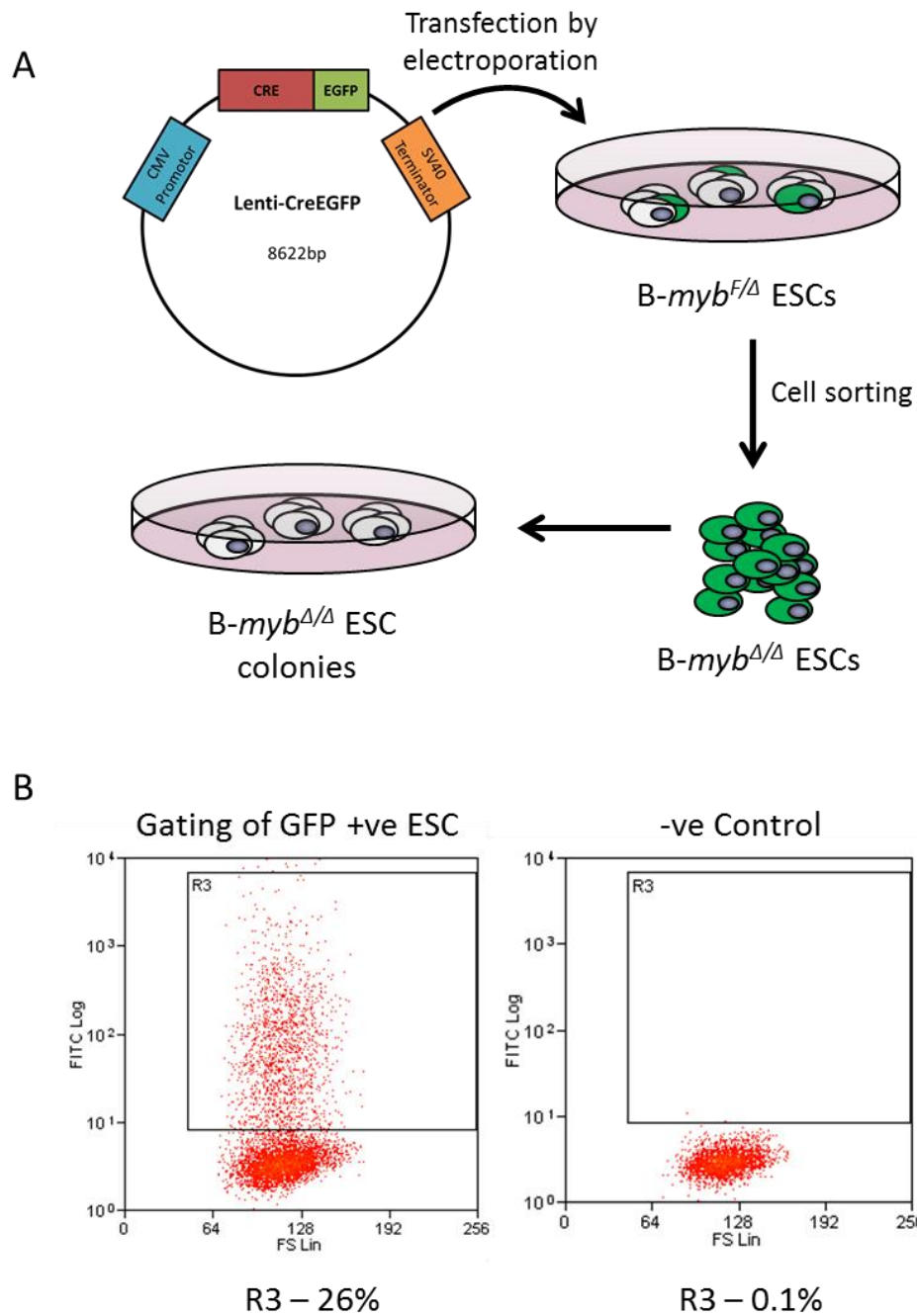


Figure 3.1 Generation of B-*myb*^{Δ/Δ} ESCs A) Schematic representing the experimental strategy. Briefly, ESCs containing modified B-*myb* alleles (B-*myb*^{F/Δ}) were transfected with a plasmid that encoded *Cre-IRES-EGFP*. GFP positive ESC were sorted, re-plated and isolated as single clones. B) Dot plot generated using flow cytometry showing expression of GFP in transfected (left panel) or un-transfected (right panel) B-*myb*^{F/Δ} ESCs.

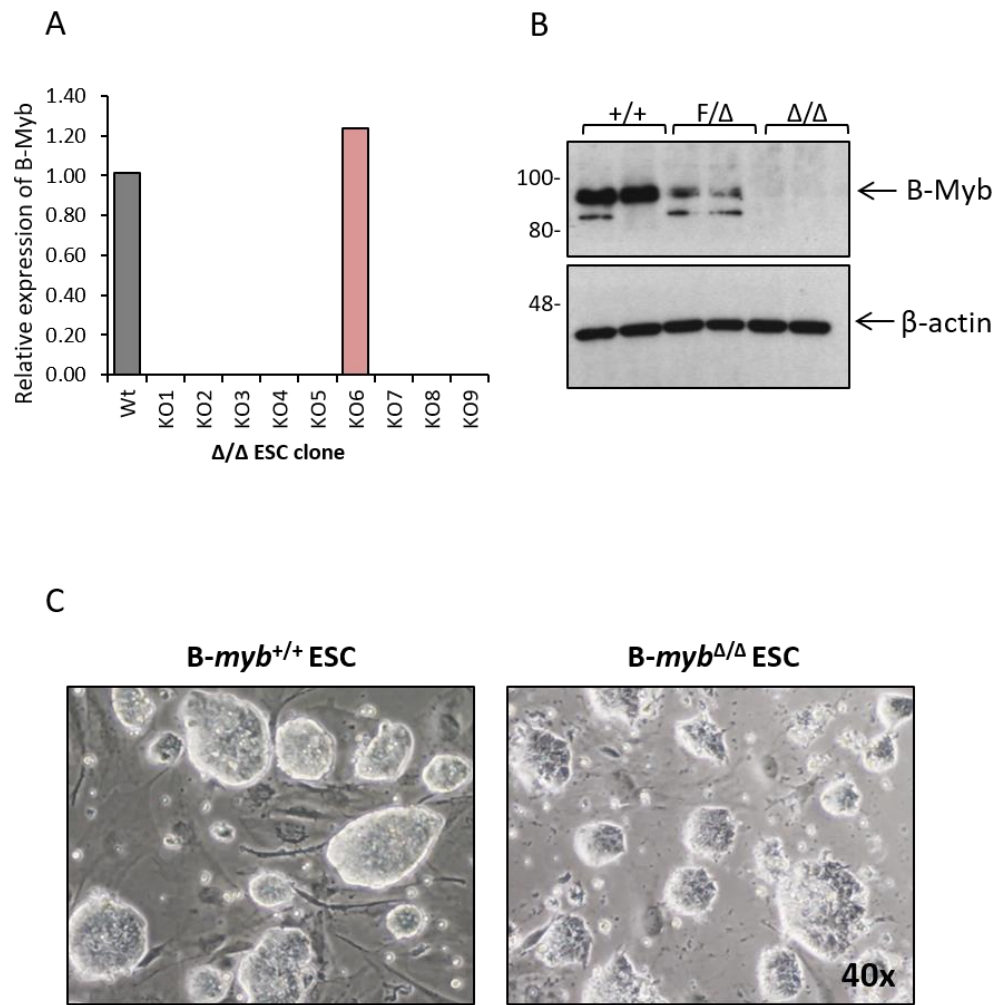


Figure 3.2 Validation of B-myb ablation in B-myb^{Δ/Δ} ESCs A) RNA was extracted from wildtype ESCs (B-myb^{+/+}) and the B-myb^{Δ/Δ} individual ESC clones. cDNA was reverse transcribed from total RNA and B-myb expression was measured by qPCR. Data was normalized against the level of β2-microglobulin housekeeping gene and presented relative to B-myb^{+/+}. Data from two technical replicates. B) Total protein was extracted from B-myb^{Δ/Δ} clone 3 and B-myb^{+/+} ESCs and 50ug was subjected to SDS-PAGE. Protein abundance was detected by western blotting with antibodies against B-Myb or Actin. The position of relevant molecular weight (kDa) standards is shown on the left side of the blot, and arrow on the right indicate the specific bands of interest. C) Representative phase contrast images from 40x magnification of B-myb^{+/+} and B-myb^{Δ/Δ} ESCs after two days in culture.

3.2.2 Analysing the effect of B-Myb loss upon replication progression in ESCs

Previous work from the Garcia lab demonstrates that ablation of *B-myb* in ESCs results in genome instability due to a replication stress phenotype (Lorvellec et al., 2010). To verify these findings, dual label DNA fibre spreading analysis was performed in the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs. This technique allows detailed qualitative and quantitative examination of replication dynamics in whole cell populations. Cells were treated sequentially with two deoxyribonucleoside analogues 5-Iodo-2'-deoxyuridine (IdU) and 5-Chloro-2'-deoxyuridine (CldU) for 20 minutes each before cell lysis and spreading of the labelled DNA onto microscope slides (Figure 3.3A). The DNA was denatured and immunofluorescence staining for the incorporated labels performed (Figure 3.3B).

Through measuring the length of replication tracks (1μm = 2.59 kilobases of DNA (Jackson and Pombo, 1998)), the replication rate in kilobases per minute was calculated. The rate of replication was considerably attenuated in the *B-myb*^{Δ/Δ} ESCs compared to *B-myb*^{+/+} ESCs (Figure 3.3C), demonstrated by a global shift to decreased rates in a speed distribution curve. In addition, the median replication rate was reduced from 1.23 (±0.05) to 0.81 (±0.08) kb per minute. This global distribution change was determined to be highly significant using the Mann Whitney U test producing a p-value less than 0.0001.

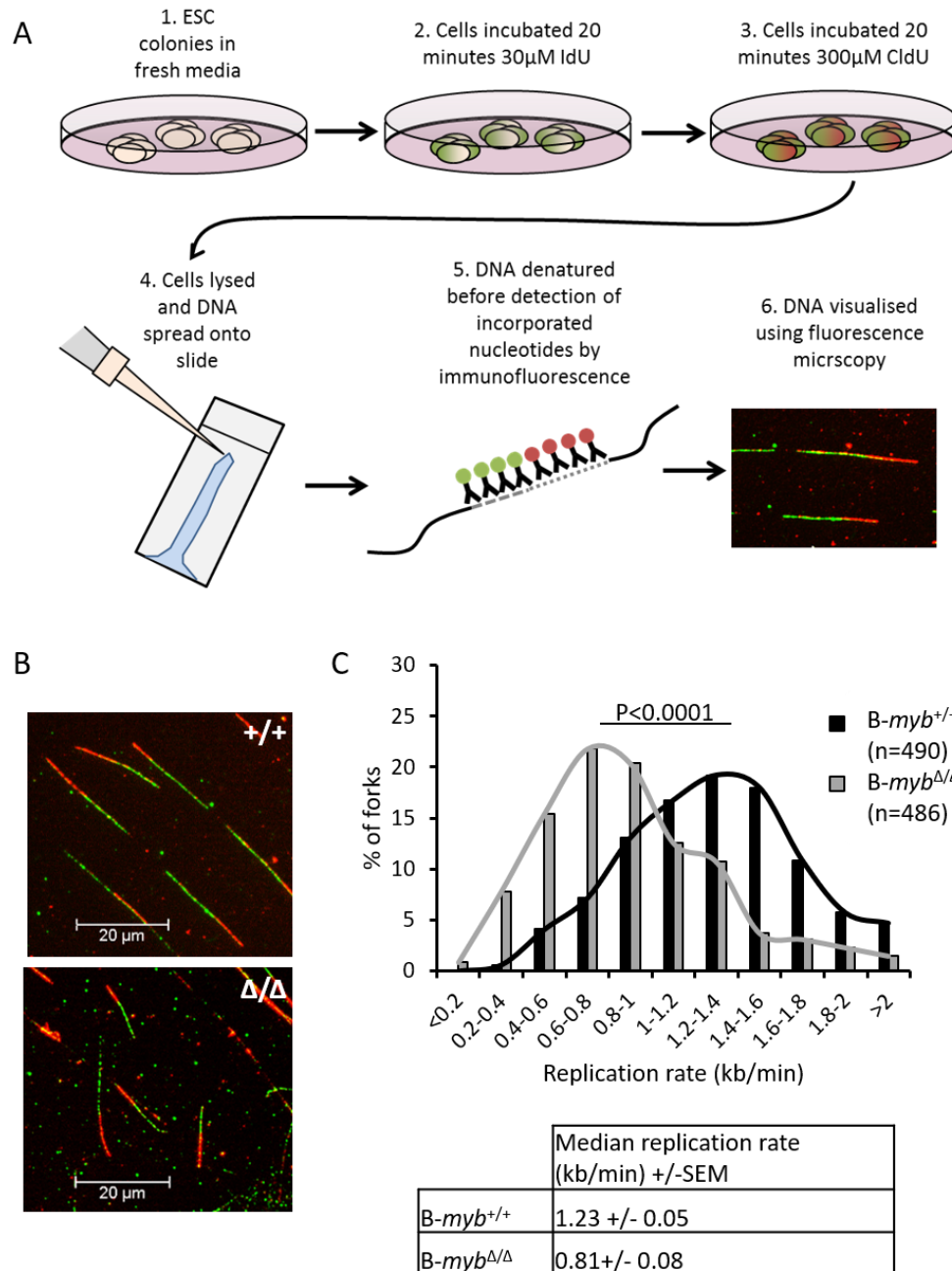


Figure 3.3 Decreased replication progression rate in B-*myb* ^{Δ/Δ} ESCs A) Schematic workflow of DNA fibre experiment. ESCs were sequentially incubated for 20 mins with IdU, then a further 20 mins with CldU, before cell lysis and DNA spreading. DNA was denatured before performing immunofluorescence staining against incorporated labels for visualisation by fluorescence microscopy. B) Images of DNA fibres were captured at 60x magnification and analysed using LasX. The length of the IdU label of elongating forks was measured in μ m before conversion to replication rate using the equation; $\text{Replication rate (kb/min)} = (\text{tract length } (\mu\text{m}) * 2.59 \text{ (kb)}) / 20 \text{ (min)}$. C) Graph represents replication rate as a distribution curve and table shows the median replication rate of B-*myb*^{+/+} versus B-*myb* ^{Δ/Δ} ESCs $\pm$ SEM from 6 independent experiments. Statistical analysis was carried out using a two-tailed Mann Whitney U test. The ‘n’ number presented represents the total number of fibres quantified per group.

To analyse the dynamics of the replication programme further, the relative percentage of various replication fork structures was calculated (Figure 3.4A). Compared to the B-*myb*^{+/+}, a significant decrease in the percentage of elongating forks in the B-*myb*^{Δ/Δ} ESCs was observed (76% vs 66%; t-test, p-value = 0.0004) (Figure 3.4B). This decrease in elongating structures was associated with an increase in newly fired origins (26% in B-*myb*^{Δ/Δ} vs 18% in B-*myb*^{+/+}) and first label only tracks representing an increase in fork slowing/stalling (8% in B-*myb*^{Δ/Δ} vs 5% in B-*myb*^{+/+}) (Figure 3.4C and D). Both increases in the B-*myb*^{Δ/Δ} were found to be statistically significant using two-tailed unpaired t-tests producing p-values of 0.0014 and 0.0122 respectively.

To further investigate potential changes in the kinetics of replication fork progression, the positive ratio between the lengths of each incorporated label of elongating forks was calculated. The B-*myb*^{Δ/Δ} ESCs exhibited significantly increased asymmetry with an average fork ratio of 1.88 versus only 1.50 in the B-*myb*^{+/+}. (Mann Whitney U test, p < 0.0001). In addition, the B-*myb*^{Δ/Δ} presented 29% of tracts with a ratio greater than two, compared to just 12% in the B-*myb*^{+/+} (Figure 3.5A). The fork length ratio around new origins was also measured. Similar to the elongating fork ratio, the B-*myb*^{Δ/Δ} displayed a strong asymmetry phenotype with an average ratio of 2.02 compared to a ratio of 1.22 in the B-*myb*^{+/+} (Mann Whitney U test, p<0.0001). In the B-*myb*^{Δ/Δ}, 24% of origins presented a ratio of above two, compared to only 2% in B-*myb*^{+/+} ESC (Figure 3.5B). Overall, this data suggests that the B-*myb*^{Δ/Δ} present increased fork instability.

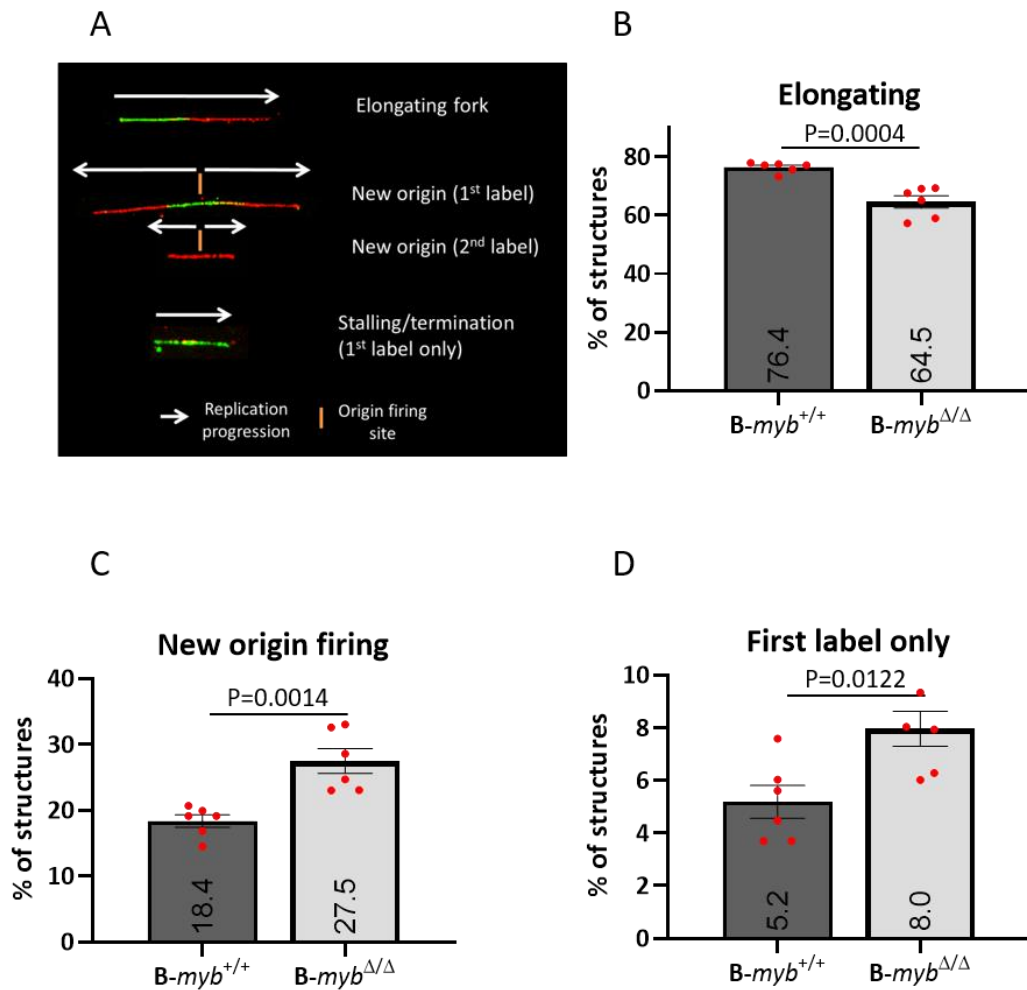


Figure 3.4 B-*myb*^{Δ/Δ} ESCs display an increase in replication stress associated fork structures A) Representative images of the different fork structures measured. B) Bar graphs representing the percentage of elongating forks, C) new origin firing and D) first label only slowing/stalling events in B-*myb*^{+/+} versus B-*myb*^{Δ/Δ} ESCs, calculated as a percent of all structures. Error bars represent SEM. Statistical analysis was carried out using two-tailed unpaired t-tests. At least 1000 total structures were counted for each condition, from 6 separate repeats.

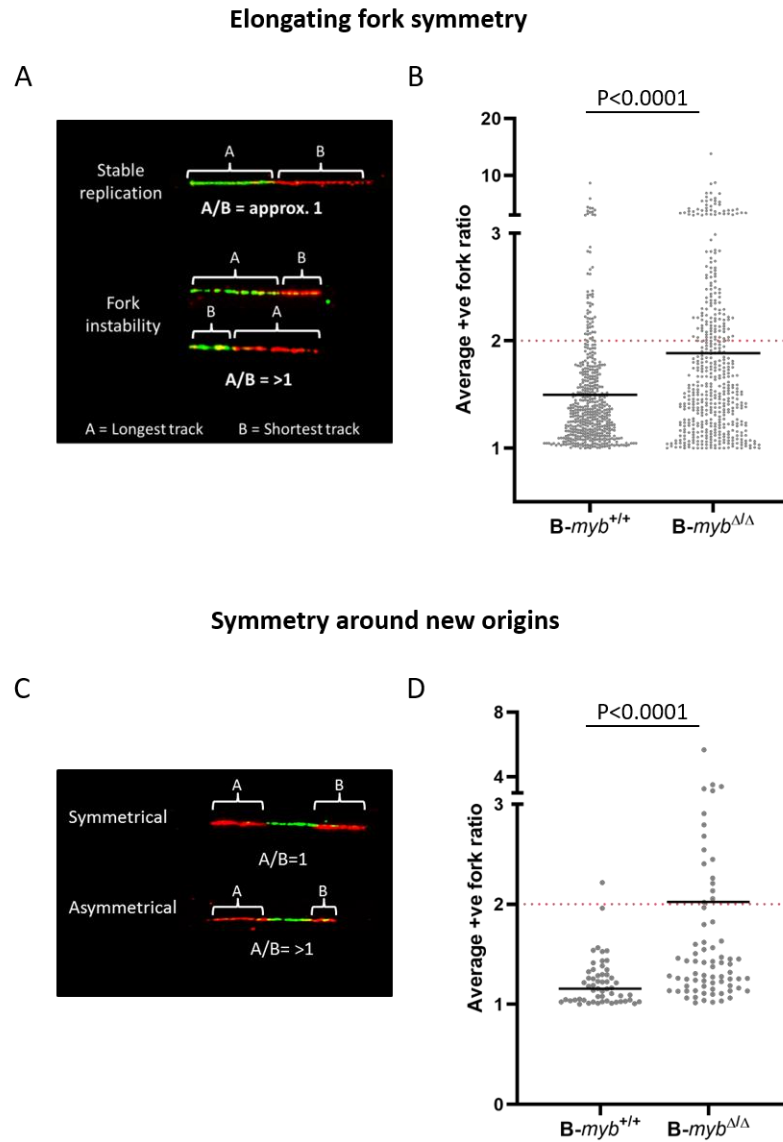


Figure 3.5 Increased replication disorganisation in B-*myb*^{Δ/Δ} ESCs A) The length of both the IdU and CldU labels of elongating forks was measured in micrometres before calculating the positive ratio between the two measurements. Symmetric forks represent stable replication progression. Whereas, those with a ratio significantly greater than 1 represent a disruption of normal fork progression, also known as fork instability. B) Dot plot presenting the average fork ratio in the B-*myb*^{+/+} (1.50) versus B-*myb*^{Δ/Δ} (1.88) ESCs. Statistical analysis was performed using the Mann Whitney U test. At least 450 ratios were recorded from 6 experimental replicates. C) The length of both CldU labels surrounding IdU tracks (newly fired origins) was measured before calculating a ratio representing symmetry around new origins D) Dot plot presenting the average label ratio in the B-*myb*^{+/+} (1.22) versus B-*myb*^{Δ/Δ} (2.02) ESCs. Statistical analysis was performed using the Mann Whitney U test. 55 and 75 ratios were recorded in the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} respectively from 3 replicates.

3.2.3 Investigating the global effect of B-Myb loss on S phase and the cell cycle in ESCs

Next, I sought to determine whether the replication defect observed at the level of single DNA fibre could have an impact on progression through the cell cycle. To investigate this possibility, the percentage of cells in each phase of the cell cycle in the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs was determined through incorporation of 50μM 5-Bromo-2'-deoxyuridine (BrdU) for one hour followed by its detection through flow cytometry. Cells were also incubated with 10μg/ml 7-Aminoactinomycin D (7-AAD) to stain DNA content (Figure 3.6A). Histograms were generated using 'FlowJo' before gating cells in each of the specific cell cycle phases (Figure 3.6B).

From the flow cytometry analysis, it was evident that ESCs spend the majority of their time replicating DNA with 68% of the *B-myb*^{+/+} positive for BrdU. Interestingly, the *B-myb*^{Δ/Δ} presented a slight increase (76%) of cells positive for BrdU compared to *B-myb*^{+/+} ESCs (Figure 3.6C). Further analysis into the percentage of cells in specific stages of S phase in the *B-myb*^{Δ/Δ} revealed that the increase in S phase cells was fairly evenly distributed with a potential increase in late S phase. This data suggests that there may be subtle changes in the cell cycle distribution in *B-myb*^{Δ/Δ}; however, they retain the capacity for unhindered passage through the cell cycle.

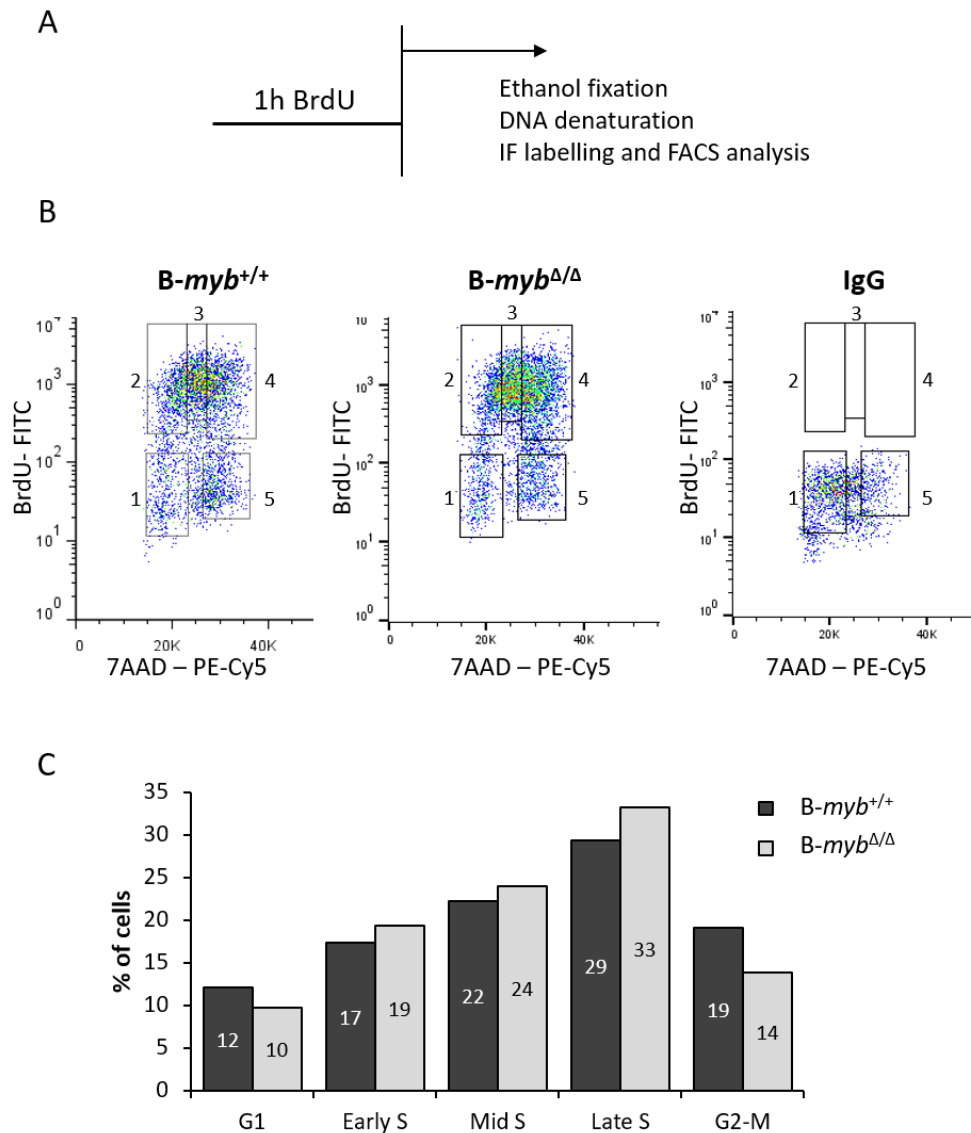


Figure 3.6 Changes in cell cycle dynamics in the B-*myb*^{Δ/Δ} A) Schematic of the labelling procedure. Briefly, cells were culture in the presence of 50μM BrdU for one hours before fixation, labelling and detection by flow cytometry. B) Histograms showing flow cytometry analysis of BrdU incorporation (vertical axis, logarithmic scale) and total DNA content by 7AAD (horizontal axis, linear scale) in B-*myb*^{+/+} (left panel), B-*myb*^{Δ/Δ} (middle) and Isotype control (right panel). Gate numbers represent cell cycle phases: 1) G1, 2) Early S, 3) Mid S, 4) Late S and 5) G2/M. C) Bar graph showing the percentage of cells gated in specific stages of the cell cycle in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs., n=2.

Replication occurs in discrete replication factories within the nucleus. It was previously demonstrated that loss of B-Myb results in an increase in the number of replication factories in ESCs (Lorvellec et al., 2010). To replicate these findings, the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated with the modified thymidine analogue, IdU, before its detection for visualisation of replication factories. During the early stages of S phase, the nucleus presents numerous small foci throughout the nucleoplasm, whereas, towards mid/late S phase, replication factories aggregate at the nuclear periphery before adopting a more clumped appearance (Dimitrova and Berezney, 2002, Ferreira and Carmo-Fonseca, 1997). For this experiment, only nuclei in early replication, determined by their staining pattern, were imaged for analysis (Figure 3.7A). Three-dimensional image stacks (z-stacks) were acquired through confocal microscopy before conversion to rendered images for automatic foci counting using 'Imaris' (Figure 3.7B). In the B-*myb*^{+/+} ESCs, the average number of replication factories per nucleus was 399 with not much variation producing a standard deviation of 70. In contrast, the B-*myb*^{Δ/Δ} ESCs presented a substantial increase in the number of replication factories producing an average of 699 factories. In addition, there was increased variation producing a standard deviation of 206 with a selection of cells having greater than 1000 factories (Figure 3.7C). The difference between the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs was determined to be highly statistically significant using a two-tailed unequal variance t-test producing a p-value less than 0.0001. Overall, this finding confirms that there is abnormal activation of replication factories during early S phase.

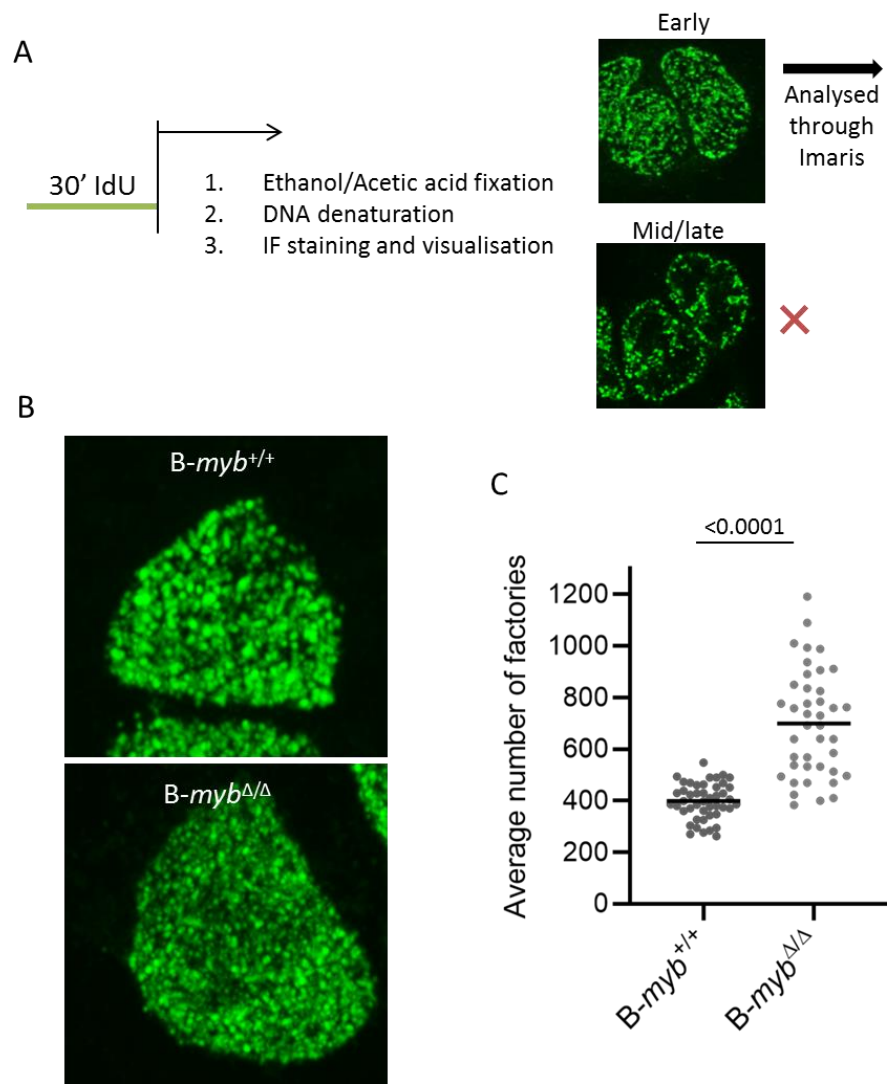


Figure 3.7 Abnormal activation of replication factories in *B-myb*^{Δ/Δ} ESCs A) A schematic workflow of the experimental procedure. Cells were treated 30 minutes with 20μM IdU before fixation, DNA denaturation and immunofluorescent antibody labelling. Cells were visualised through confocal microscopy and z-stack images of early replicating nuclei were acquired at 100x magnification. B) Example images of maximum intensity projections acquired from collating the z-stack images together C) Using ‘Imaris’ the z-stacks were converted to 3D rendered images before adopting an automatic foci counting tool. Dot plot demonstrating the average number of replication factories, with each dot representing one nucleus. The average number in the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs was 399 and 699 respectively. At least 40 nuclei were analysed per group from two experimental repeats. Statistical analysis was performed using a two-tailed unpaired unequal variances t-test.

The B-*myb*^{Δ/Δ} ESCs display chronic replication problems which would potentially be expected to induce a G2-M checkpoint response; despite this, they retain the capacity to proliferate limitlessly. B-Myb has been increasingly associated with entry into mitosis with a role in G2-M checkpoint control (Henrich et al., 2017, Katzen et al., 1998, Sadasivam et al., 2012, Saldivar et al., 2018, Tarasov et al., 2008). Therefore, I questioned whether B-Myb was required for G2/M checkpoint control in ESCs. To investigate this, the percentage of cells passing into mitosis in untreated, and CPT treated conditions was measured. Colcemid, a microtubule depolymerising agent, was also used to arrest cells which pass into mitosis (Rieder and Palazzo, 1992). Serine 10 phosphorylation of histone H3 (H3-pS10) is associated with chromatin condensation, thus can be used as a surrogate marker for cells in mitosis (Li et al., 2005). Therefore, mitotic cells were detected through immunostaining for H3-pS10 (Figure 3.8A).

In the absence of treatment, the percentage of cells positive for H3-pS10 was minimal in both the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs. However, after colcemid treatment, the percentage of cells positive for H3-pS10 was increased to 23.3% in the B-*myb*^{+/+} ESCs and 17.6% in the B-*myb*^{Δ/Δ} ESCs (Figure 3.8B+C). As expected, activation of G2/M checkpoint was achieved by addition of CPT in the B-*myb*^{+/+} ESCs resulting in a significant reduction in H3-pS10 positive cells from 23.3% to 4.5%. Interestingly, CPT treatment in the B-*myb*^{Δ/Δ} ESCs resulted in a much more modest reduction in the percentage of positive cells from 17.6 to 12.6% (Figure 3.8B+C). Therefore, this data presents evidence for a weaker G2-M checkpoint response to CPT in the B-*myb*^{Δ/Δ} ESCs.

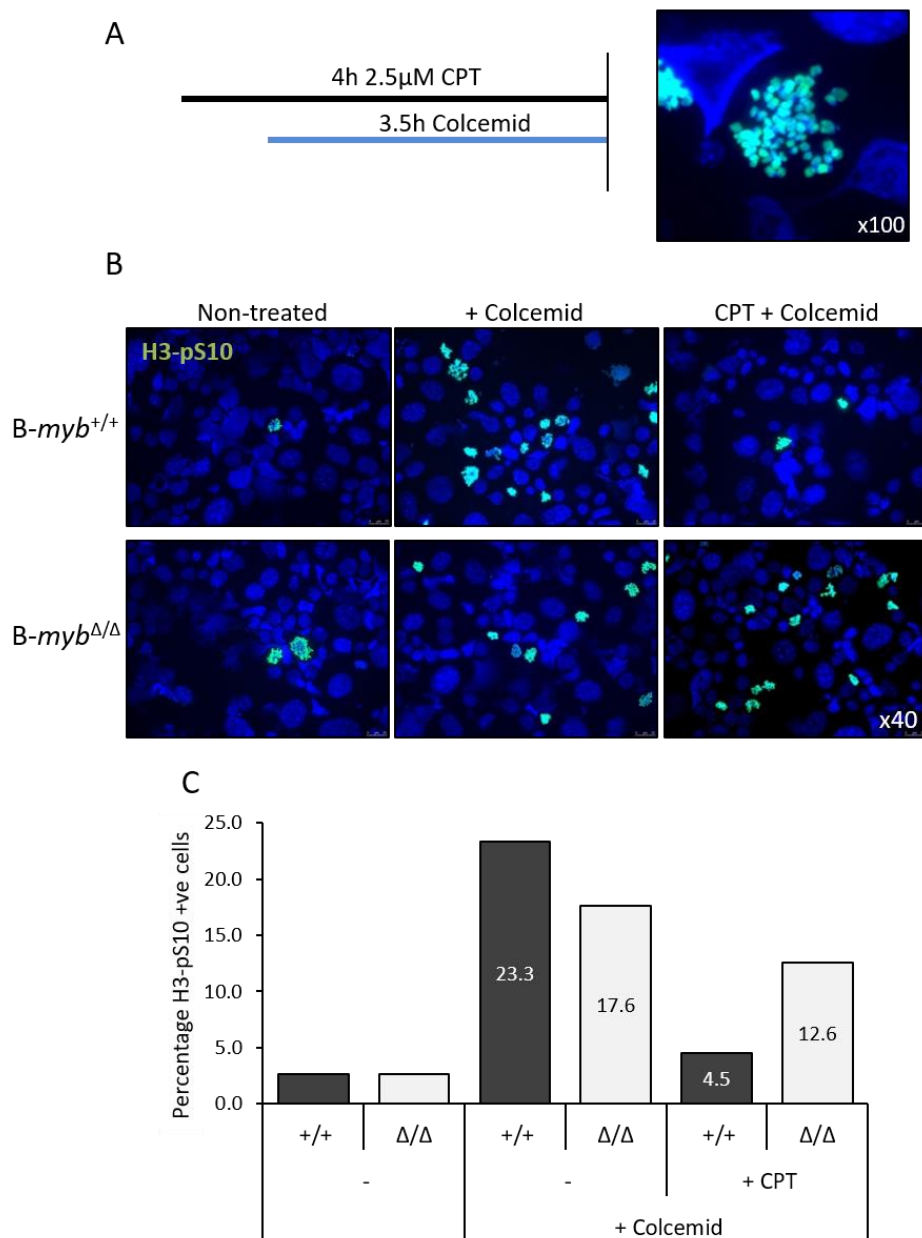


Figure 3.8 Weak G2-M checkpoint control in the B-*myb*^{Δ/Δ} ESCs after CPT treatment A) Schematic of experiment workflow. Briefly, ESCs were treated with or without CPT for 4 hours before 0.1μg/ml (0.27μM) colcemid was added during the final 3.5 hours of treatment. Cells were fixed before immunostaining for H3-pS10, a marker of mitotic cells. Images were taken at x40 magnification B) Shown are representative images of H3-pS10 positive cells (green) in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs; presented, without treatment (left panels), plus colcemid (centre panels) and plus colcemid and CPT treatment (right panels). C) The number of H3-pS10 positive cells were counted and calculated as a percentage of total cells per image in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs. Bar graph shows the average percentage of H3-pS10 positive cells from two experimental repeats.

Another indication of prolonged replication stress is the formation of topological DNA entanglements during anaphase due to presence of under-replicated DNA structures known as ultra-fine bridges (UFBs) (Chan et al., 2018). Although UFBs consist of DNA, they are not detectable using conventional DNA staining methods, therefore, detection of PICH, a DNA translocase known to coat ultra-fine bridges during anaphase, was utilised (Baumann et al., 2007, Chan and Hickson, 2011). To determine the presence of these structures in the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs, immunofluorescence staining designed to detect PICH was carried out on synchronous cell populations. Then, using fluorescence microscopy, anaphase cells were located before scoring them for UFB positivity (Figure 3.9A). In the *B-myb*^{+/+}, only 20% of anaphases presented UFBs. Strikingly, there was a highly significant increase (60%) in the percentage of UFB positive anaphases in the *B-myb*^{Δ/Δ} compared to *B-myb*^{+/+} (two-tailed unpaired t-test, $p < 0.0001$) (Figure 3.9B). This data reveals the presence of under-replicated DNA in mitosis upon the loss of B-Myb, which may reflect their inability to efficiently replicate DNA.

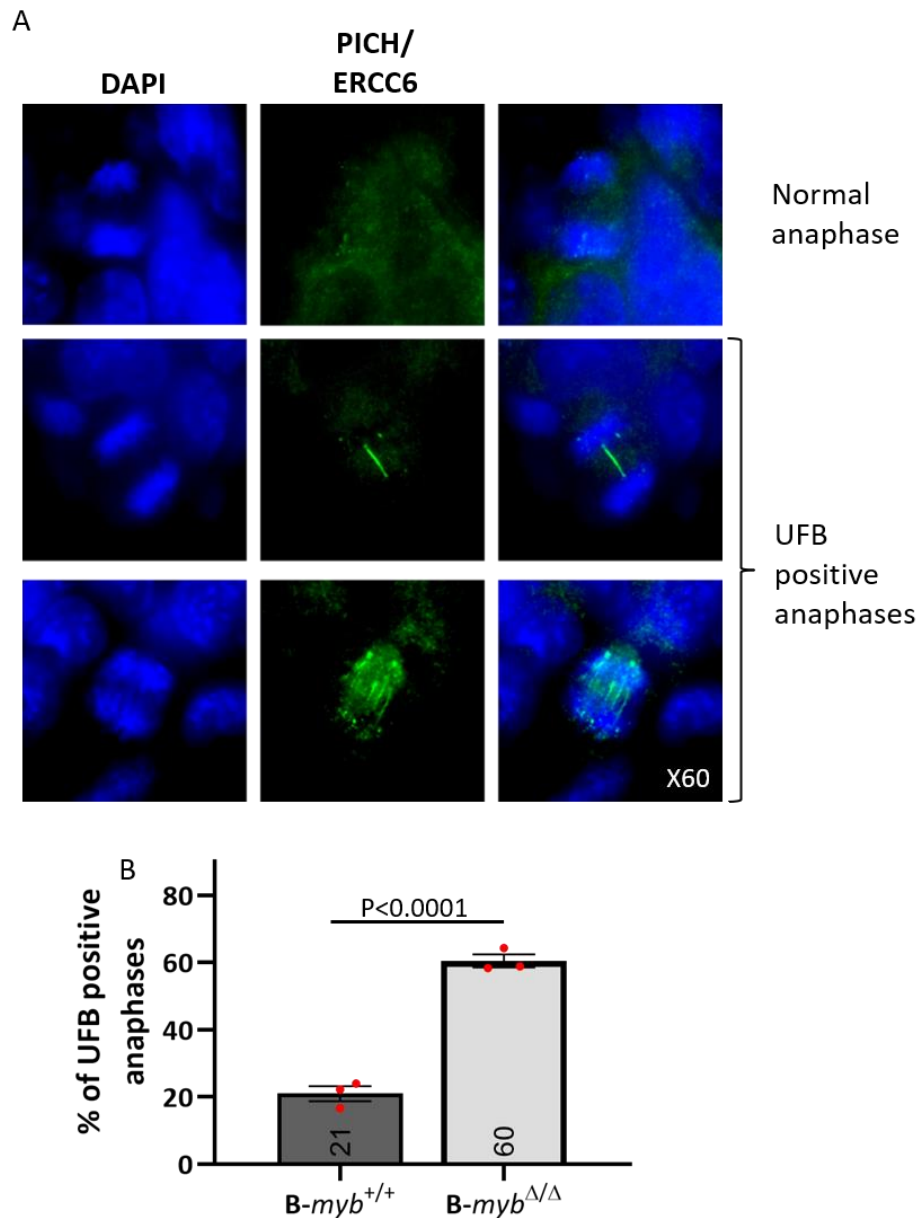


Figure 3.9 Formation of ultra-fine bridges in the *B-myb*^{Δ/Δ} ESCs during mitosis A) Whole ESC colonies were fixed before performing immunostaining for PICH (UFBs) as well as DAPI (DNA content). Using fluorescence microscopy, the anaphases were located before scoring for UFB positivity. Images show examples of anaphases, negative for UFBs (top panel), and positive for UFBs (middle and bottom panel). B) Bar graph shows the average percentage of anaphases positive for UFBs, quantified relative to all anaphases in the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs. At least 75 anaphases were counted per experimental group from 3 independent experiments. Statistical analysis was performed using an unpaired two-tailed t-test.

3.2.4 Investigating the influence of B-Myb loss upon genome stability

Cells under chronic replication stress display a susceptibility to DNA damage (Zeman and Cimprich, 2014), therefore, I questioned whether the replication stress observed in the B-*myb*^{Δ/Δ} ESCs is accompanied by DNA damage accumulation. In response to the formation of DSBs, 53BP1 is recruited and plays a vital role in regulating DNA damage repair pathway choice (Stewart, 2009, Daley and Sung, 2014). It is possible to measure the recruitment of 53BP1, through its formation of discrete nuclear foci at the site of damage in S phase cells which are visible by microscopy (Schultz et al., 2000). In addition, large nuclear bodies containing 53BP1 (53BP1 bodies) have been shown to arise during G1. However, these structures are attributed to unrepaired damage acquired during the previous S phase (Harrigan et al., 2011).

Cells were first treated with 5-Ethynyl-2'-deoxyuridine (EdU), a thymidine analogue which incorporates into nascent DNA, moreover, labelling of EdU can be used to identify S phase/replicating cells. Cells were also treated with CPT as a positive control for damage. After treatment, cells were fixed and permeabilised before immunofluorescence staining for 53BP1. For the quantification of 53BP1, nuclei were differentiated into EdU positive (S phase) and EdU negative (G1 and G2/M) (Figure 3.10A). In the EdU positive B-*myb*^{Δ/Δ} ESCs, increased recruitment of 53BP1 was apparent with 28% of nuclei presenting more than 6 53BP1 foci, a two-fold increase compared to only 11% in the B-*myb*^{+/+} ESCs. This increase was statistically significant using an unpaired t-test producing a p-value of 0.0010 (Figure 3.10B). In response to CPT treatment, the B-*myb*^{+/+} ESCs presented a strong increase from 11% to 49% in the percentage of cells with over 6 foci (unpaired t-test, p=0.0024). 53BP1 was increased even further after CPT treatment in the B-*myb*^{Δ/Δ} ESCs from 28% to 62% (p=0.0006). There was a 13% difference in the percentage of positive cells between the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs after CPT suggesting potentially increased sensitivity to CPT treatment, although, this difference was determined to be non-statistically significant (p= 0.1439) (Figure 3.10B).

53BP1 recruitment was also quantified in the EdU negative nuclei despite being a more infrequent occurrence in ESC populations. For analysis, nuclei with more than 3 53BP1 bodies were considered positive. There was a mild increase in the percentage of cells with over 3 53BP1 bodies in the *B-myb*^{Δ/Δ} ESCs (23%) compared to the *B-myb*^{+/+} (17%), although, this difference was not statistically significant (two-tailed unpaired t-test, (p=0.2480) (Figure 3.10C). Treatment with CPT resulted in a strong increase in nuclei positive for 53BP1 bodies, increasing from 17% to 47% in the *B-myb*^{+/+} ESCs and from 23% to 51% in the *B-myb*^{Δ/Δ} ESCs. Both increases were statistically significant using two-tailed unpaired t-tests producing p-values of 0.0055 and 0.0021 respectively. There was little to no difference in the percentage of 53BP1 body positive cells between the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs after CPT treatment (p=0.5215) (Figure 3.10C). These findings suggest that the *B-myb*^{Δ/Δ} ESCs have an increased susceptibility to DSB formation which is mostly incurred in replicating cells.

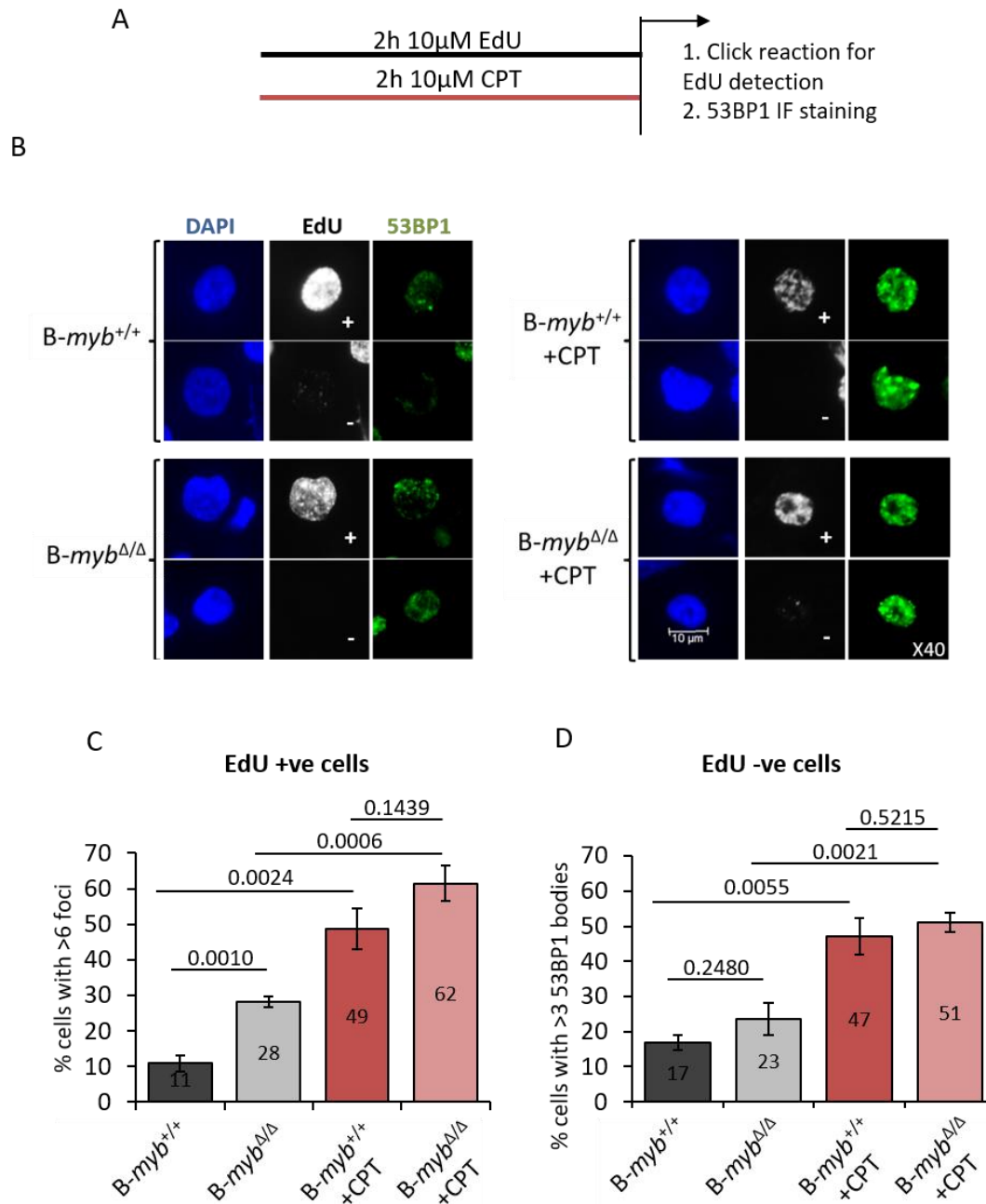


Figure 3.10 Increased recruitment of 53BP1 in B-*myb*^{Δ/Δ} ESCs A) To label replicating cells, 10µM EdU was incorporated for 2 hours in culture. After cell collection and fixation, incorporated EdU was detected using the ‘click’ reaction. For a measure of double strand breaks, immunofluorescence staining for 53BP1 was performed. Cells were also treated with 10µM CPT. B) Representative images for B-*myb*^{+/+}, B-*myb*^{Δ/Δ} untreated and B-*myb*^{+/+} and B-*myb*^{Δ/Δ} plus CPT are shown. Staining for DAPI (blue), EdU (white) and 53BP1 (green). C) Bar graph of the percentage of EdU positive cells with over 6 53BP1 foci in B-*myb*^{+/+}, B-*myb*^{Δ/Δ} untreated and B-*myb*^{+/+} and B-*myb*^{Δ/Δ} plus CPT. D) Bar graph of the percentage of EdU negative cells with over 3 53BP1 bodies in all four groups. For bar graphs, error bars represent SEM. Statistical analysis using unpaired two-tailed t-test. At least 200 EdU positive and 100 EdU negative nuclei were analysed from 4 experimental repeats.

To strengthen these findings, an alkali comet assay or single cell gel electrophoresis was performed. This technique relies on the electrophoretic migration of genomic material through agarose, moreover, cells with increased DNA breakage/smaller DNA fragments travel further producing a 'comet' like structure (Ostling and Johanson, 1984, Singh et al., 1988). Comets were stained by DAPI and visualised using immunofluorescence microscopy. Resulting images were analysed using 'Casplab' producing a value for olive tail moment (OTM) (see materials and methods, section 2.12).

The B-*myb*^{Δ/Δ} ESCs presented an increase in DNA breakage with an average OTM of 3.6 compared to 2.6 in the B-*myb*^{+/+}. This was also clear when analysing the percentage of cells with an OTM greater than 6; with 20% in the B-*myb*^{Δ/Δ} compared to only 8.7% in the B-*myb*^{+/+}. Statistical analysis using a Mann Whitney U test revealed that this increase was statically significant (p<0.0001) (Figure 3.11). Treatment with 2 hours 10μM CPT in the B-*myb*^{+/+} ESCs resulted in a statistically significant increase in average OTM up to 5.2 (p<0.0001), whilst, the B-*myb*^{Δ/Δ} ESCs presented an even stronger increase in average OTM up to 7.8 (p<0.0001) (Figure 3.11). Comparison of the average OTMs in the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs after CPT treatment suggested that the B-*myb*^{Δ/Δ} ESCs display an increased sensitivity to DNA breakage (P<0.0001). To support this theory, the B-*myb*^{Δ/Δ} present a massive 41% of comets with an OTM greater than 6 after CPT treatment compared to only 25% in the B-*myb*^{+/+} ESCs (Figure 3.11). These findings not only support the hypothesis of increased DNA damage susceptibility in the B-*myb*^{Δ/Δ} ESCs, but it also suggests that they display an increased sensitivity to CPT treatment.

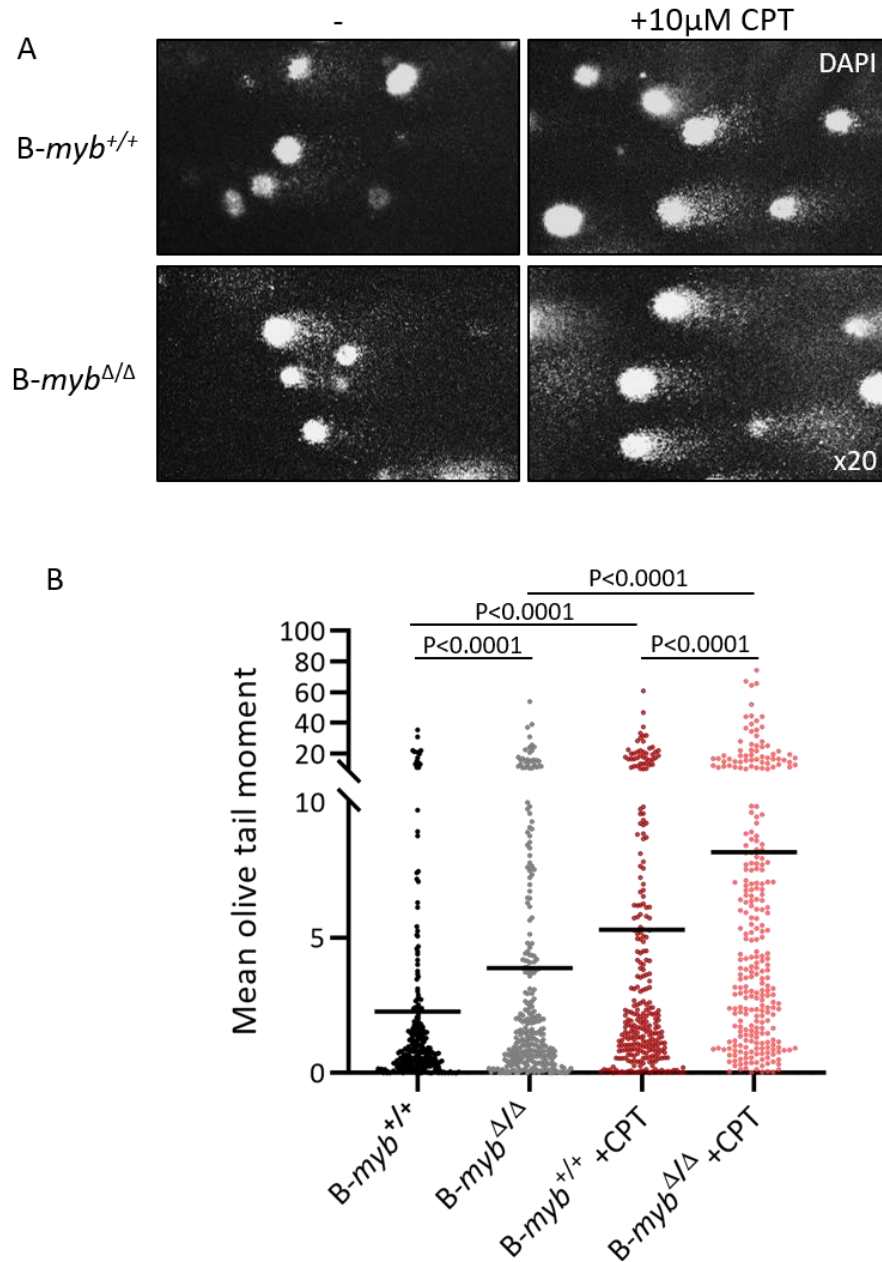


Figure 3.11 Increased DNA breakage in the *B-myb*^{Δ/Δ} by alkaline comet assay A) The cells were either untreated or treated with 10μM CPT for 2 hours before harvesting. Cells were embedded in agarose on microscope slides before performing cell lysis under alkaline conditions. Slides were subjected to electrophoresis to promote comet formation before staining with DAPI and visualisation using a fluorescence microscope at x20 magnification. Images are representative of resulting comets in *B-myb*^{+/+} and *B-myb*^{Δ/Δ} untreated and *B-myb*^{+/+} and *B-myb*^{Δ/Δ} plus CPT. B) Dot plot showing the mean and distributions of olive tail moments in *B-myb*^{+/+} and *B-myb*^{Δ/Δ} untreated and *B-myb*^{+/+} and *B-myb*^{Δ/Δ} plus CPT. Mean OTMs were 2.6, 3.6, 5.2 and 7.8 in the order presented. Statistical analysis using Mann Whitney U tests. At least 300 comets were measured for each group from 5 experimental replicates.

3.2.5 Examining the sensitivity of B-*myb*^{Δ/Δ} ESCs to chemical inhibitors

It is clear that loss of B-Myb results in formation of DNA damage, however, it is not clear whether the damage is due to a replication associated mechanism, or whether there is a direct effect upon DNA repair. Testing the sensitivity to certain chemical inhibitors is a useful way to reveal defects in certain cellular pathways. Therefore, to reveal more information concerning the DNA damage defect observed in the B-*myb*^{Δ/Δ} ESCs, cell viability after 24 hours treatment with a range of inhibitors was measured. 0.1% saponin was added to the cells as a positive control for cell death. After 24 hours of treatment, cell viability was measured in triplicate using resazurin, which undergoes a detectable change in emission after being converted to resorufin in the presence of live cells (Czekanska, 2011).

Cells were treated with caffeine, a non-specific inhibitor of the kinases instrumental for activation of the DNA damage response, including ATM and ATR kinases (Blasina et al., 1999, Wang et al., 2004, Zelensky et al., 2013). Cells were treated with a range of concentrations and a dose-response curve was constructed. The IC₅₀ was similar in both the B-*myb*^{+/+} at 0.76mM and the B-*myb*^{Δ/Δ} ESCs at 0.70mM, moreover, this difference was determined non-statistically significant using non-linear regression analysis producing a p-value of 0.7268 suggesting there is no difference in the response to caffeine treatment.

Cells were treated with CPT, a replication stress inducing agent which functions by fixing topoisomerase 1 (TOP1) on chromatin forming an obstruction (Pommier, 2013). The IC₅₀ in the B-*myb*^{+/+} was 1.43μM, whereas, the B-*myb*^{Δ/Δ} ESCs presented a remarkably reduced IC₅₀ of 0.24μM, a 5-fold reduction compared to the control. Non-linear regression analysis revealed the difference to be highly statistically significant producing a p-value of 0.0002. Overall, this data, as well as the increased DNA damage sensitivity after CPT observed previously by alkaline comet assay (Figure 3.11), suggests that the B-*myb*^{Δ/Δ} ESCs present a strong sensitivity to CPT toxicity.

Hydroxyurea was also tested, an inhibitor of ribonucleotide reductase in which treatment causes replication fork stalling (Yarbro, 1992). There was a slight decrease in cell viability after 0.25mM hydroxyurea from 57% in the B-myb^{+/+} to 48% in the B-myb^{Δ/Δ} ESCs which could represent some dysfunction in the response to replication inhibition, therefore, this treatment would benefit from more repeats to investigate this further. An inhibitor of PARP1, Olaparib, was also employed, for which sensitivity has been shown to result from an inability to repair DSBs by homologous recombination (Golan et al., 2019). After 24 hours of 2μM Olaparib treatment, there was little to no difference in cell viability between the B-myb^{+/+} at 58% and B-myb^{Δ/Δ} and 55%. WEE1, the kinase which inhibits Cdk1 activity (Sorensen and Syljuasen, 2012) was also targeted using the drug MK-1775. 24 hours of 5μM MK-1775 was highly detrimental to cell viability producing values of 16% in the B-myb^{+/+} and 13% in the B-myb^{Δ/Δ} ESCs highlighting the importance of CDK control for cell survival. However, there was not much difference in the response to MK-1775 between the two groups. Moreover, both of these treatments provide some evidence to suggest that the defect in the B-myb^{Δ/Δ} ESCs is not due to a defect in the DSB repair pathways, although, both experiments would benefit from further repeats.

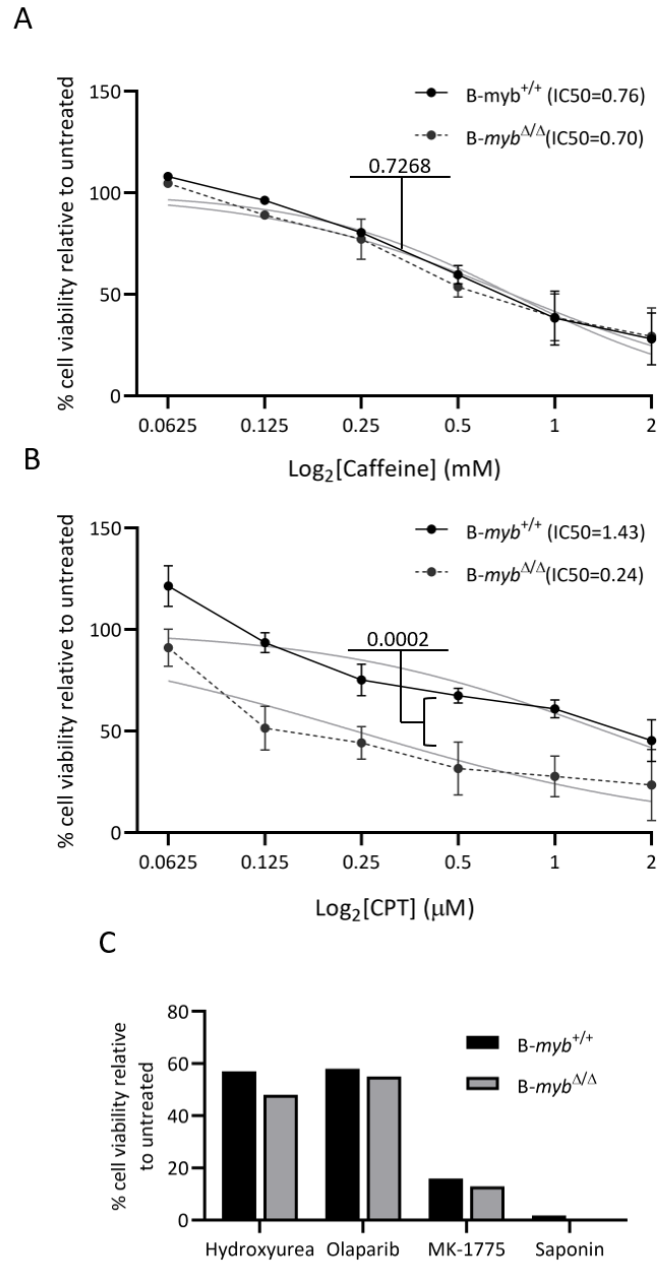


Figure 3.12 Decreased cell viability after CPT treatment in the B-myb^{Δ/Δ} ESCs Cells were treated with various chemical inhibitors for 24 hours before measuring cell viability. 0.1% saponin was used as a positive control for death in each experiment. A) Dose-response curve in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs after treatment with Caffeine presenting percentage cell viability relative to untreated. X-axis is log₂ scale of caffeine concentration (mM). Data from 3 independent experiments carried out in triplicate and error bars represent SEM B) Dose-response curve in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs after treatment with CPT presenting percentage cell viability relative to untreated. X-axis is log₂ scale of CPT concentration (μM). Error bars are SEM. Representative of 3 independent experiments carried out in triplicate C) Bar graph shows percentage cell viability relative to untreated in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs after treatment with 1) 0.25mM hydroxyurea 2) 2μM Olaparib 3) 5μM MK-1775 and 4) 0.1% saponin (positive control), from 1 experimental repeat carried out in triplicate.

3.3 Discussion

3.3.1 Generation of a constitutive B-Myb knockout ESC line

Work from the Garcia lab proposed that B-Myb has no influence upon expression of pluripotency transcription factors, contradicting previous findings (Tarasov et al., 2008). Instead, B-Myb was shown to play an uncharacterised role during S phase in ESCs with a specific influence on replication progression (Lorvellec et al., 2010). To recapitulate these findings and to further explore the roles of B-Myb during S phase, total B-*myb* knockout ESCs (B-*myb*^{Δ/Δ}) were generated by transfecting B-*myb* conditional knockout ESCs (B-*myb*^{F/Δ}) with Cre recombinase. To determine the effectiveness of the B-*myb* knockout, both the expression of the gene as well as the levels of B-Myb protein was analysed (Figure 3.2); both experiments confirmed the successful ablation of B-*myb*. As previously described (Lorvellec et al., 2010), the B-*myb*^{Δ/Δ} ESCs were able to propagate continuously in culture, supporting the theory that loss of B-Myb does not alter their capacity to maintain pluripotent stem cell identity.

Previous studies investigating the role of B-Myb have utilised partial or transient knockdown systems (Tarasov et al., 2008, Werwein et al., 2012). These strategies analyse the short-term effect of B-Myb loss, therefore, the phenotypes produced represent the initial cellular reaction to its loss which may encompass activation of non-specific responses such as a stress response. The B-*myb*^{Δ/Δ} ESCs used in this study represent a good model to examine the effects of B-Myb loss upon ESC growth and function after several cell generations, once the mutant ESCs are established in culture. In addition, B-Myb ablated embryos die at E4.5-6 just prior to maturation of the inner cell mass (Tanaka et al., 1999), therefore, this model also represents a good system to study the role of B-Myb during this developmental period.

A limitation of the model used is that the floxed allele contained LoxP sites surrounding the N-terminal DNA binding domain and did not incorporate the rest of the B-*myb* gene. The western

blot performed to confirm loss of B-Myb protein utilised an antibody against the N-terminal region containing the DNA binding domain. Therefore, it cannot be ruled out that the cell could be expressing and producing a truncated version of B-Myb protein missing the DNA binding domain. The qPCR performed to analyse the levels of B-*myb* expression found loss of expression specifically of a region within the exon boundary of 6-7, suggesting that this specific exon outside of the Cre-deleted region was not expressed. However, through looking at the expression of the B-*myb* gene from RNA sequencing analysis in the B-*myb*^{Δ/Δ} ESCs, it was clear that the remaining exons of the B-*myb* gene, including exon 6 and 7, were still expressed (Figure 6.7). A possible way to explain this conflicting data is that the qPCR was performed immediately after producing the knockout, whereas, the RNA sequencing analysis was performed on cells after several passages. Therefore, it could be that the remaining exons start to be expressed some time after the knockout.

From this data, it is unclear whether the knockout system used results in loss of the protein. Therefore, an important future experiment would be to perform westerns using antibodies against the C-terminal region of B-Myb. If there was no signal, this would demonstrate that the protein is not able to be produced without the region encoding the DNA binding domain. Alternatively, if the C terminal region was detected, this would suggest that the phenotypes observed are dependent upon loss of the DBD of B-Myb specifically.

3.3.2 Investigating replication dynamics in B-*myb*^{Δ/Δ} ESCs

In this chapter, various techniques were used to confirm and validate the effect loss of B-Myb has upon S phase progression. Through analysis of single DNA fibres, it was observed that the B-*myb*^{Δ/Δ} ESCs present a global reduction in replication speed; agreeing with the previous work from the lab proposing B-Myb as an important factor in promoting normal replication progression (Lorvellec et al., 2010). Slowdown of active replication is well characterised as a response to cellular stress induced through activation of a checkpoint response (Paulovich and

Hartwell, 1995, Iyer and Rhind, 2017). However, checkpoint activation is also characterised by a global shutdown of origin firing and arrest of cell cycle progression (Merrick et al., 2004), whereas, the B-*myb*^{Δ/Δ} ESCs presented a contrasting increase in new origin firing and continued to proliferate in these conditions. This data suggests that the replication speed defect observed in the B-*myb*^{Δ/Δ} ESCs does not represent the result of a normal checkpoint response, but likely represents unusual replication behaviour.

The B-*myb*^{Δ/Δ} ESCs also presented increased fork instability, represented by asymmetry of both elongating forks and forks around new origins. Asymmetry of elongating forks represents a number of different potential causes. A shortening of the second incorporated label, which covers most of the asymmetric forks analysed, is representative of the slowing or the complete stalling of a replication fork. Whereas, shortening of the first label represents a slowing/stalling event which then recovers/restarts during the second label. Additionally, the increased asymmetry around newly fired origins demonstrated that the fork instability is not limited to elongating forks. Overall, this data suggests there is global deregulation of replication dynamics in the B-*myb*^{Δ/Δ} ESCs. The distinction between whether this phenotype represents fork slowing or actual stalling of the replication fork is difficult to determine from this data. Analysis of the frequency of first label only tracks, which has been used as a marker for increased fork stalling (Reynolds et al., 2017), presented a subtle (3%) increase in these structures in the B-*myb*^{Δ/Δ} ESCs possibly reflecting an increase in fork stalling which may contribute to the instability observed. Although, an increase in first label only forks may alternatively represent an increase in replication termination events, questioning the validity of this method for measuring fork stalling.

Replication dynamics in the B-*myb*^{Δ/Δ} ESCs was analysed further through visualisation of replication factories revealing a highly significant increase in the number of factories per cell during early S phase. It has been demonstrated that in response to replicative stress, activation

of a checkpoint response results in inhibition of new replication factory activation and firing of new origins within the same factory (Ge and Blow, 2010). A more recent study demonstrated that this response was highly dependent on the level of stress, with low levels of replication stress resulting in a contrasting increase in replication factory activation (Rybak et al., 2015). In the *B-myb*^{Δ/Δ} ESCs, it may be that the chronic replication stress results in the increase in factories observed. An alternative explanation is that there could be a deregulation of pathways involved in controlling replication factories; a potential example being the ATR-Chk1 pathway, shown to play a direct role in suppressing replication factory activation in response to stress (Ge and Blow, 2010).

3.3.3 The influence of B-Myb loss upon the cell cycle

A potential caveat of analysing replication speeds by the DNA fibre technique is the potential for differences in the distribution of cells in S phase. It is known that the dynamics of replication initiation differs depending on the stage of S phase, more specifically, the number of initiation events per length of DNA increases until mid S phase and then sharply decreases towards termination of replication (Goldar et al., 2008, Goldar et al., 2009). This potential issue was addressed through tracking incorporated BrdU, a deoxynucleoside analogue, through the cell cycle and analysis of its distribution by flow cytometry. Although there was an increase in the proportion of S phase cells in the *B-myb*^{Δ/Δ} ESCs, possibly reflecting their reduced replication efficiency, there was no significant difference in the proportion of cells in early, mid and late replication. Therefore, this ruled out a potential role for cell cycle distribution in the replication speed defect.

The replication stress observed also led to under-replicated DNA passing into the next phase of the cell cycle indicated by the formation of ultra-fine bridges (UFBs) (Figure 3.9) (Chan et al., 2007). Several types of UFBs have been characterised in the literature. Centromeric UFBs are the most common type which form from double-stranded catenanes in centromeric regions

which are found during normal mitosis (Chan et al., 2018). UFBs also form from inappropriate processing of late replication intermediates commonly seen under conditions of replication stress (Naim et al., 2013). This mechanism may be responsible for the increased UFBs present in the B-*myb*^{ΔΔ} ESCs. Another mechanism for UFB formation is through abnormal replication of telomeres (Nera et al., 2015). Moreover, B-Myb loss has been associated with telomeric fragility (Bayley et al., 2018a), presenting another potential mechanism for the increased UFBs in B-*myb*^{ΔΔ} ESCs.

Recent work suggests that B-Myb also plays a role in the downregulation of pro-mitotic genes in response to stress and therefore is important for G2-M checkpoint control (Henrich et al., 2017). The B-*myb*^{ΔΔ} ESCs are able to transition through the cell cycle in the presence of replication stress, albeit with a reduced proliferation rate, potentially supporting this theory. The capacity of the B-*myb*^{ΔΔ} ESCs to activate a G2/M checkpoint response was tested further after treatment with CPT. Moreover, the checkpoint response appeared to be much weaker in the B-*myb*^{ΔΔ} ESCs supporting the theory that the G2/M checkpoint is defective. Overall, this data suggests that B-Myb plays a role in activation of the G2/M checkpoint in response to stress.

3.3.4 Accumulation of DNA damage in the B-*myb*^{ΔΔ} ESCs

Previous work in the lab showed an increase in γ H2AX recruitment in B-*myb* ablated ESCs, nonetheless, γ H2AX is not a reliable marker of DSBs (Lorvellec et al., 2010). In stem cells, γ H2AX presents at abnormally high levels in the absence of DNA damage attributed to the changes in chromatin structure associated with the pluripotent state (Banath et al., 2009, Chuykin et al., 2008). To investigate in more detail whether loss of B-Myb results in DNA damage, staining for 53BP1, a signalling protein recruited to sites of DSBs, was performed. 53BP1 was significantly increased in the B-*myb*^{ΔΔ}. Separation of replicating and non-replicating cells through EdU incorporation revealed that the increase in 53BP1 levels was mostly specific to cells undergoing replication. This data suggests that the B-*myb*^{ΔΔ} ESCs

present increased levels of DNA damage which could be dependent on a replication coupled mechanism.

Although recruitment of 53BP1 suggests an increase in double strand breaks, a caveat of this detection method is that it relies on cellular response pathways, therefore, an alkali comet assay was performed to further analyse total levels of DNA damage in single cells. Based on this assay, there was a significant increase in DNA damage in the *B-myb*^{Δ/Δ} ESCs. A caveat of the alkaline comet assay is that it does not detect DSBs exclusively but detects all alkali labile sites including DNA double-strand breaks, single-strand breaks, DNA-protein cross-linking, and incomplete excision repair sites (Pu et al., 2015). Nonetheless, this assay in combination with the 53BP1 result provides strong evidence for increased DNA damage accumulation after B-Myb loss.

Another question was whether the DNA damage accumulation in the *B-myb*^{Δ/Δ} is due to a defect in the response to and repair of DNA damage in ESCs. There is evidence in the literature for a role of B-Myb in the response to DNA damage (Bayley et al., 2018a, Henrich et al., 2017). To test this possibility, cells were treated with different inhibitors of key response pathways and their sensitivity after 24 hours was assessed through measuring cell viability.

Cells were treated with caffeine, a non-specific inhibitor of the DNA damage response, for which treatment has been demonstrated to increase DNA damage sensitivity after ionizing irradiation (Blasina et al., 1999). Moreover, if the response to DSBs was defective in the *B-myb*^{Δ/Δ} ESCs, it may be expected that the *B-myb*^{Δ/Δ} would be less sensitive to caffeine treatment compared to *B-myb*^{+/+} ESCs. However, there was little difference in the cell viability response between the *B-myb*^{+/+} and the *B-myb*^{Δ/Δ}. Caffeine treatment was originally thought to be an inhibitor of DDR associated kinases such as ATM and ATR, however, recent work has suggested that its mechanism of action is not strictly related to the DDR but plays influences in

multiple processes including homologous recombination, Rad51 function, and end resection (Tsabar et al., 2015, Wang et al., 2004, Zelensky et al., 2013). Therefore, to investigate a potential role in the DDR, cell viability assays after treatment with more specific inhibitors of the DDR components would be beneficial.

Cells were also treated with hydroxyurea, an inhibitor of ribonucleotide reductase (Yarbro, 1992). Sensitivity could result from an inability to respond to and process stalled forks and DNA damage. The B-*myb*^{ΔΔ} displayed some sensitivity to hydroxyurea, indicating that these mechanisms could be defective, although, more repeats of this experiment would be necessary to further explore this idea. Sensitivity to Olaparib, an inhibitor of PARP1 activity, was also tested. Moreover, Olaparib is used clinically to target cancer cells with a homologous recombination defect (Ledermann et al., 2014), therefore, sensitivity can indicate a problem with the capacity to repair DSBs. Inhibition of WEE1 through MK-1775 treatment was also performed; sensitivity to WEE1 inhibition has also been shown to selectively target DNA repair deficient cells (Aarts et al., 2015). The B-*myb*^{ΔΔ} ESCs presented no substantial change in sensitivity to both treatments providing some evidence to suggest that the B-*myb*^{ΔΔ} ESCs do not present a defect in the DNA repair pathways in ESCs. Again, to acquire more reliable data concerning the response to these agents, more experimental repeats would be required.

3.3.5 Increased sensitivity to camptothecin in B-*myb*^{ΔΔ} ESCs

The sensitivity of the B-*myb*^{ΔΔ} ESCs to camptothecin (CPT), an inhibitor of topoisomerase 1, was investigated. Topoisomerase 1 is vital for alleviating topological constraints during replication, transcription, and chromatin remodelling (Champoux, 2001, Wang, 2002). It binds as a cleavage complex (TOP1cc) which cleaves one strand of the DNA to allow its relaxation, before migrating away allowing re-ligation of the DNA (Pommier, 2013). CPT binds to TOP1cc and reversibly fixes it onto chromatin (Hsiang et al., 1985, Pommier et al., 2010). Moreover, the fixed TOP1cc complex present a barrier to replication and transcription activity through an

inability to deal with excess supercoiling but also potentially through direct collisions (Sordet et al., 2009, Pommier et al., 2016, Liu et al., 2000a, Liu and Wang, 1987, Strumberg et al., 2000).

Strikingly, the B-*myb*^{Δ/Δ} presented a highly significant reduction in cell viability at a range of concentrations of CPT treatment. Supporting this, the B-*myb*^{Δ/Δ} also presented significantly increased levels of DNA damage by alkaline comet assay after short-term CPT treatment. The increased DNA damage after CPT was not as apparent through measuring the recruitment of 53BP1, however, this may be attributed to limitations with the analysis which does quantify those cells with greater than 6 53BP1 foci. Overall, this data strongly suggests that the B-*myb*^{Δ/Δ} ESCs present increased sensitivity to CPT treatment.

Toxicity to CPT has been shown to be caused by multiple different potential mechanisms. Firstly, sensitivity can arise from a defect in the removal of the TOPO1cc or repair of the resulting lesion. Moreover, Mus81 dependent end-resection and homologous recombination repair have been demonstrated to be vital in the processing and subsequent repair of the double strand ends (DSEs) which remain after TOPO1cc removal (Regairaz et al., 2011, van Waardenburg et al., 2004). Therefore, it is possible that the B-*myb*^{Δ/Δ} ESCs are defective in the processing and repair of DSEs, although, previous data would argue against a direct role for B-Myb in DNA repair (Henrich et al., 2017).

There is a fine balance between the level of DNA processing, which alters the topological state of DNA, and topoisomerase activity, which relieves the topological stress. Disruption of this balance can result in excess topological stress and the accumulation of DNA damage. (Teves and Henikoff, 2014). Cancer cells, which present increased topological stress due to an inability to appropriately control DNA processing, display a resulting sensitivity to topoisomerase inhibition (Bermejo et al., 2012, Dwarakanath et al., 2004, Fry et al., 1991). In addition,

cytotoxicity to CPT was shown to result from disruption of cell cycle associated signalling pathways involved in regulating S phase progression (Shao et al., 1997, Furuta et al., 2006).

The B-*myb*^{Δ/Δ} ESCs present increased DNA processing indicated by elevated origin firing, as well as weaker checkpoint control in response to CPT, highlighting additional potential mechanisms for the sensitivity to CPT observed. The increased DNA processing observed in the B-*myb*^{Δ/Δ} ESCs would result in an increase in topoisomerase activity. Therefore, CPT treatment would result in an increase in the number of TOPO1cc's trapped on chromatin, which would elevate the topological stress and increase DNA damage accumulation further (Pommier, 2006). CPT sensitivity was also recently linked to the levels of chromatin modifications such as H4 K16 acetylation, highlighting another potential mechanism for sensitivity in the B-*myb*^{Δ/Δ} ESCs (Puddu et al., 2017). Therefore, further work is required to pinpoint the exact mechanisms behind the sensitivity to CPT in the B-*myb*^{Δ/Δ} ESCs.

3.3.6 Explaining the replication defect in the B-*myb*^{Δ/Δ} ESCs

In this chapter, it was established that the B-*myb*^{Δ/Δ} ESCs present decreased replication rates and increased DNA damage accumulation, however, this is accompanied by an increase in replication initiation and loss of G2-M checkpoint control. In addition, despite displaying a strong replication stress phenotype and increased DNA damage, the B-*myb*^{Δ/Δ} ESCs are able to continue proliferating. This behaviour is similar to cancer cells which exploit and deregulate the cell cycle to ignore anti-proliferation signals and to favour limitless proliferative capacity (Jones and Petermann, 2012, Kotsantis et al., 2016). It is also important to recognise that this study is in ESCs, which already possess a rapid and unrestricted cell cycle. Therefore, it may be their identity as stem cells which allows them to tolerate the detrimental effects upon genome stability after loss of B-Myb.

**Chapter 4 Deregulation of cell cycle signalling as a
mechanism for the replication stress in the B-*myb*^{Δ/Δ}
ESCs**

4.1 Introduction

In the previous chapter, it was established that B-Myb ablated ESCs display replication stress and genome instability, yet also present elevated replication initiation and lack normal G2/M checkpoint control in the presence of stress. Multiple mechanisms for replication stress have been identified and often there can be an accumulating combination of causes which can culminate in severe replication toxicity and DNA damage. Moreover, replication stress is commonly observed in cancer cells and is a major hallmark of carcinogenesis (Zeman and Cimprich, 2014).

Cancer cells exhibit the capacity for unscheduled proliferation in the presence of an unstable genome. Moreover, this has been partly attributed to deregulation of cyclin dependent kinases (CDKs) and their upstream regulators (Malumbres and Barbacid, 2005). Deregulation of B-Myb has been associated with multiple different cancer entities including leukaemia and multiple solid tumour such as breast and colon cancer (Bayley et al., 2018a, Ramsay and Gonda, 2008). In addition, B-Myb has been linked to cell cycle control; firstly, through its involvement in transcription of cell cycle progression associated genes (Georlette et al., 2007, Sadasivam and DeCaprio, 2013), but also more recently, its binding partner FOXM1 was suggested to be a transcriptional target of CDK1 activity in control of entry into G2 from S phase (Saldivar et al., 2018). In this chapter, I aimed to determine whether the influence of B-Myb loss upon replication control is due to changes in cell cycle associated regulation of the replication programme.

4.2 Results

4.2.1 Investigating the influence of B-Myb loss on CDK1 activity in ESCs

To examine the activity of CDK1 in the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs, whole cell protein was extracted before performing western blot analysis to quantify the levels of inhibitory phosphorylation of CDK1 (tyr15). The *B-myb*^{Δ/Δ} ESC presented a reduction in the levels of P-CDK1 compared to *B-myb*^{+/+} ESCs suggesting this inactivating mark was reduced. Additional densitometry analysis of P-CDK1 relative to loading control uncovered that the level of phosphorylation averaged at 68% of the *B-myb*^{+/+}, a difference which was determined to be statistically significant using a two-tailed unpaired t-test (p-value = 0.0106) (Figure 4.1B). To rule out a potential reduction in the total levels of CDK1 protein in the *B-myb*^{Δ/Δ} ESCs, immunoblotting against CDK1 protein was performed revealing no obvious variations in levels compared to the *B-myb*^{+/+} (Figure 4.1A).

To analyse the effect of additional stress upon CDK1 activity in the *B-myb*^{Δ/Δ} ESCs, cells were treated with CPT for 4 hours. The *B-myb*^{+/+} ESCs presented a slight increase in P-CDK1 levels possibly reflecting an inhibition of CDK1 activity. However, the CPT-treated *B-myb*^{Δ/Δ} ESCs did not display any obvious increase in P-CDK1, suggesting they potentially lack this response to CPT treatment (Figure 4.1A). Overall, this data proposes that chronic loss of B-Myb in ESCs results in abnormal CDK1 activity.

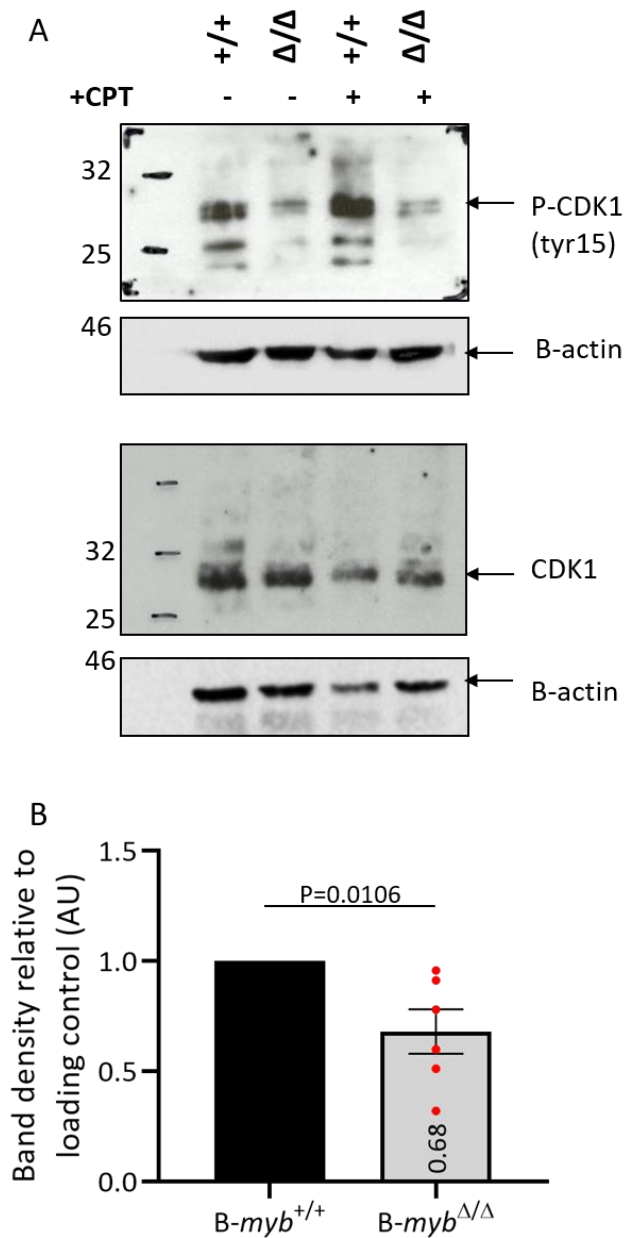


Figure 4.1 Reduced levels of P-CDK1 (tyr15) in B-*myb*^{Δ/Δ} ESCs B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated for 2 hours with 10μM CPT before protein extraction, separation by SDS-PAGE and protein detection by western blotting. A) An example of a western blot for B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs with or without CPT treatment. Immunoblotting performed using antibodies against P-CDK1 (tyr15), CDK1 and Beta-actin as a loading control. Numbers represent the molecular weight (kDa) determined through a protein ladder and arrows point to the specific bands examined B) Bar graph represents average band density of P-CDK1 in B-*myb*^{Δ/Δ} ESCs relative to loading control and relative to the B-*myb*^{+/+} from 6 repeats. Error bars represent SEM. Statistical analysis was carried out using a two-tailed unpaired t-test.

4.2.2 The effect of CDK1 inhibition upon replication function in B-*myb*^{Δ/Δ} ESCs

Based on the results from the protein analysis, the next question was whether the atypical CDK1 activity contributes to the replication dysfunction observed in the B-*myb*^{Δ/Δ} ESCs. To investigate this, RO3306, an ATP-competitive inhibitor which presents highly selective inhibition of CDK1 and low selectivity (10x less) for CDK2 was utilised (Vassilev et al., 2006). The B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated with the inhibitor for 1.5 hours before addition of modified thymidine analogues IdU and CldU for 20 minutes each; DNA fibres were prepared from the isolated cells and visualised as previously described (Figure 4.2A).

Inhibition of CDK1 in B-*myb*^{+/+} ESCs resulted in a global reduction in replication rate, with the median rate reduced from 1.42 ±0.06 to 1.22 ±0.07 kilobases per minute (Figure 4.2B+D). This decrease was determined to be statistically significant using the Mann Whitney U test producing a p-values less than 0.0001. In contrast, inhibition of CDK1 in the B-*myb*^{Δ/Δ} ESCs resulted in an increase in the replication speed, with median rate increasing from 0.92 ±0.04 to 1.22 ±0.04 kilobases per minute, to similar levels seen in the B-*myb*^{+/+} ESCs after CDK1 inhibition (Figure 4.2C+D) (Mann Whitney U test p<0.0001). Overall, short-term inhibition of CDK1 activity appeared to produce mild toxicity in normal replicating ESCs, however, in the B-*myb*^{Δ/Δ} ESCs there was a partial rescue of the replication speed defect.

To investigate further the influence of CDK1 inhibition upon replication dynamics in the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs, the frequency of newly fired origins was measured (Figure 4.3A). Inhibition of CDK1 in B-*myb*^{+/+} ESCs resulted in a mild increase in new origin firing from 23% to 26%, whereas, in the B-*myb*^{Δ/Δ} ESCs, CDK1 inhibition resulted in a reduction in new origin firing from 33% to 26%, levels close to that in the B-*myb*^{+/+}. However, both differences were not statistically significant using two-tailed unpaired t-tests producing p-values of 0.4714 and 0.1141 respectively (Figure 4.3B).

The level of fork instability was also investigated through analysis of the elongating fork ratio (Figure 4.3A). In the B-*myb*^{+/+} ESCs, there was an increase in the average fork ratio from 1.50 to 1.74 after CDK1 inhibition. Treatment with CDK1 inhibitor in the B-*myb*^{Δ/Δ} ESCs resulted in a mild rescue of the elevated fork ratio from 1.98 to 1.85. However, both of these differences were determined to be not statistically significant producing p-values of 0.1115 and 0.3792 respectively (Figure 4.3C). Overall, although inhibition of CDK1 appears to partially rescue the replication fork speed defect observed in the B-*myb*^{Δ/Δ}, the influence upon replication initiation and fork dynamics was inconsistent. This may potentially be attributed to the additional toxicity produced through CDK1 inhibition which was observed in the B-*myb*^{+/+} ESCs.

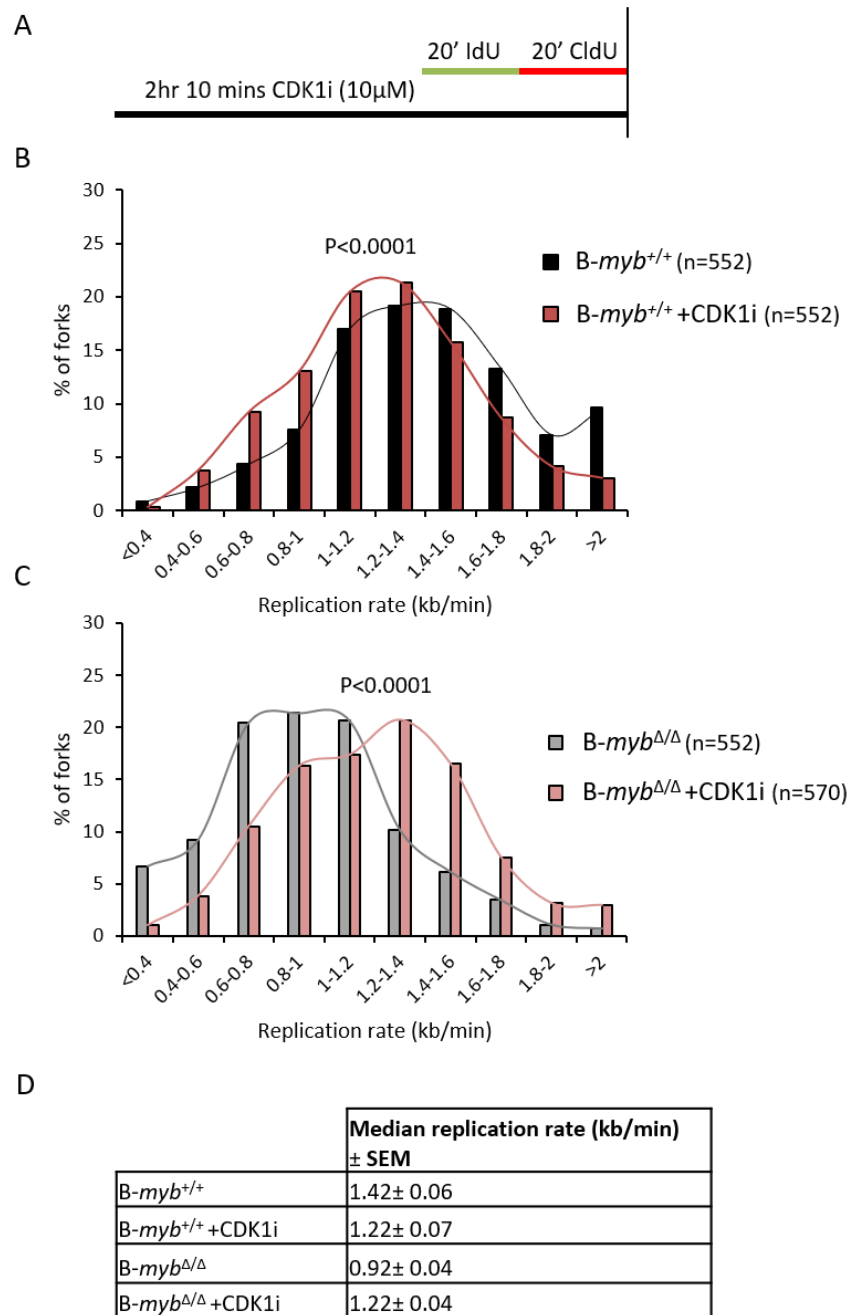


Figure 4.2 Partial rescue of the replication speed defect after CDK1 inhibition in B-*myb*^{Δ/Δ} ESCs

A) Schematic representation of the procedure. Briefly, B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated for 1.5 hours with RO3306, an inhibitor of CDK1, before sequential addition of deoxynucleoside analogues IdU and CldU for 20 minutes each. DNA was treated and visualised as previously described. B) Distribution curve demonstrating the influence of CDK1 inhibition upon B-*myb*^{+/+} ESC replication speed. C) Distribution curve for CDK1 inhibition in B-*myb*^{Δ/Δ} ESCs. Statistical analysis of distributions was carried out using an unpaired Mann Whitney U test. D) Table showing median replication speed in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs with or without CDK1 inhibitor treatment. n=4. Number of forks quantified for each of the genotypes and conditions are shown on the respective distribution curves.

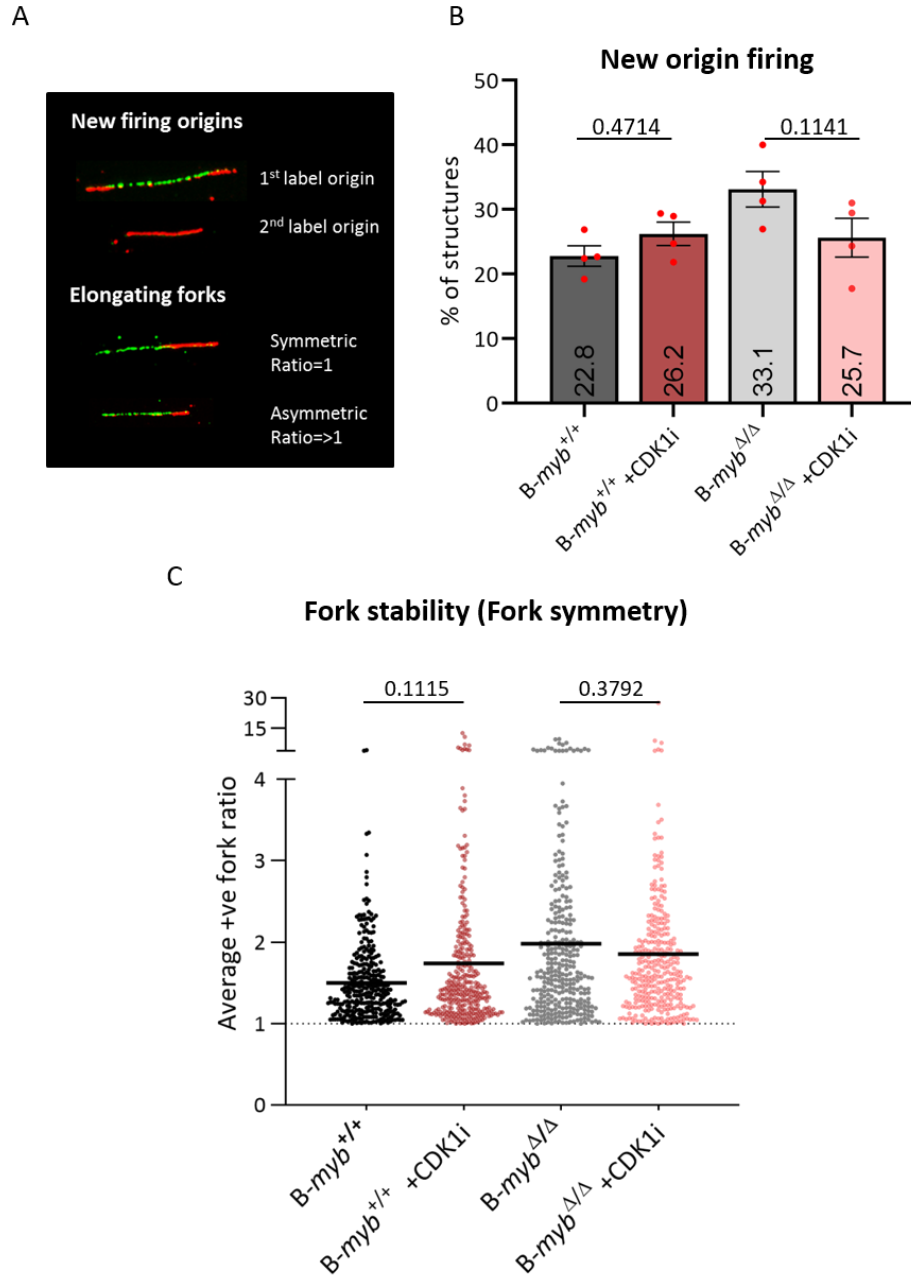


Figure 4.3 Failure to rescue new origin firing and replication instability after CDK1 inhibition in B-myb^{Δ/Δ} ESCs B-myb^{+/+} and B-myb^{Δ/Δ} ESCs were treated for 1.5 hours with 10μM CDK1 inhibitor (RO3306) before performing DNA replication labelling. A) The staining patterns were used to identify elongating forks and newly fired origins as shown. B) Bar graph representing the percentage of new fired origins relative to total number of structures counted. Error bars represent SEM. Statistical analysis was carried out using two-tailed unpaired t-test. At least 500 replication structures were counted per treatment group from 4 separate repeats. C) As a measure of replication instability, the length of both labels for each elongating fork was measured in μm and the positive ratio calculated. Dot plot showing the distribution and the average fork ratio in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs with or without CDK1 inhibitor treatment. Average ratios were 1.5, 1.74, 1.98 and 1.85 in the order presented. Statistical analysis was carried out using the Mann Whitney U test. Over 300 ratios were calculated per treatment group, n=4.

4.2.3 Investigating the influence of elevated CDK1 activity upon replication through inhibition of WEE1 kinase

Inhibition of CDK1 activity resulted in a partial rescue of replication progression in the B-*myb*^{Δ/Δ} ESCs, despite also presenting replication toxicity in normal wildtype ESCs. Next, the effect of amplification of CDK1 activity was examined through inhibition of WEE1, the kinase responsible for inhibitory phosphorylation of CDK1 (Lim and Kaldis, 2013). As CDK1 is potentially overactive in the B-*myb*^{Δ/Δ} ESCs, it was hypothesised that WEE1 inhibition could mimic the replication stress observed.

Cells were treated with 10μM MK-1775, a chemical inhibitor of WEE1 kinase, for two hours before protein extraction, and western blot analysis. Examination of total levels of WEE1 protein revealed no change in the B-*myb*^{Δ/Δ} compared to B-*myb*^{+/+}. In addition, treatment with the WEE1 inhibitor had no obvious influence on the protein levels of WEE1 in both groups (Figure 4.4). Interestingly, although the levels of P-CDK1 in the B-*myb*^{Δ/Δ} were reduced compared to B-*myb*^{+/+}, treatment with the WEE1 inhibitor reduced P-CDK1 to exceedingly low levels in both groups, highlighting the importance of WEE1 for phosphorylation of CDK1 activity in ESCs (Figure 4.4).

The effect of WEE1 inhibition upon replication dynamics was investigated; cells were treated with MK1775 for 1.5 hours before labelling replicating DNA and performing fibre analysis (Figure 4.5A). Interestingly, WEE1 inhibition was highly detrimental to replication progression in both the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs (Figure 4.5B+C); with the B-*myb*^{+/+} median speed reduced from 1.40 ± 0.21 to 0.69 ± 0.14 kb/min, and from 0.89 ± 0.15 to 0.66 ± 0.14 kb/min in the B-*myb*^{Δ/Δ} ESCs (Figure 4.5D). Moreover, the effects seen was statistically significant in both the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs producing p-values of less than 0.0001. Thus, demonstrating that inactivation of WEE1 kinase activity is highly detrimental to replication progression.

Analysis of replication structures after WEE1 inhibition revealed a potential increase in new origin firing in both the B-*myb*^{+/+} (20% to 34%) and the B-*myb*^{Δ/Δ} ESCs (30% to 38%). (Figure 4.6B). The presence of fork instability was also investigated through measuring the elongating fork ratio. The B-*myb*^{+/+} ESCs presented a substantial increase in fork ratio with the mean ratio increasing from 1.45 to 2.45. In addition, the percentage of forks with a ratio greater than 2 also increased from 10% to 44%, highlighting a strong increase in fork instability (Mann Whitney U test, $p < 0.0001$) (Figure 4.6C). Interestingly, the already elevated fork instability observed in the B-*myb*^{Δ/Δ} was increased even further after WEE1 inhibition with the mean ratio increasing from 2.01 to 2.47 (Mann Whitney U test, $p < 0.0001$). This was also reflected in the percentage of forks with a ratio greater than 2, increasing from 34% to 50% (Figure 4.6C). Overall, these findings demonstrate that loss of WEE1 activity results in severe deregulation of the replication programme and exacerbates the replication stress observed in the B-*myb*^{Δ/Δ} ESCs.

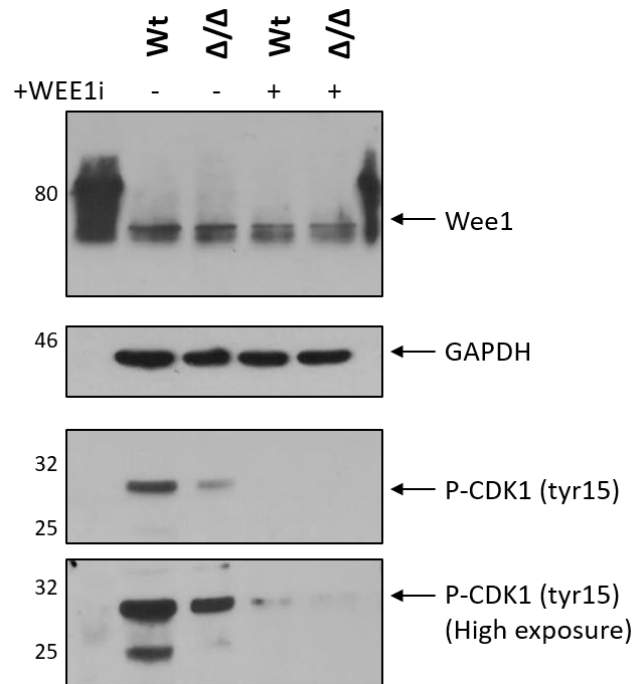


Figure 4.4 Loss of P-CDK1 (tyr15) levels after inhibition of WEE1 kinase *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs were treated for 1.5 hours with 10μM WEE1 inhibitor before harvesting cells and extracting protein. Samples were separated by SDS-PAGE and specific protein targets were detected by immunoblotting. Image shows resulting photographic film generated through chemiluminescent detection of conjugated HRP. Shown is immunoblotting for WEE1, phospho-CDK1 (tyr15), and GAPDH loading control. Numbers represent molecular weight (kDa) determined through protein ladder.

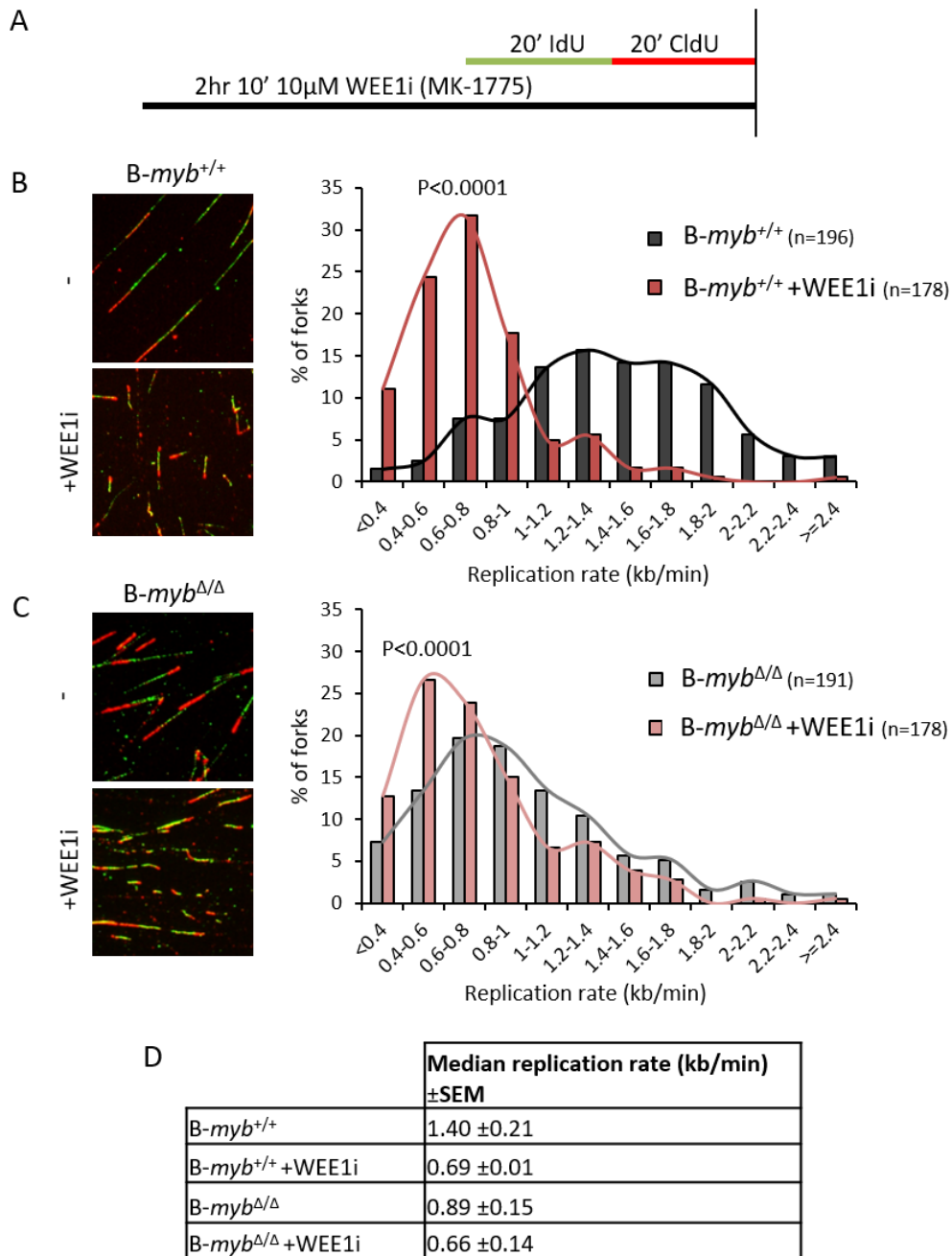


Figure 4.5 Inhibition of WEE1 results in severe replication slowdown in both *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs A) ESCs were treated for 1.5 hours with 10μM MK-1775 an inhibitor of WEE1, before sequential addition of deoxynucleoside analogues IdU and CldU for 20 minutes each. DNA was treated and visualised as previously described. B) Example fluorescence images of DNA fibres in *B-myb*^{+/+} in the presence or absence of WEE1 inhibitor, plus, distribution curve demonstrating the influence of WEE1 inhibition upon *B-myb*^{+/+} ESC replication speed. C) Fluorescence images of DNA fibres in *B-myb*^{Δ/Δ} in the presence or absence of WEE1 inhibitor, plus, distribution curve for WEE1 inhibition in *B-myb*^{Δ/Δ} ESCs. Statistical analysis of distributions was carried out using an unpaired Mann Whitney U test. D) Table showing median replication speed in *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs with or without WEE1 inhibitor treatment. Data acquired from 4 slides per group from 2 independent experiments. Number of forks quantified for each of the genotypes and conditions are shown on the respective distribution curves

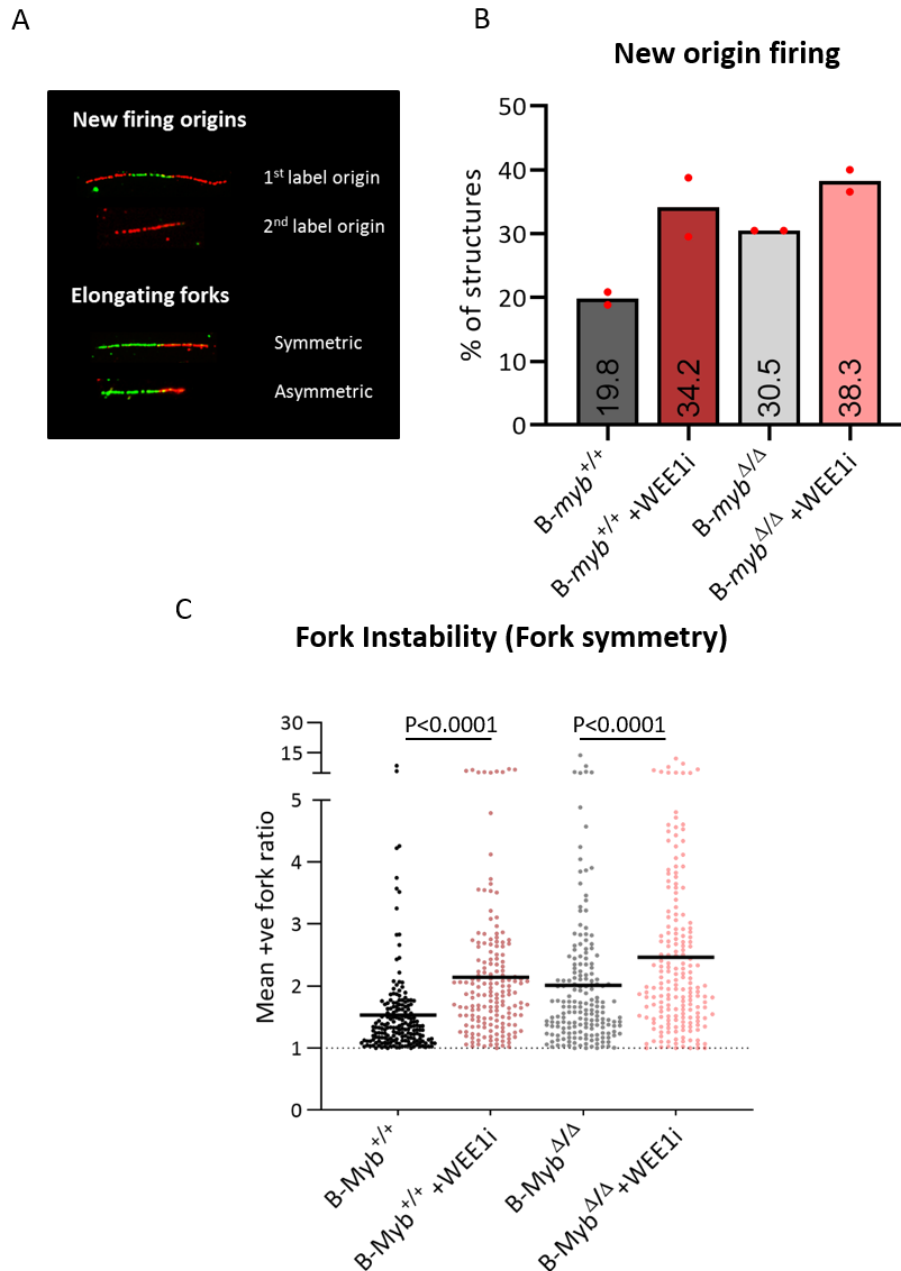


Figure 4.6 Inhibition of WEE1 kinase results in replication stress associated fork behaviour. B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated for 1.5 hours with 10μM MK-1775 an inhibitor of WEE1 before performing DNA replication labelling. A) The staining patterns were used to identify elongating forks and newly fired origins as shown. B) Bar graph representing the percentage of new fired origins in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs with or without WEE1 inhibitor treatment. At least 200 replication structures were counted per treatment group from 2 experimental repeats. C) The length of both incorporated labels for each elongating fork was measured in μm and the positive ratio was calculated. Dot plot demonstrating the average fork ratio in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs with or without WEE1 inhibitor treatment. Average ratios were 1.53, 2.38, 2.01 and 2.47 in the order presented. Statistical analysis was carried out using the Mann Whitney U test. At least 170 ratios were calculated per treatment group from 2 separate repeats.

4.2.4 The potential role for origin firing deregulation in replication dysfunction observed in *B-myb*^{Δ/Δ} ESCs

To investigate more closely the role of origin firing in the replication stress observed, more specific regulators were targeted. PHA-767491 is a dual chemical inhibitor of cdc7 and CDK9, moreover, Cdc7 is directly involved in activation of origin firing throughout S phase as a component of the DDK complex (Jackson et al., 1993, Yeeles et al., 2015). Cells were treated for 1.5 hours with PHA-767491, an inhibitor of Cdc7 kinase, before labelling active replication forks (Figure 4.7A). In the *B-myb*^{+/+}, there was little change in replication progression speed with median speeds from 1.32 (±0.07) to 1.35 (±0.08) after treatment (Mann Whitney U test, $p=0.4503$) (Figure 4.7B+D). Interestingly, the percentage of forks with a speed greater than 2 kilobases per minute was increased from 6% to 14%, reflecting abnormally accelerated replication of some forks to compensate for the lack of firing (Figure 4.7B). Remarkably, treatment in the *B-myb*^{Δ/Δ} ESCs resulted in a complete rescue of replication fork speed with median speed increasing from 0.89 (±0.11) to 1.44 (±0.01) kilobases per minute. This shift in distribution was statistically significant using a Mann Whitney U test producing a p-value less than 0.0001 (Figure 4.7C+D). Overall, this data suggests that inhibition of new origin firing is enough to rescue the replication speed defect observed in *B-myb*^{Δ/Δ} ESCs.

The frequency of new origin firing after Cdc7 inhibition was also investigated. Treatment with 10μM Cdc7 inhibitor in the *B-myb*^{+/+} ESCs resulted in a significant decrease in new origin firing from 22% to 16% (two-tailed unpaired t-test $P=0.0360$) (Figure 4.8B), confirming that the inhibitor was disrupting firing of new origins. Interestingly, the elevated origin firing observed in the *B-myb*^{Δ/Δ} was considerably decreased from 29% to 16%, to levels comparable to the *B-myb*^{+/+} after Cdc7 inhibition. This substantial rescue was determined to be statistically significant producing a p-value of 0.0088 using a two-tailed unpaired t-test (Figure 4.8B). To investigate further into the effect of Cdc7 inhibition of replication fork dynamics, the elongating

fork ratio was measured. In the B-*myb*^{+/+}, the average ratio was marginally decreased from 1.58 to 1.48 after Cdc7 inhibition (Mann Whitney U test, p=0.2936). Also, the percentage of forks with a ratio greater than 2 decreased from 16% to 11%. (Figure 4.8C). Interestingly, treatment with the Cdc7 inhibitor in the B-*myb*^{Δ/Δ} ESCs resulted in a complete rescue of the elevated elongating fork ratio, with the average ratio decreasing from 1.95 to 1.60 and the percentage of cells with a ratio above 2 decreasing from 35% to 14%. This substantial rescue was determined to be highly statistically significant producing a p-value less than 0.0001. Overall, direct inhibition of origin firing by Cdc7 inhibition in the B-*myb*^{Δ/Δ} ESCs results in a complete rescue of the replication progression defect and the observed increased fork instability.

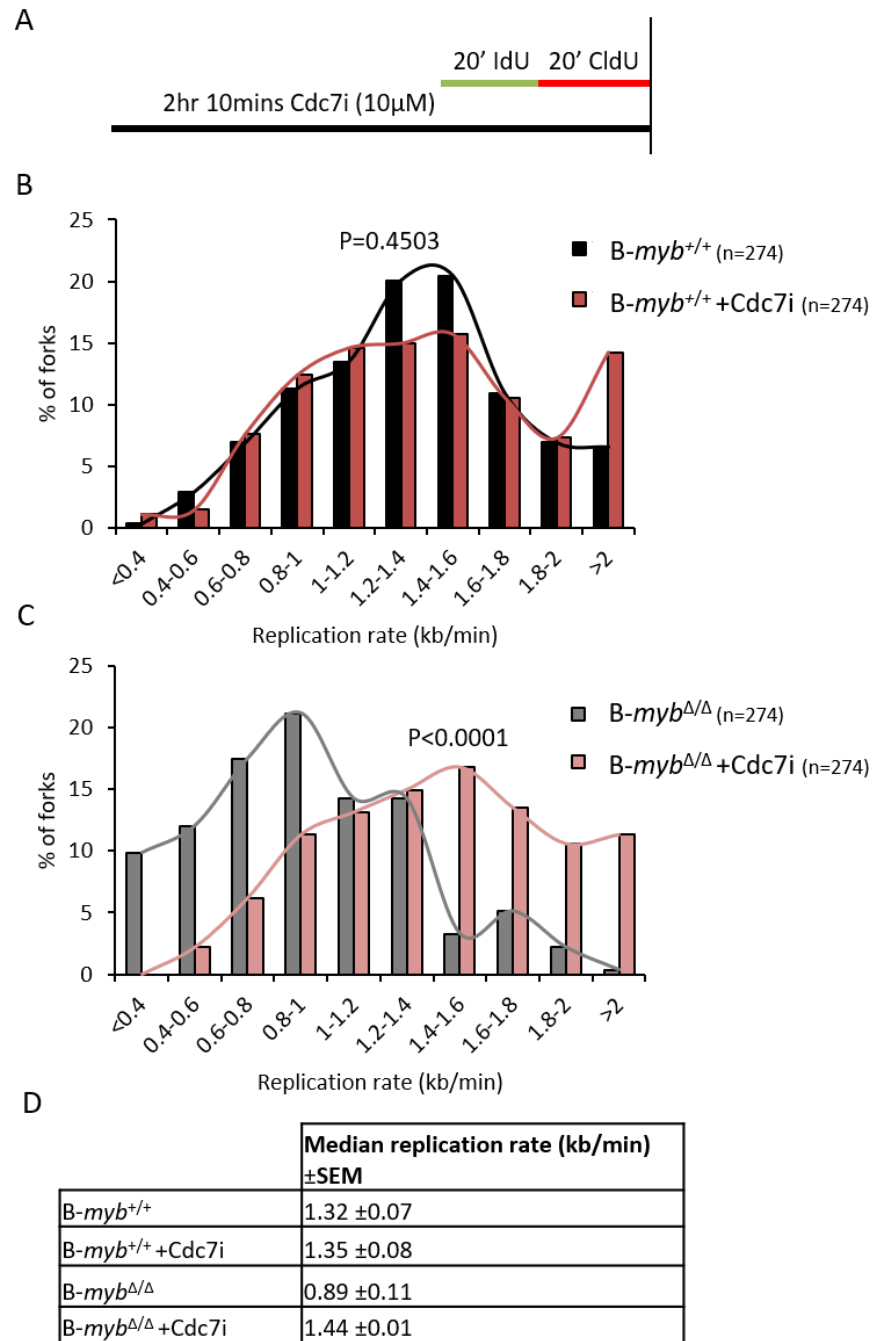


Figure 4.7 Inhibition of Cdc7 results in rescue of the replication speed defect observed in B-*myb*^{Δ/Δ} ESCs. A) ESCs were treated for 1.5 hours with 10µM PHA-767491, an inhibitor of Cdc7, before sequential addition of deoxynucleoside analogues IdU and CldU for 20 minutes each. DNA was treated and visualised as previously described. B) Distribution curve demonstrating the influence of Cdc7 inhibition upon B-*myb*^{+/+} ESC replication speed. C) Replication speed distribution curve for Cdc7 inhibition in B-*myb*^{Δ/Δ} ESCs. Statistical analysis of distributions was carried out using an unpaired Mann Whitney U test. D) Table showing median replication speed in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs untreated or treated with Cdc7i inhibitor. Data is representative of 6 slides from 3 independent experiments. Number of forks quantified for each of the genotypes and conditions are shown on the respective distribution curves.

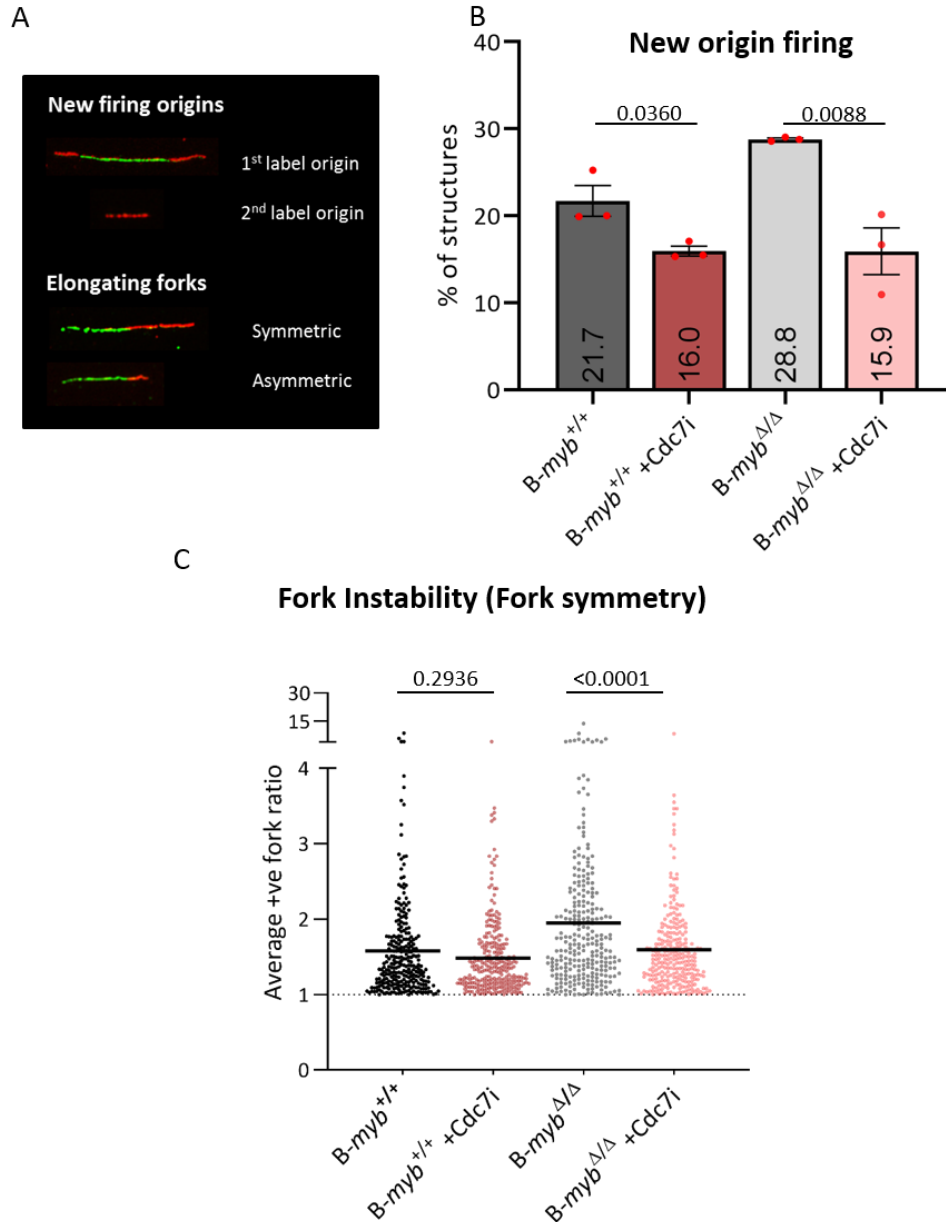


Figure 4.8 Inhibition of Cdc7 rescues elevated origin firing and fork instability in B-myb^{Δ/Δ} ESCs
 B-myb^{+/+} and B-myb^{Δ/Δ} ESCs were treated for 1.5 hours with 10μM Cdc7 inhibitor (PHA-767491) before performing DNA replication labelling. A) The staining patterns were used to analyse the behaviour of elongating forks and new firing origins B) Bar graph representing the percentage of new fired origins in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs untreated or treated with Cdc7 inhibitor. Error bars represent SEM. Statistical analysis was carried out using two-tailed unpaired t-tests. At least 500 replication structures were counted per treatment group from 3 separate repeats. C) The length of both incorporated labels for each elongating fork was measured in μm and the positive ratio was calculated. Dot plot demonstrating the average fork ratio in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs with or without Cdc7 inhibitor treatment. Average ratios were 1.58, 1.48, 1.95 and 1.60 in the order presented. Statistical analysis was carried out using the Mann Whitney U test. At least 270 ratios were calculated per treatment group from 3 experimental repeats.

4.2.5 The effect of CDK1 and Cdc7 inhibitor treatment upon the levels of DNA damage in the *B-myb*^{Δ/Δ} ESCs

It has been established that short-term inhibition of CDK1 and Cdc7 results in a partial and full rescue of the replication slowdown phenotype observed in the *B-myb*^{Δ/Δ} ESCs. Therefore, I sought to determine whether inhibition of these targets could also rescue the increased DNA damage observed in the *B-myb*^{Δ/Δ} ESCs. Cells were first treated for 2 hours with a CDK1 inhibitor (RO3306) before processing and performing immunofluorescence staining and imaging for detection of 53BP1 recruitment (Figure 4.9A). Acquired images were analysed by quantifying the percentage of cells with over 6 53BP1 foci. In the *B-myb*^{+/+} ESCs, CDK1 inhibition increased the level of 53BP1 from 2% up to 14% of cells with more than 6 53BP1 foci, moreover, this increased sensitivity was statistically significant producing a p-value of 0.0334 (two-tailed unpaired t-test). Inhibition of CDK1 in the *B-myb*^{Δ/Δ} ESCs resulted in a contrasting mild decrease in 53BP1 positivity from 23% to 20%, although, this difference was not statistically significant (P=0.6876) (Figure 4.9B).

Accumulation of 53BP1 was also measured after incubation with an inhibitor of Cdc7 (PHA-767491) for 2 hours. In this experiment, cells were also treated with EdU to analyse the accumulation of 53BP1 in replicating cells specifically (Figure 4.10A). In the *B-myb*^{+/+} ESCs, Cdc7 inhibition resulted in an increase in the percentage of 53BP1 positive cells from 15% to 31%. Interestingly, treatment with Cdc7 inhibitor in the *B-myb*^{Δ/Δ} ESCs had little effect on the already elevated percentage of positive cells remaining at 29% (Figure 4.10B). Overall, short-term inhibition of CDK1 and Cdc7 results in increased DNA damage in normal replicating ESCs, however, the same treatments appear to have no additional influence upon already elevated DNA damage in the *B-myb*^{Δ/Δ} ESCs.

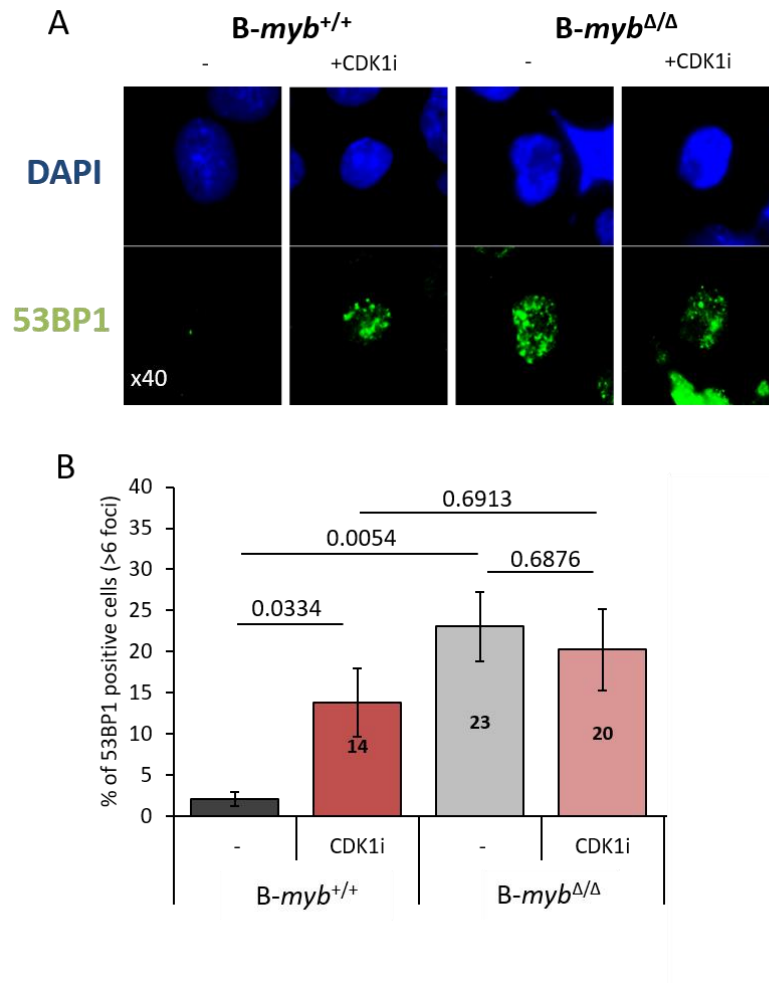


Figure 4.9 53BP1 recruitment in response to inhibition of CDK1 For a measure of double strand breaks, immunofluorescence staining for 53BP1 was performed. A) Representative images for B-*myb*^{+/+}, B-*myb*^{Δ/Δ} untreated and B-*myb*^{+/+} and B-*myb*^{Δ/Δ} plus 2 hours 10μM CDK1 inhibitor (RO3306) are shown. Staining for DAPI (blue) and 53BP1 (green). B) Bar graph of the percentage of cells with over 6 53BP1 foci in B-*myb*^{+/+}, B-*myb*^{Δ/Δ} untreated and B-*myb*^{+/+} and B-*myb*^{Δ/Δ} plus CDK1 inhibitor. Error bars represent SEM. Statistical analysis using two-tailed unpaired t-test. At least 200 nuclei were analysed from 4 experimental repeats.

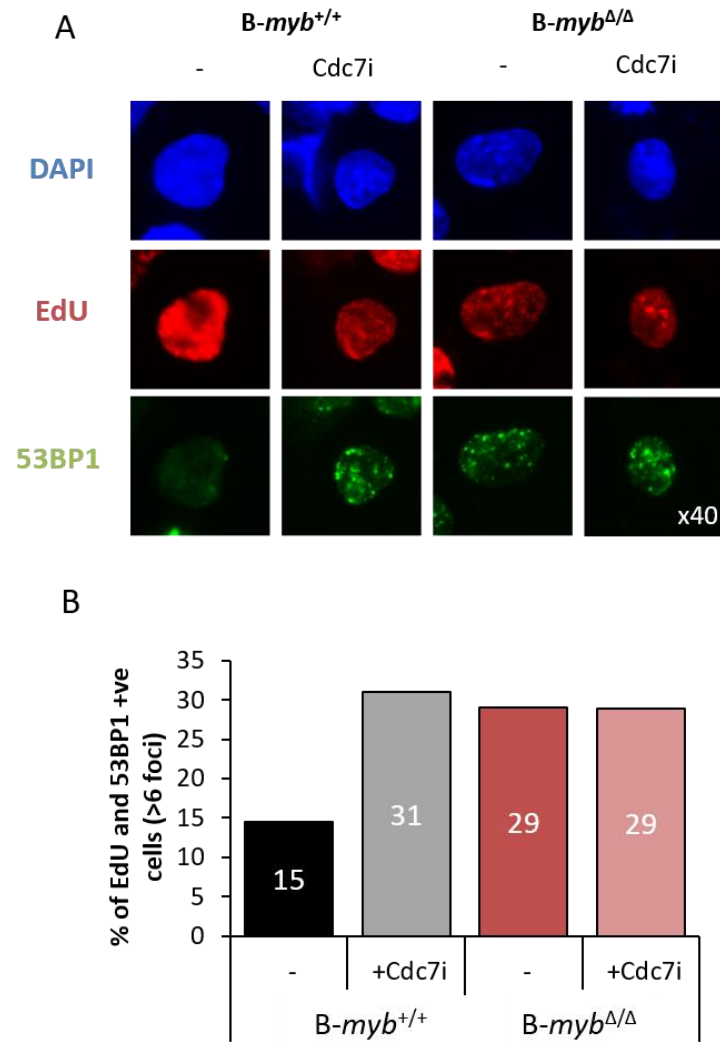


Figure 4.10 53BP1 recruitment in replicating cells in response to inhibition of Cdc7 A) To label replicating cells, 10μM EdU was incorporated for 2 hours in culture. After cell collection and fixation, incorporated EdU was detected using the ‘click’ reaction. For a measure of double strand breaks, immunofluorescence staining for 53BP1 was performed. Representative images for *B-myb*^{+/+}, *B-myb*^{Δ/Δ} untreated and *B-myb*^{+/+} and *B-myb*^{Δ/Δ} plus 2 hours 10μM Cdc7 inhibitor (PHA-767491) are shown. Staining for DAPI (blue), EdU (red) and 53BP1 (green). B) Bar graph of the percentage of EdU positive cells with over 6 53BP1 foci in *B-myb*^{+/+}, *B-myb*^{Δ/Δ} untreated and *B-myb*^{+/+} and *B-myb*^{Δ/Δ} plus Cdc7 inhibitor. At least 50 EdU positive nuclei were analysed from 1 experimental repeat.

4.3 Discussion

4.3.1 Deregulation of CDK1 activity as a mechanism for replication stress in *B-myb*^{Δ/Δ} ESCs

The activity of CDK1 is vital for control of replication initiation as a downstream target of the DDR (Anda et al., 2016, Petermann et al., 2010, Sorensen and Syljuasen, 2012, Syljuasen et al., 2005), plus, in mouse stem cells specifically, it was demonstrated that CDK1 is the only essential CDK for normal stem cell proliferation and behaviour (Santamaria et al., 2007). For these reasons, the activity of CDK1 in *B-myb*^{Δ/Δ} ESCs was investigated. Interestingly, the levels of CDK1 tyrosine 15 phosphorylation were significantly reduced in the *B-myb*^{Δ/Δ} ESCs compared to the *B-myb*^{+/+} ESCs. In addition, examining the levels of unmodified CDK1 protein revealed no change in the *B-myb*^{Δ/Δ} ESCs ruling out a potential role in CDK1 transcription. The reduction of this post translational modification provides some evidence that the activity of CDK1 is potentially elevated in the *B-myb*^{Δ/Δ} ESCs.

Due to the highly complex network of signalling involved in controlling CDK1 activity, it is difficult to determine the precise mechanism responsible for the silencing of CDK1 tyrosine 15 phosphorylation (Malumbres et al., 2009). CDK1 activity is in part governed by its phosphorylation of threonine 14 and tyrosine 15 by two kinases, MYT1 and WEE1 respectively (Parker and Piwnica-Worms, 1992, Malumbres, 2014). The activity of WEE1 in ESCs was confirmed in this study through chemical inhibition of WEE1 resulting in complete ablation of CDK1 tyrosine 15 phosphorylation. It was observed that WEE1 protein levels were constant in the control and *B-myb*^{Δ/Δ} ESCs, ruling out the possibility that loss of B-Myb was influencing WEE1 transcription. However, the data presented did not rule out a potential defect in WEE1 activity, as control of WEE1 is also multifaceted encompassing various post-translational modifications (Watanabe et al., 2005). The suppression of CDK1 by WEE1 is also balanced by the activity of CDC25 phosphatases (Roshak et al., 2000), highlighting another mechanism by

which CDK1 may be deregulated. Therefore, the mechanism for increased CDK1 activity observed in the B-*myb*^{Δ/Δ} ESCs could be a result of multiple different mechanisms and would require further investigation.

It has been demonstrated in cases where there is replication stress characterised by elevated origin firing, inhibition of CDK activity can result in a rescue of the stress (Beck et al., 2012, Petermann et al., 2010, Syljuasen et al., 2005). Therefore, cells were treated with a chemical inhibitor of CDK1 before examining its effect upon the replication stress in the B-*myb*^{Δ/Δ} ESCs. Inhibition of CDK1 resulted in a partial rescue of the replication stress indicated by an increase in replication speed and some reduction in the elevated origin firing. However, CDK1 inhibition failed to fully rescue the fork instability observed in the B-*myb*^{Δ/Δ}. Inhibition of CDK1 in the B-*myb*^{+/+} ESCs resulted in a contrasting decrease in replication rate accompanied by a mild increase in new origin firing and fork instability, therefore indicating an increase in replication stress. The sensitivity of the control cells to the CDK1 inhibitor could explain why the rescue was not complete in the B-*myb*^{Δ/Δ}. Moreover, it may be that the strong requirement for CDK1 signalling for other roles outside of origin firing, for example, its roles in transcription control (Lim and Kaldis, 2013, Santamaria et al., 2007), meant that its inhibition was highly toxic to replication.

Inhibition of WEE1 resulted in complete loss of P-CDK1 (tyr15) levels, therefore, it was of interest whether this treatment mimicked the replication stress observed in the B-*myb*^{Δ/Δ} ESCs, in which CDK1 also appears overactive. Inhibition of WEE1 resulted in a strong replication stress phenotype characterised by almost complete abrogation of replication progression, plus an increase in origin firing and fork instability in control ESC. This agrees with previous studies demonstrating that inhibition of WEE1 results in loss of replication progression due to CDK1 dependent changes in origin firing (Beck et al., 2012). Interestingly, in the B-*myb*^{Δ/Δ}, the replication stress observed after WEE1 inhibition was even more severe than seen in untreated

B-myb^{ΔΔ}. Moreover, WEE1 inhibition had an additive effect upon the replication defect, possibly reflecting the change from mild CDK1 deregulation in the *B-myb*^{ΔΔ} to complete loss of its control after WEE1 inhibition.

The findings presented provide support for a potential influence of CDK1 deregulation in the replication stress phenotype observed in the *B-myb*^{ΔΔ} ESCs, however, they do not reveal how B-Myb is interacting with CDK1. It was recently shown that B-Myb may be a downstream transcriptional target of CDK1. Moreover, inhibition of CDK1 abrogated FOXM1 phosphorylation, which is recruited to B-Myb to carry out its role in transcription (Saldivar et al., 2018, Down et al., 2012). Therefore, it is possible that the increased CDK1 activity observed in the *B-myb*^{ΔΔ} ESCs could be a reaction to loss of B-Myb transcriptional activity. It is clear from the western blot analysis that the *B-myb*^{ΔΔ} ESCs present some level of P-CDK1 (tyr15), therefore, the *B-myb*^{ΔΔ} ESCs are still able to activate CDK1 to some degree. Increases in the activity of CDKs is typical in cancer cells which to promote proliferation to increase acquisition of genetic changes (Malumbres et al., 2009, Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). Therefore, loss of B-Myb could function in a similar way, resulting in deregulation of pathways involved in normal cell cycle and proliferation control, explaining the elevated CDK1 activity observed.

4.3.2 Origin firing as a mechanism for replication stress in *B-myb*^{ΔΔ} ESCs

Although CDK1 inhibition resulted only in a partial rescue of the replication speed defect and the elevated origin firing in the *B-myb*^{ΔΔ}, it was also clear that the same treatment was toxic to normal wildtype ESCs. To look more closely at origin firing in this system, Cdc7, which plays a specific role in origin firing (Yeeles et al., 2015), was targeted through use of a chemical inhibitor. In wildtype ESCs, inhibition of Cdc7 resulted in an increase in abnormally long

replication tracks, likely reflecting the cell having to compensate for an inability to fire new origins, confirming the efficacy of this drug.

Remarkably, the B-*myb*^{Δ/Δ} presented a complete rescue of the replication progression defect after Cdc7 inhibition to the same level as the control, accompanied by a strong rescue of the increased origin firing. Moreover, this data strongly suggests that global changes in origin firing is a major contributing factor to the replication slowdown observed in the B-*myb*^{Δ/Δ} ESCs. In addition, this strengthens the idea that replication progression rate is equivalent to origin firing in ESCs, a relationship which has been described in the literature (Zhong et al., 2013). The B-*myb*^{Δ/Δ} also presented a highly significant rescue of the fork instability after Cdc7 inhibition, suggesting that the abnormal origin firing also causes the increased fork instability phenotype observed.

There are multiple different potential mechanisms for why increased origin firing causes problems with replication progression. Firstly, an abnormal increase in replication sites on chromatin has the potential to increase the chance of collisions with other processes taking place on chromatin such as transcription (Bermejo et al., 2012, Jones et al., 2013, Neelsen et al., 2013). Unscheduled increases in DNA processing also has the potential to result in unmanageable levels of torsional stress exacerbating the replication progression problems further (Bermejo et al., 2012). Depletion of replication factors, including the levels of nucleotides, is another outcome of increased origin firing. Moreover, supplementation with nucleotides has been shown to rescue replication stress caused by elevated origin firing after WEE1 inhibition (Beck et al., 2012). An interesting experiment would be to supplement the B-*myb*^{Δ/Δ} ESCs with nucleosides and to assess the influence on replication dynamics, to determine whether nucleotide depletion is a direct cause of the replication slowdown and fork instability observed.

4.3.3 Inhibition of origin firing fails to rescue DNA damage in B-*myb*^{Δ/Δ} ESCs

It was of interest whether the rescue of the replication progression defect after inhibition of origin firing was also reflected in the level of DNA damage. In response to CDK1 inhibition, there was an increase in 53BP1 positive cells demonstrating that just short-term loss of CDK1 activity is detrimental to genome stability in normal ESCs. Interestingly, although there was no rescue of 53BP1 in the B-*myb*^{Δ/Δ}, the levels of 53BP1 did not increase like in the B-*myb*^{+/+}. Despite being a more specific inhibitor of origin firing, the Cdc7 inhibitor also caused a substantial increase in 53BP1 recruitment in the B-*myb*^{+/+} after just 2 hours treatment. Moreover, this suggest that specific inhibition of origin firing is detrimental to genome stability. Similar to the CDK1 inhibition, the Cdc7 inhibitor did not increase 53BP1 levels in the B-*myb*^{Δ/Δ} (Figure 4.10).

One way to interpret this data is there could potentially be a decrease in the sensitivity of the B-*myb*^{Δ/Δ} to CDK1 and Cdc7 inhibition compared to the B-*myb*^{+/+}. However, this idea is challenged by the fact that in the DNA fibre analysis, through looking at new origin firing, the Cdc7 inhibitor results in a reduction in new origin firing to the same level in both the B-*myb*^{+/+} and the B-*myb*^{Δ/Δ} (Figure 4.8), therefore it would be expected to increase DNA damage in both.

A more likely explanation is that the data is limited by the analysis technique. Positivity for 53BP1 was determined by measuring the percentage of cells with greater than 6 foci. Moreover, the B-*myb*^{Δ/Δ} may have already reached a threshold of positive cells, and an increase in the number of damage sites beyond 6 would not be picked up using this technique. A better way to investigate whether inhibition of origin firing rescues the DNA damage phenotype would be to perform alkaline comet assays which would give a direct quantification of the total level of DNA damage which accumulates in the cell after the treatments. It may also be that the short treatment times are enough to rescue replication fork speeds but isn't enough time to rescue

global DNA damage. Therefore, longer treatment times with the inhibitors may produce more meaningful results.

4.3.4 Could changes in cell cycle regulation of replication explain CPT sensitivity?

In chapter 3, it was demonstrated that the B-*myb*^{Δ/Δ} ESCs present an increase in sensitivity to CPT in terms of DNA damage accumulation and cell viability. In this chapter, the B-*myb*^{Δ/Δ} ESCs were shown to present elevated CDK1 activity which could contribute to this sensitivity. CPT treatment resulted in a potential increase in phosphorylated CDK1 levels by western blot in the B-*myb*^{+/+} which may reflect activation of a checkpoint response. However, the levels of phosphorylated CDK1 in the B-*myb*^{Δ/Δ} remained low, potentially suggested that the B-*myb*^{Δ/Δ} lack the normal response to CPT. This is supported by their inability to activate the G2/M checkpoint in response to CPT treatment. Increased CPT sensitivity has been shown to result from inhibition of protein kinase C resulting partly from an influence upon CDK1 kinase activity and loss of checkpoint activation (Shao et al., 1997). This provides support for changes in cell cycle control, including CDK1 activity, as a potential explanation for the CPT sensitivity in the B-*myb*^{Δ/Δ} ESCs. It would be interesting to determine whether inhibition of CDK1 or Cdc7 could rescue the sensitivity of the B-*myb*^{Δ/Δ} ESCs to CPT treatment.

**Chapter 5 An epistatic relationship between B-Myb
activity and the DNA damage response for control of
replication progression in ESCs**

5.1 Introduction

The intra S phase checkpoint encompasses a system of signalling pathways, including the DDR pathways, which modulate DNA replication dynamics to ensure genome stability is maintained (Jones and Petermann, 2012). In the previous chapter, the replication progression defect observed in the *B-myb*^{ΔΔ} ESCs was demonstrated to arise from deregulation of origin firing control through an influence upon cell cycle associated signalling. Manipulation of origin firing is identified as an important target of the DDR to promote appropriate S phase progression (Beck et al., 2012, Petermann et al., 2010, Shechter et al., 2004, Syljuasen et al., 2005).

B-Myb activity has long been known vital for maintaining genome stability (Ahlbory et al., 2005, Garcia and Frampton, 2006, Lorvellec et al., 2010, Manak et al., 2007, Shepard et al., 2005), but more recent work has highlighted potential roles for B-Myb associated with DDR signalling (Bayley et al., 2018a, Henrich et al., 2017, Saldivar et al., 2018). A recent study demonstrated that B-Myb interacts directly with the MRN complex which is responsible for recruitment of ATM kinase. Moreover, phosphorylation of B-Myb was abrogated in response to ATM inhibition highlighting an interaction with ATM dependent DDR signalling specifically (Henrich et al., 2017). It was also shown that activation of FOXM1, a key player in B-Myb transcriptional activity, is governed by ATR dependent signalling (Saldivar et al., 2018). Therefore, in this chapter, I aimed to uncover whether the role for B-Myb in maintaining normal replication progression and genome stability in ESCs is dependent on signalling through the DDR.

5.2 Results

5.2.1 B-Myb plays no obvious role in activation of the ATR dependent signalling

It was predicted that B-Myb could be implicated in ATR dependent signalling due to the important roles for ATR-Chk1 activity in control of the replication programme (Petermann et al., 2010, Syljuasen et al., 2005). Whole cell protein was extracted from the *B-myb*^{+/+}, *B-myb*^{+/ Δ} (one copy of *B-myb*) and *B-myb* ^{Δ/Δ} ESCs before performing western blot analysis. Firstly, the levels of unmodified Chk1 protein were demonstrated to remain constant in the *B-myb*^{+/+}, *B-myb*^{+/ Δ} and *B-myb* ^{Δ/Δ} ESCs in unperturbed conditions, as well as after 5Gy ionizing irradiation and in the presence of ATR and ATM inhibitors (Figure 5.1C). In response to replication stress, Chk1 is recruited to chromatin by ATR and is activated by its phosphorylation at serine 345 (Liu et al., 2000b, Zhao and Piwnica-Worms, 2001). To investigate a potential defect in Chk1 activation in the *B-myb* ^{Δ/Δ} ESCs, the levels of P-Chk1 (ser345) were analysed. In unperturbed conditions, the level of P-Chk1 in the *B-myb* ^{Δ/Δ} was slightly increased compared to *B-myb*^{+/+} ESCs (Figure 5.1A). Cells were also treated for 4 hours with CPT as a positive control for replication stress and DNA damage. The level of P-Chk1 in both the *B-myb*^{+/+} and *B-myb* ^{Δ/Δ} ESCs was increased substantially suggesting there is no defect in activation of Chk1 in response to stress in the *B-myb* ^{Δ/Δ} ESCs (Figure 5.1A).

To further investigate the mechanism for Chk1 activation in ESCs, cells were treated with inhibitors of ATR (AZ20) and ATM (KU55933) for two hours before subjecting the cells to 5Gy ionizing radiation to induce DNA damage. Combined ATR and ATM inhibition resulted in complete abrogation of P-Chk1 levels in control and B-Myb mutant cell types (Figure 5.1B). As expected, treatment with the ATR inhibitor alone reduced the levels of P-Chk1 in all groups (Figure 5.1C). Interestingly, the treatment with the ATM inhibitor alone appeared to also slightly reduce the levels of P-Chk1 in the *B-myb*^{+/+} and *B-myb* ^{Δ/Δ} ESCs (Figure 5.1C).

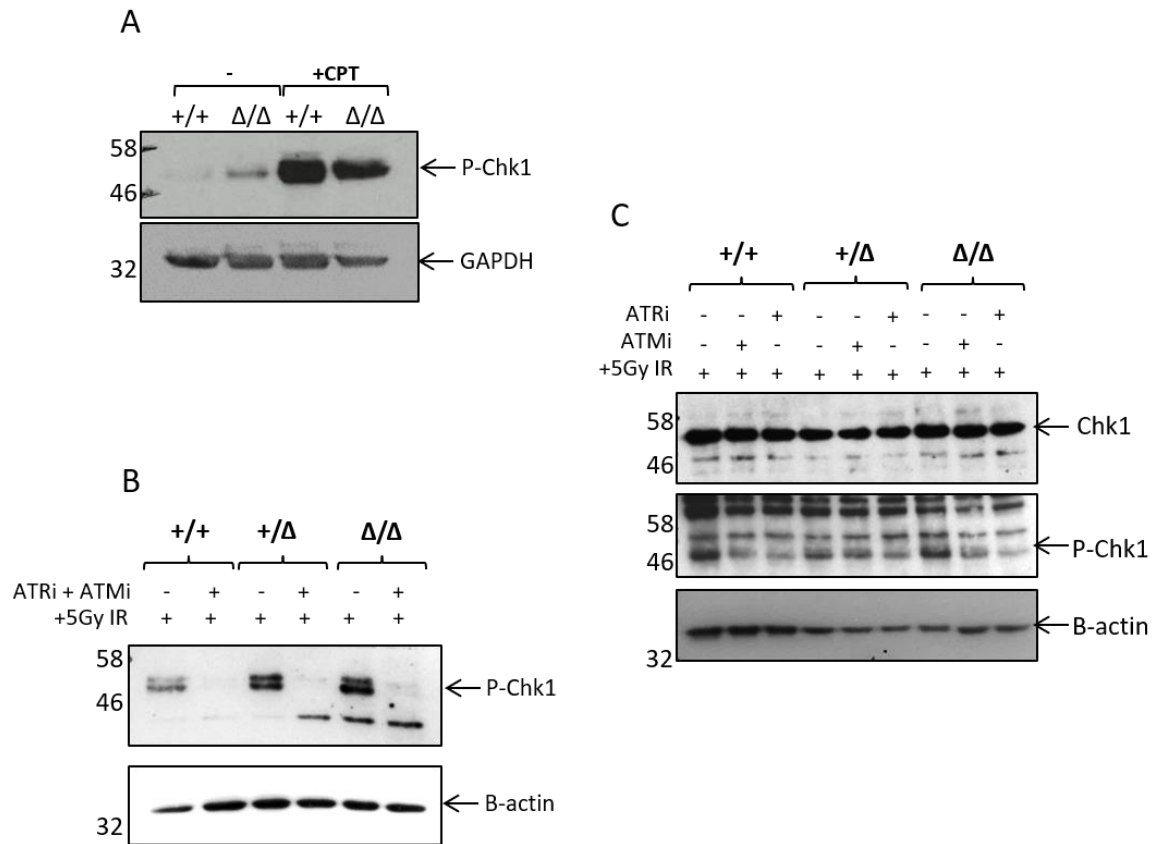


Figure 5.1 ATR-Chk1 activation after replication stress and DNA damage in *B-myb*^{+/+}, *B-myb*^{+/ Δ} and *B-myb* ^{Δ/Δ} ESCs A) Cells were untreated or treated with 2.5 μ M CPT for 4 hours before performing protein extraction and western blot analysis. Blot shows the levels of phosphorylated Chk1 (ser345) and GAPDH as a loading control in the *B-myb*^{+/+} and *B-myb* ^{Δ/Δ} ESCs with or without CPT treatment. B) Cells were treated with an inhibitor of ATR (AZ20, 5 μ M) and ATM (Ku55933, 10 μ M) in combination for 3 hours. Cells were also exposed to 5Gy irradiation before the final hour of inhibitor treatment. Blot shows levels of P-Chk1 (ser345) and β -actin in irradiated *B-myb*^{+/+}, *B-myb*^{+/ Δ} and *B-myb* ^{Δ/Δ} ESCs with or without inhibitor treatment. C) *B-myb*^{+/+}, *B-myb*^{+/ Δ} and *B-myb* ^{Δ/Δ} ESCs were treated with inhibitors of ATR (AZ20, 5 μ M) or ATM (Ku55933, 10 μ M) kinases for 2 hours before 5Gy irradiation followed by 1 hour further incubation. Immunoblotting for Chk1, P-Chk1 (ser345), and β -actin are depicted. In each blot, numbers represent the molecular weight (kDa) determined through a protein ladder.

5.2.2 Inhibition of ATR signalling results in severe replication dysfunction

The B-*myb*^{Δ/Δ} ESCs were able to phosphorylate Chk1 in unperturbed and CPT treated conditions, however, this did not rule out a potential role for B-Myb downstream of this pathway influencing replication. To address this, the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated with a chemical inhibitor of ATR (AZ20) for 1.5 hours before labelling active replication and performing DNA fibre analysis (Figure 5.2A). In both the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs, ATR inhibition resulted in a highly significant decrease in replication progression, producing p-values of less than 0.0001 using a Mann-Whitney U test (Figure 5.2B+C). In the B-*myb*^{+/+} ESCs, median replication speed was reduced to 0.44±0.00 from 1.29±0.02 kb/min and in the B-*myb*^{Δ/Δ} ESCs was reduced even further down to just 0.29±0.02 from 0.81±0.05 kb/min (Figure 5.2D), representing almost complete abrogation of replication progression.

In the B-*myb*^{+/+} ESCs, inhibition of ATR kinase also induced a substantial increase in new origin firing from 24% to 40%. In the B-*myb*^{Δ/Δ} ESCs, the already elevated origin firing was increased further from 33% to 40% (Figure 5.3B). Further analysis through a measure of replication fork ratio was also performed. The average ratio in the B-*myb*^{+/+} ESCs was increased from 1.42 to 2.67; moreover, the percentage of forks with a ratio above 2 was increased from 10% to 40%. This substantial increase in fork ratio was determined to be statistically significant using Mann Whitney U test producing a p-value of less than 0.0001 (Figure 5.3C). The average fork ratio in B-*myb*^{Δ/Δ} ESCs was only marginally increased from 1.76 to 1.92, much less than in the control. Statistical analysis revealed that the difference was not significant with a p-value of 0.0899 (Figure 5.3C). These findings demonstrate that inhibition of ATR is highly detrimental to replication progression and exacerbates further the replication stress observed in the B-*myb*^{Δ/Δ} ESCs.

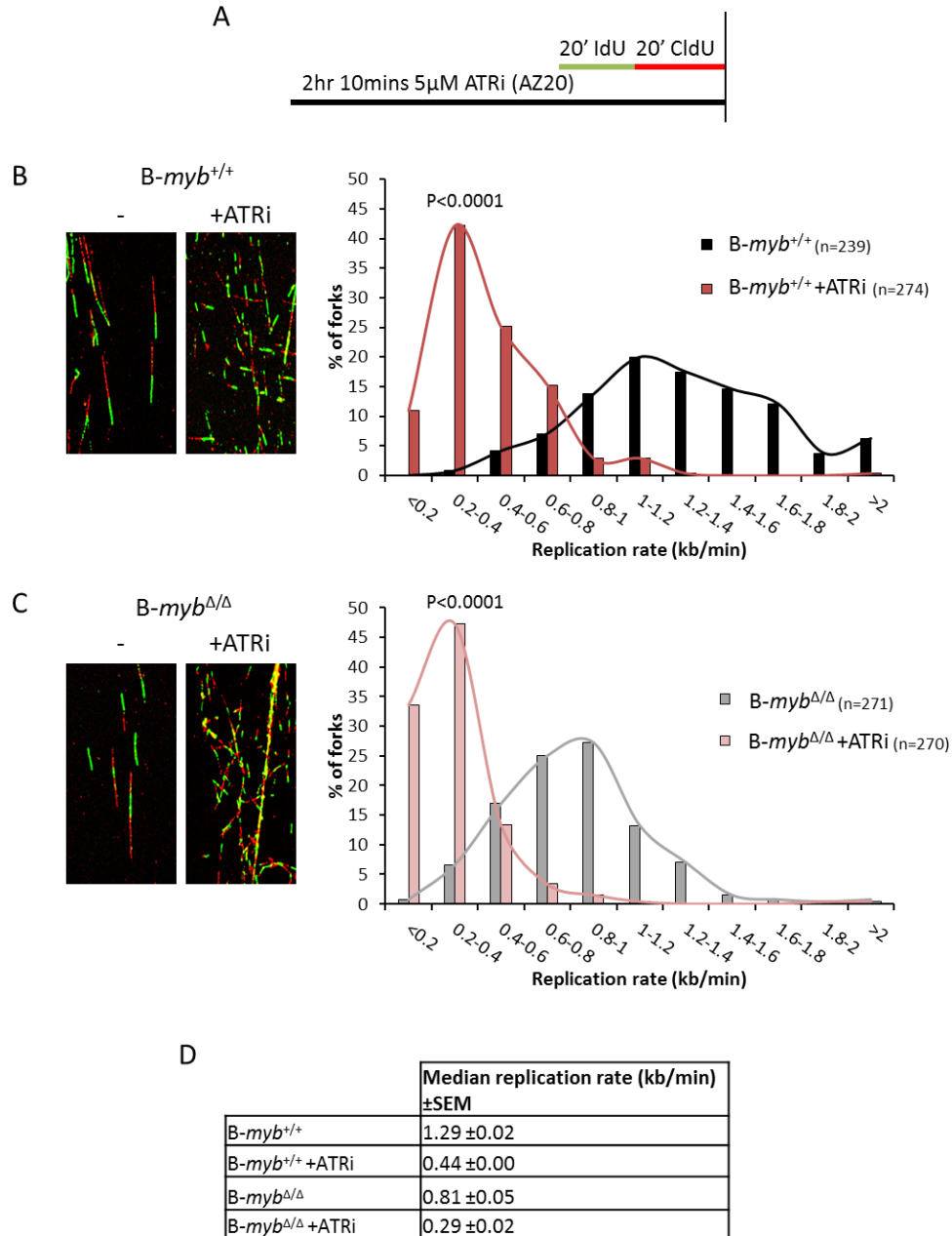


Figure 5.2 Inhibition of ATR kinase results in severe replication slowdown in *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs A) Cells were treated for 1.5 hours with 5μM AZ20, an inhibitor of ATR kinase, before sequential addition of deoxynucleoside analogues IdU and CldU for 20 minutes each. DNA was treated and visualised as previously described. B) Example images of fibres plus distribution curve representing the effect of ATR inhibition upon replication rate in *B-myb*^{+/+} ESC. C) Example images plus distribution curve showing the effect of ATR inhibition upon replication rate in *B-myb*^{Δ/Δ} ESCs. Statistical analysis of distributions was carried out using unpaired Mann Whitney U tests. D) Table showing median replication speed in *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs untreated or treated with ATR inhibitor. Data was acquired from 4 slides from 2 independent repeats. The number of forks quantified for each of the genotypes and conditions are shown on the respective distribution curves.

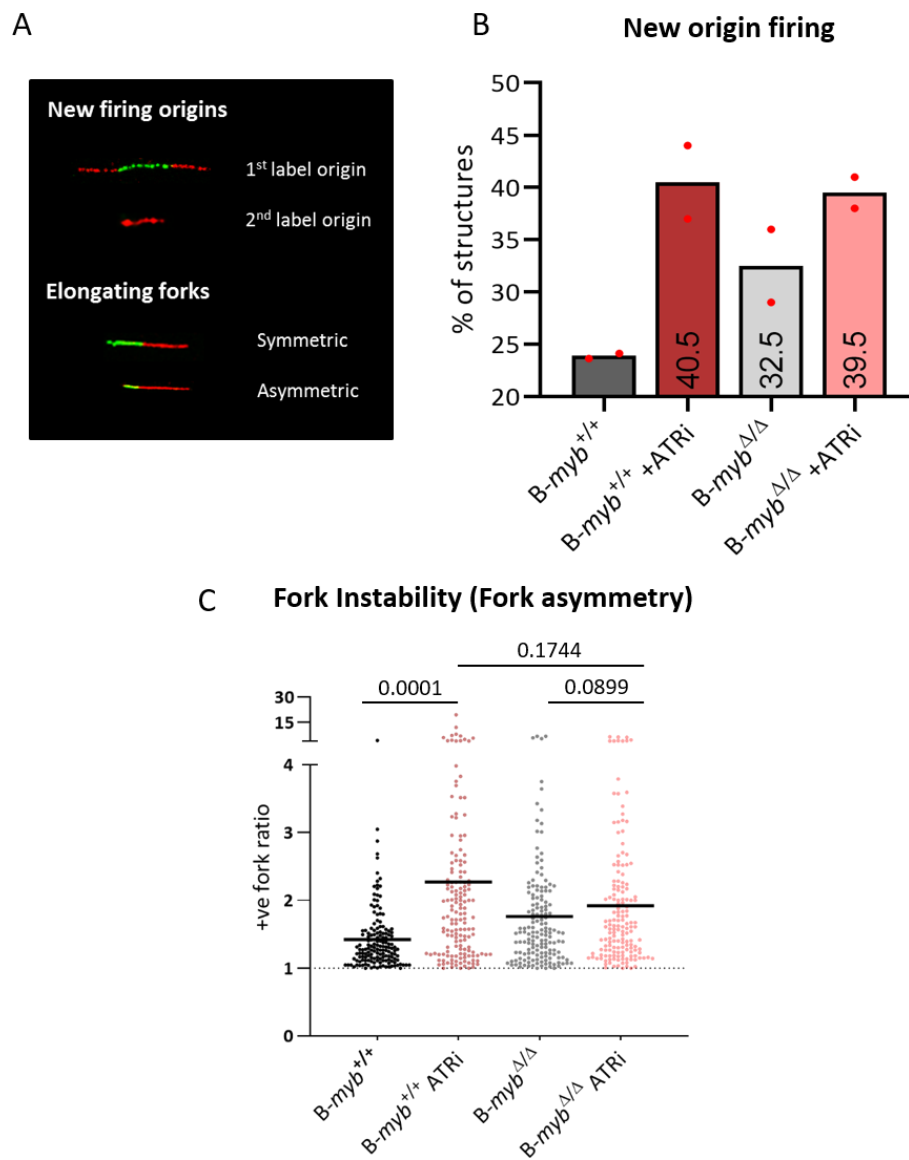


Figure 5.3 Inhibition of ATR kinase results in an increase in new origin firing and fork instability in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs Cells were treated with AZ20 and DNA fibres performed as described in Figure 5.2. A) The staining patterns were used to identify elongating forks (symmetric and non-symmetric) and newly fired origins as shown B) The percentage of new fired origins was quantified relative to total structures counted. Bar graph representing the percentage of new fired origins in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs with or without ATR inhibitor treatment At least 500 replication structures were counted per treatment group from 2 separate repeats. C) The length of both incorporated labels for each elongating fork was measured in μm and the positive ratio was calculated. Dot plot presents the distribution and the average fork ratio in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs with or without ATR inhibitor treatment. Average ratios were 1.42, 1.76, 2.27 and 1.92 in the order presented. Statistical analysis was carried out using the Mann Whitney U test. At least 150 ratios were calculated per treatment group from 2 independent repeats.

5.2.3 ATM kinase is epistatic to B-Myb for its replication function

B-Myb has been recently been implicated in ATM dependent activity (Bayley et al., 2018a, Henrich et al., 2017). For these reasons, the potential contribution of ATM dependent activity in the replication stress defect in the B-*myb*^{Δ/Δ} ESCs was investigated. The B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated with a chemical inhibitor of ATM (KU60019) for 1.5 hours before addition of IdU and CldU for 20 minutes each to label actively replicating DNA. DNA fibres were prepared and visualised as previously described (Figure 5.4A). Firstly, analysis of replication speed revealed that the B-*myb*^{+/+} ESCs were surprisingly sensitive to ATM inhibition, with the median replication rate presenting a statistically significant decrease from 1.33 ±0.07 to 0.88 ±0.08 kb/min (Mann Whitney U test, p<0.0001) (Figure 5.4B+D). The rate of replication after ATM inhibition in the B-*myb*^{+/+} ESCs (0.88 ±0.08 kb/min) was comparable to that observed in the untreated B-*myb*^{Δ/Δ} ESCs (0.86 ±0.12 kb/min). Interestingly, ATM inhibition in the B-*myb*^{Δ/Δ} ESCs had very little to no influence upon replication progression rate at 0.83 ±0.08 kb/min (Mann Whitney U test, P= 0.3068) (Figure 5.4C+D).

Next, replication fork structures were analysed to investigate the effect of ATM inhibition upon the replication programme further. Interestingly, the B-*myb*^{+/+} ESCs presented a significant increase in new origin firing after ATM inhibition from 18 to 29% (two-tailed unpaired t-test p=0.0178) up to levels comparable to that seen in the B-*myb*^{Δ/Δ} (28%) (Figure 5.5B). In the B-*myb*^{Δ/Δ} ESCs, the already increased origin firing was virtually unaffected after inhibition of ATM kinase, from 28% to 29% (two-tailed unpaired t-test p=0.6484) (Figure 5.5B). To examine potential changes in fork stability, a measure of elongating fork ratio was performed. The B-*myb*^{+/+} ESCs presented a statistically significant increase in average fork ratio from 1.47 to 1.86 after ATM inhibition, with the percentage of forks with a ratio greater than 2 increasing from 12% to 24% (Mann Whitney U, p= 0.0004) (Figure 5.5C). Similar to the result of the replication rate and frequency of origin firing, the already elevated fork ratio in the B-*myb*^{Δ/Δ}

ESCs was not increased further with the average ratio from 1.82 to 1.80 and the percentage of ratios greater than 2 remaining at 27% (Figure 5.5C). Overall, although ATM inhibition inflicted detrimental effects upon DNA replication in wildtype ESCs, the same treatment had no additional influence upon the pre-existing replication stress observed in the B-*myb*^{Δ/Δ} ESCs, highlighting a potential epistatic relationship between ATM and B-Myb activity.

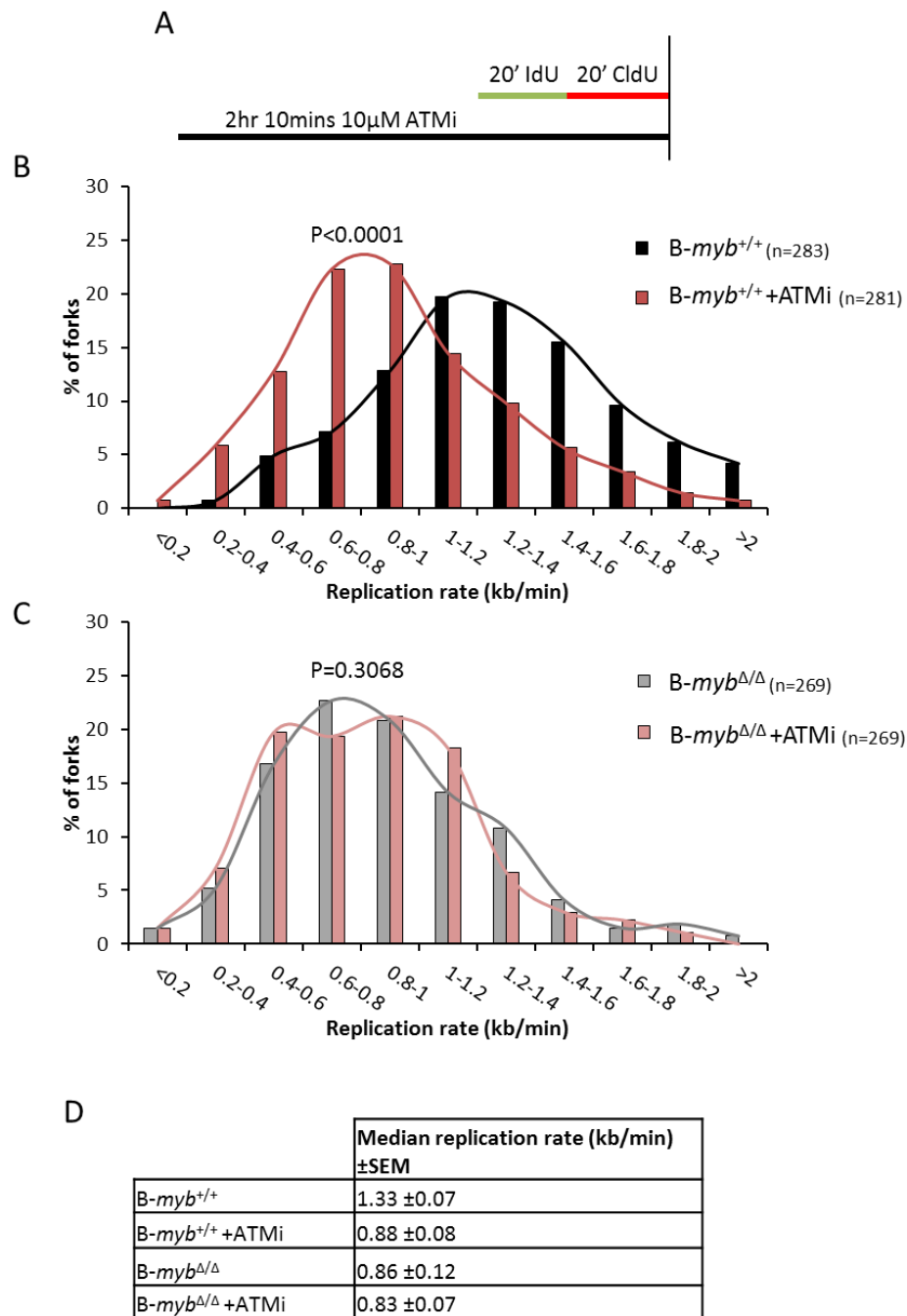


Figure 5.4 Inhibition of ATM kinase is epistatic to B-Myb loss for replication progression in ESCs

A) B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated for 1.5 hours with 10µM KU60019, an inhibitor of ATM kinase, before sequential addition of deoxynucleoside analogues IdU and CldU for 20 minutes each. DNA was treated and visualised as previously described. B) Distribution curve demonstrating the influence of ATM inhibition upon B-*myb*^{+/+} ESC replication speed. C) Distribution curve for ATM inhibition in B-*myb*^{Δ/Δ} ESCs. Statistical analysis of distributions was carried out using an unpaired Mann Whitney U test. D) Table showing median replication speed in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs with or without ATM inhibitor treatment. n=4. Number of forks counted for each condition is shown on the respective distribution curves.

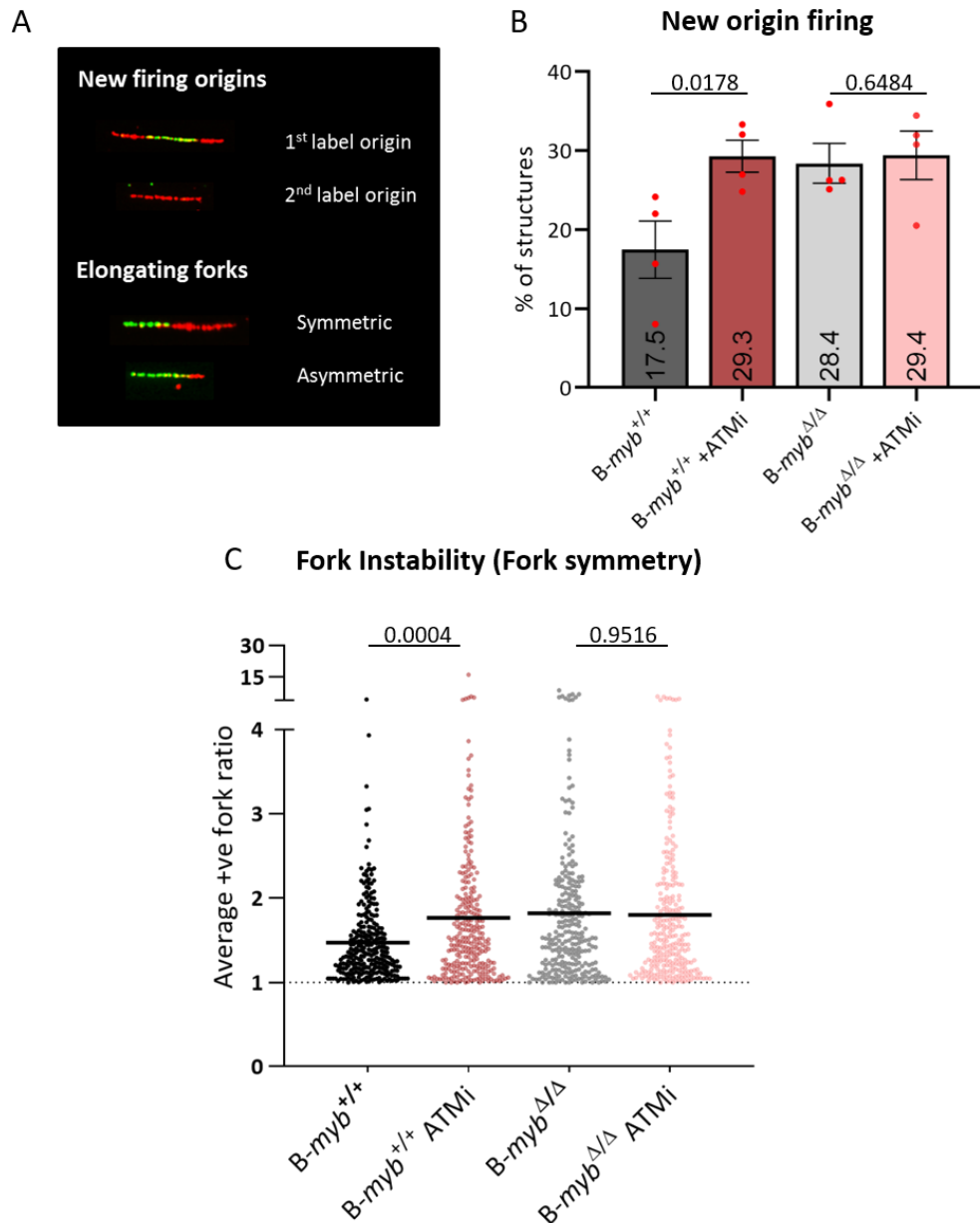


Figure 5.5 Inhibition of ATM kinase is epistatic to B-Myb loss for origin firing control and maintained fork stability B-myb^{+/+} and B-myb^{Δ/Δ} ESCs were treated for 1.5 hours with 10μM Ku60019, an inhibitor of ATM kinase, before sequential addition of deoxynucleoside analogues IdU and CldU. DNA fibres were prepared and visualised as previously described. A) The staining patterns were used to identify elongating forks (symmetric and non-symmetric) and newly fired origins as shown. B) The percentage of new fired origins was quantified relative to total structures counted. Bar graph representing the percentage of new fired origins in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs with or without ATM inhibitor treatment. Error bars represent SEM. Statistical analysis was carried out using unpaired t-test. At least 500 replication structures were counted per treatment group from 4 separate repeats. C) The length of both incorporated labels for each elongating fork was measured in μm and the positive ratio was calculated. Dot plot demonstrating the distribution and the average fork ratio in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs with or without ATM inhibitor treatment. Average ratios were 1.47, 1.86, 1.82 and 1.80 in the order presented. Statistical analysis was carried out using the Mann Whitney U test. At least 260 ratios were calculated per treatment group from 4 separate experiments.

It was demonstrated in chapter 3 that the *B-myb*^{Δ/Δ} present increased activation of replication factories. Therefore, due to the potential epistatic influence between B-Myb loss and ATM inhibition upon replication forks locally, it was of interest to determine whether ATM inhibition also mimicked the effect upon the distribution of replication factories. Cells were treated with an inhibitor of ATM (KU60019, 10μM) for 2 hours and 20μM IdU was added to the cultures for the final 30 minutes of treatment. After fixation and permeabilization, DNA was denatured before immunofluorescence detection of the incorporated label. Nuclei in early replication, determined by their staining pattern, were imaged for analysis (Figure 5.6A). Three-dimensional image stacks (z-stacks) were acquired using confocal microscopy before conversion to three dimensional rendered images for automatic foci counting using Imaris (Figure 5.6B).

As shown in Figure 3.7, the *B-myb*^{Δ/Δ} present a significant increase in the number of replication factories in early replication cells with an average of 699 factories compared to just 399 in the *B-myb*^{+/+} ESCs. Treatment with the ATM inhibitor also resulted in a significant increase in replication factories compared to the *B-myb*^{+/+} ESCs (Mann Whitney U test, $p < 0.0001$), however, the average number of factories (567 factories) was considerably lower than seen in the *B-myb*^{Δ/Δ} ESCs. The difference between the *B-myb*^{Δ/Δ} untreated and the *B-myb*^{+/+} after ATM inhibition was also statistically significant (Mann Whitney U test, $p = 0.0017$) (Figure 5.6C). Therefore, short-term ATM inhibition results in a substantial increase in replication factories in early replicating ESCs, but the levels are not comparable to that seen in the *B-myb*^{Δ/Δ} ESCs.

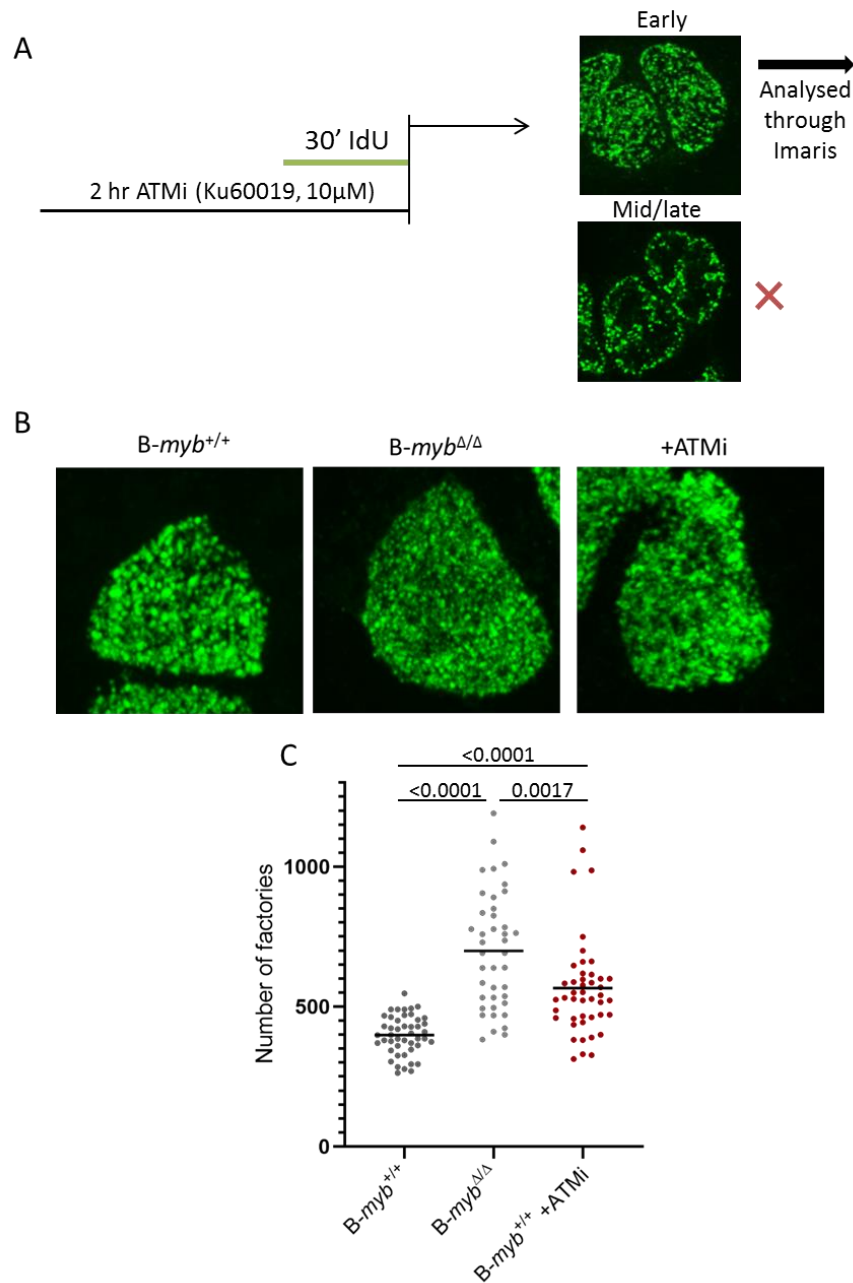


Figure 5.6 Increased replication factories in early replicating cells after inhibition of ATM A) The *B-myb*^{+/+} were untreated or treated with an inhibitor of ATM (KU60019, 10µM) for 2 hours. The *B-myb*^{+/+}, with or without ATM inhibition, plus the *B-myb*^{Δ/Δ} ESCs were then treated for 30 minutes with 20µM IdU (during the final 30 minutes of ATM inhibition). Early replicating cells were imaged to generate z-stacks using confocal microscopy. B) Example images of maximum intensity projections acquired from collating the z-stack images together in the *B-myb*^{+/+}, with or without ATM inhibition, plus the *B-myb*^{Δ/Δ} ESCs. C) Using ‘Imaris’ the z-stacks were converted to 3D rendered images before adopting an automatic foci counting tool. Dot plot demonstrating the average number of replication factories, average values were 399 and 699 and 567 in the order presented in the graph. At least 40 nuclei were analysed per group. Statistical analysis was performed using the Mann Whitney U test. The same data for the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} was also presented in Figure 3.7, n=2.

5.2.4 Is the replication stress phenotype in B-*myb*^{ΔΔ} ESCs due to a defect in MRE11 dependent end-resection?

Recently, it was demonstrated that a slowdown of DNA replication can be caused by MRE11 dependent nucleolytic processing, moreover, inhibition of MRE11 resulted in a rescue of this phenotype (Schmid et al., 2018). Therefore, it was of interest whether the replication rate defect observed in the B-*myb*^{ΔΔ} ESCs could be attributed to a similar mechanism. To test this, mirin, an inhibitor of the 3' to 5' exonuclease activity associated with Mre11 (Dupre et al., 2008), was utilised. The B-*myb*^{+/+} and B-*myb*^{ΔΔ} ESCs were treated with or without Mirin for 1.5 hours before treatment with IdU and CldU to label nascent DNA synthesis. DNA fibre analysis was performed, and fibres were visualised as previously described (Figure 5.7A). Firstly, the effect of mirin upon replication rate was analysed. In the B-*myb*^{+/+} ESCs, there was a substantial decrease in replication rate with median rate decreased from 1.19 ±0.09 to 0.79 ±0.15 kb/min (Figure 5.7B+D). Statistical analysis using the Mann Whitney U test revealed that the decrease was highly significant producing a p-value less than 0.0001. Interestingly, treating the B-*myb*^{ΔΔ} ESCs with mirin resulted in little effect upon the already reduced replication rate, with the median rate from 0.69 ±0.10 to 0.72 ±0.08 kb/min (Figure 5.7C+D). Statistical analysis identified this difference as not statically significant producing a p-value of 0.8868.

Further analysis of replication dynamics revealed a similar phenotype in terms of the frequency of replication initiation. New origin firing was increased after mirin treatment from 21% to 32% in the B-*myb*^{+/+} ESCs to similar levels as that seen in the untreated B-*myb*^{ΔΔ} ESCs (two-tailed unpaired t-test, p=0.0280) (Figure 5.8B). However, mirin treatment induced little to no change in origin firing in the B-*myb*^{ΔΔ} ESCs from 30% to 31%, producing a non-significant p-value of 0.6028 (Figure 5.8B). The B-*myb*^{+/+} ESCs also displayed an increase in fork instability represented by an increase in elongating fork ratio from 1.55 to 2.14 (Mann Whitney U, p<0.0001). In addition, the percentage of ratios greater than 2 increased substantially from 12%

to 38% (Figure 5.8C). Treatment with mirin in the B-*myb*^{ΔΔ} ESCs increased the pre-existing fork instability with fork ratio increasing from 1.97 to 2.19 (Mann Whitney U, p=0.0130) (Figure 5.8C). Interestingly, the effect of mirin treatment in the control ESCs appeared worse than the effect of B-Myb loss for provoking fork instability. This data suggests that inhibition of MRE11 activity is highly detrimental to replication in ESCs, and that mirin treatment does not rescue the replication stress observed after loss of B-Myb.

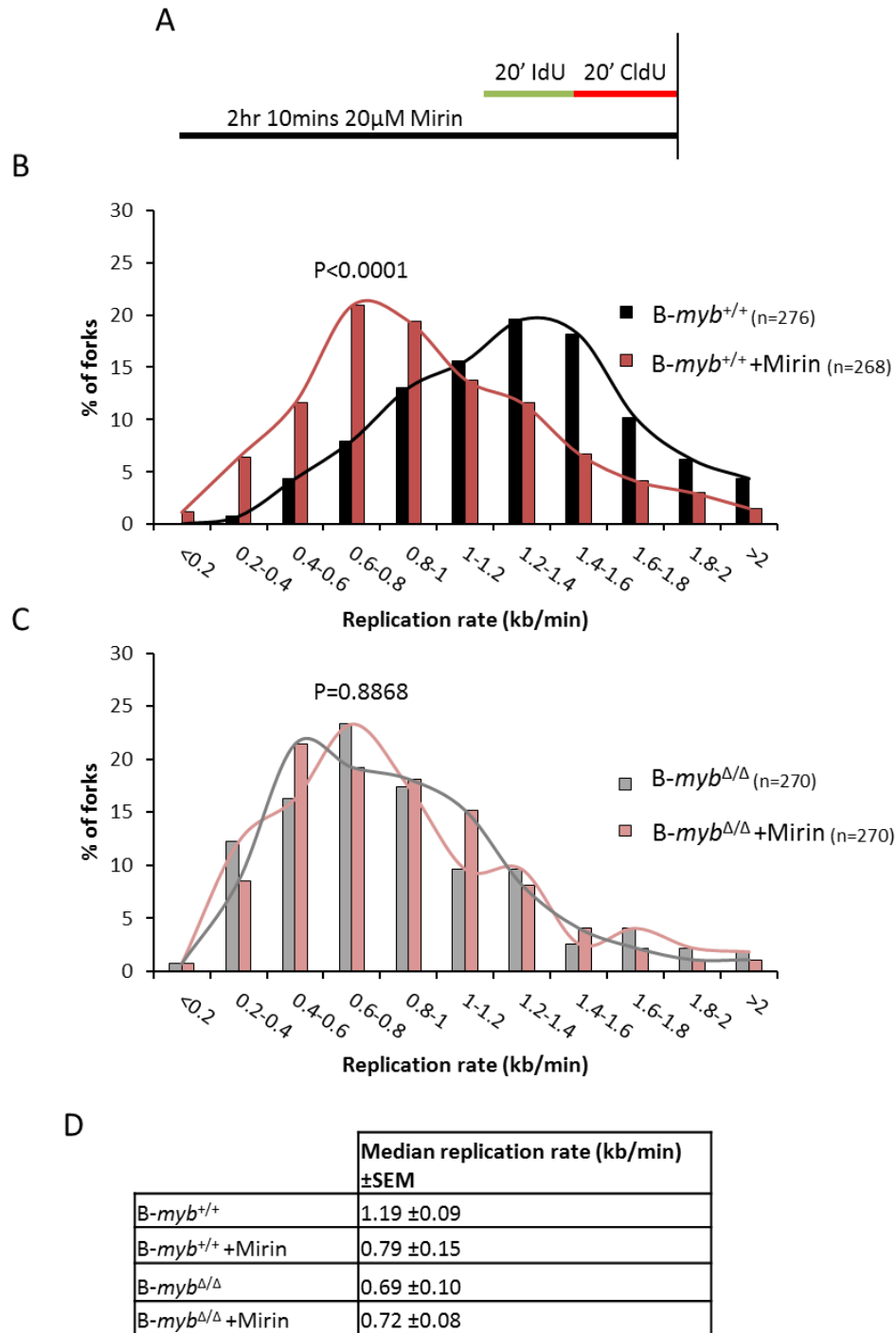


Figure 5.7 Inhibition of MRE11 is epistatic to B-Myb loss for replication progression in ESCs A) B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated for 1.5 hours with 20 μ M mirin, an inhibitor of MRE11, before sequential addition of deoxynucleoside analogues IdU and CldU. DNA was treated and visualised as previously described. B) Distribution curve demonstrating the influence of mirin treatment upon B-*myb*^{+/+} ESC replication speed. C) Distribution curve for ATM inhibition in B-*myb*^{Δ/Δ} ESCs. Statistical analysis of distributions was carried out using an unpaired Mann Whitney U test. D) Table showing median replication speed in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs with or without mirin treatment. n=3. Number of forks measured for each condition is shown on the respective distribution curves.

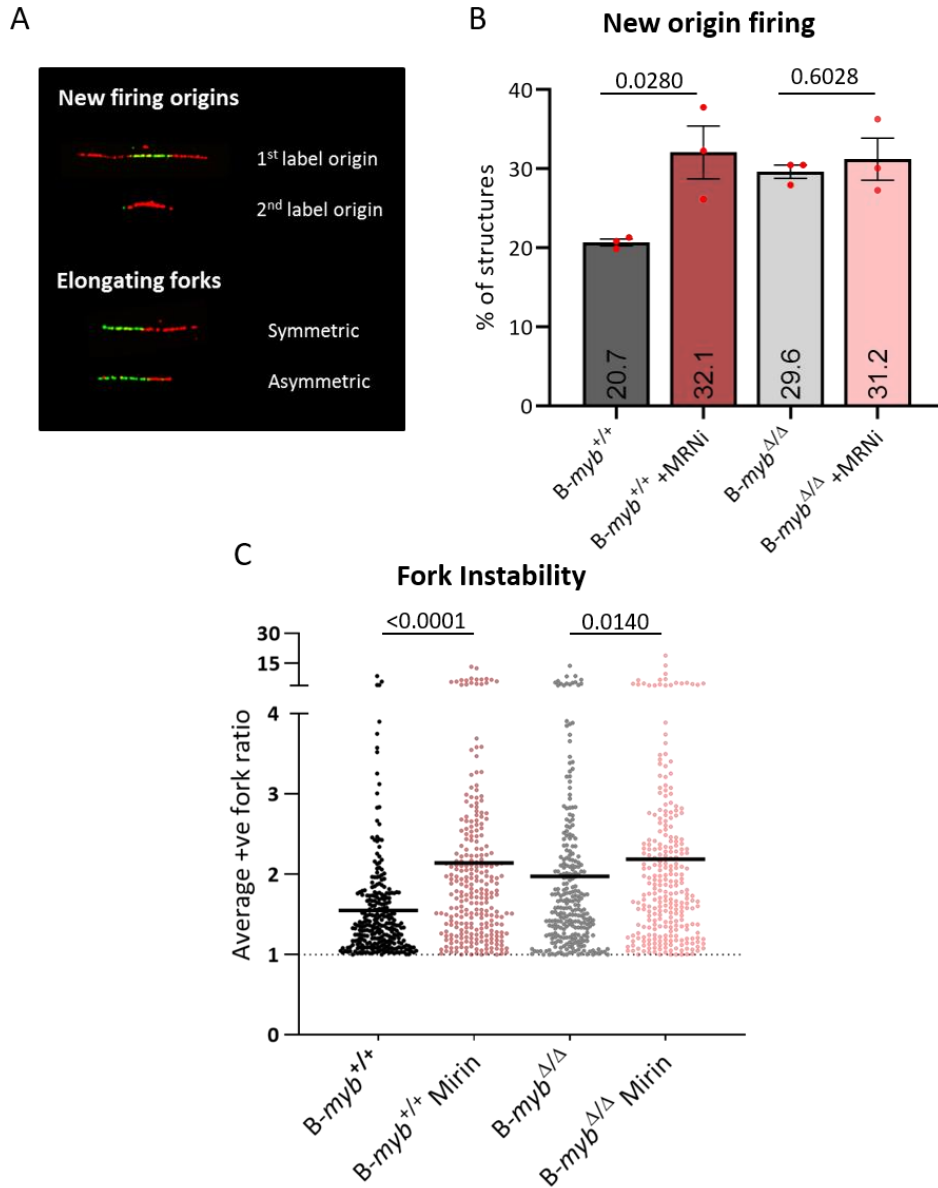


Figure 5.8 Inhibition of MRE11 activity is epistatic to B-Myb loss for origin firing control but has additional detrimental influence upon fork stability B-myb^{+/+} and B-myb^{Δ/Δ} ESCs were treated for 1.5 hours with 20μM mirin, an inhibitor of MRE11, before sequential addition of IdU and CldU for 20 minutes each. A) The staining patterns were used to identify elongating forks (symmetric and non-symmetric) and newly fired origins as shown B) The percentage of new fired origins was quantified relative to total structures. Bar graph representing the average percentage of new fired origins in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs with or without mirin treatment. Error bars represent SEM. Statistical analysis using two-tailed unpaired t-test. At least 450 replication structures were counted per treatment group from 3 separate repeats. C) The length of both incorporated labels for each elongating fork was measured in μm and the positive ratio was calculated. Dot plot demonstrating the average fork ratio in B-myb^{+/+} and B-myb^{Δ/Δ} ESCs with or without mirin treatment. Average ratios were 1.55, 2.14, 1.97 and 2.19 in the order presented. Statistical analysis was carried out using the Mann Whitney U test. At least 260 ratios were calculated per treatment group from 3 separate experiments.

5.2.5 Is inhibition of ATM activity epistatic with B-Myb loss for DNA damage accumulation?

B-Myb loss is shown to result in a replication defect which is accompanied by an increase in DNA damage. Therefore, it was of interest to determine whether the DNA damage in the B-*myb*^{Δ/Δ} ESCs is mimicked by ATM inhibition in ESCs. To test this, B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated with an ATM inhibitor (KU60019) for 2 hours before performing immunofluorescence staining for 53BP1. Cells were treated with 10μM EdU allowing the detection of 53BP1 in replicating cells specifically. In addition, treatment with a replication stress inducing agent, CPT, was used as a positive control for damage (Figure 5.9A). Inhibition of ATM in the B-*myb*^{+/+} ESCs resulted in an increase in 53BP1 recruitment in EdU positive cells with the percentage of cells with more than 6 foci increasing from 11% to 28% (two-tailed unpaired t-test, P=0.0036); although, this level of damage was not as severe as the 46% positive for 53BP1 observed after treatment with CPT (P=0.0081) (Figure 5.9B+C). Interestingly, ATM inhibition in the B-*myb*^{Δ/Δ} had little additional effect upon the already elevated 53BP1 recruitment (28% to 27%, P=0.7678) (Figure 5.9B+C).

An alkaline comet assay was also performed to examine the total level of DNA damage induced through ATM inhibition. Cells were treated for 2 hours with an ATM inhibitor (10μM) or with CPT (10μM) as a positive control for damage. Cells were harvested before performing single cell gel electrophoresis (Figure 5.10A). Firstly, treatment with CPT resulted in a substantial increase in the average OTM from 1.08 to 5.83, confirming that the assay was successful (Figure 5.10B). Treatment with the ATM inhibitor resulted in an increase in average OTM from 1.08 to 2.66 in the B-*myb*^{+/+} ESCs, whereas, the B-*myb*^{Δ/Δ} ESCs presented a slight decrease of the elevated OTM after ATM inhibition from 2.67 to 2.37 (Figure 5.10B). Overall, these findings support that ATM inhibition is epistatic with B-Myb loss in the formation of DNA damage during DNA replication in ESCs.

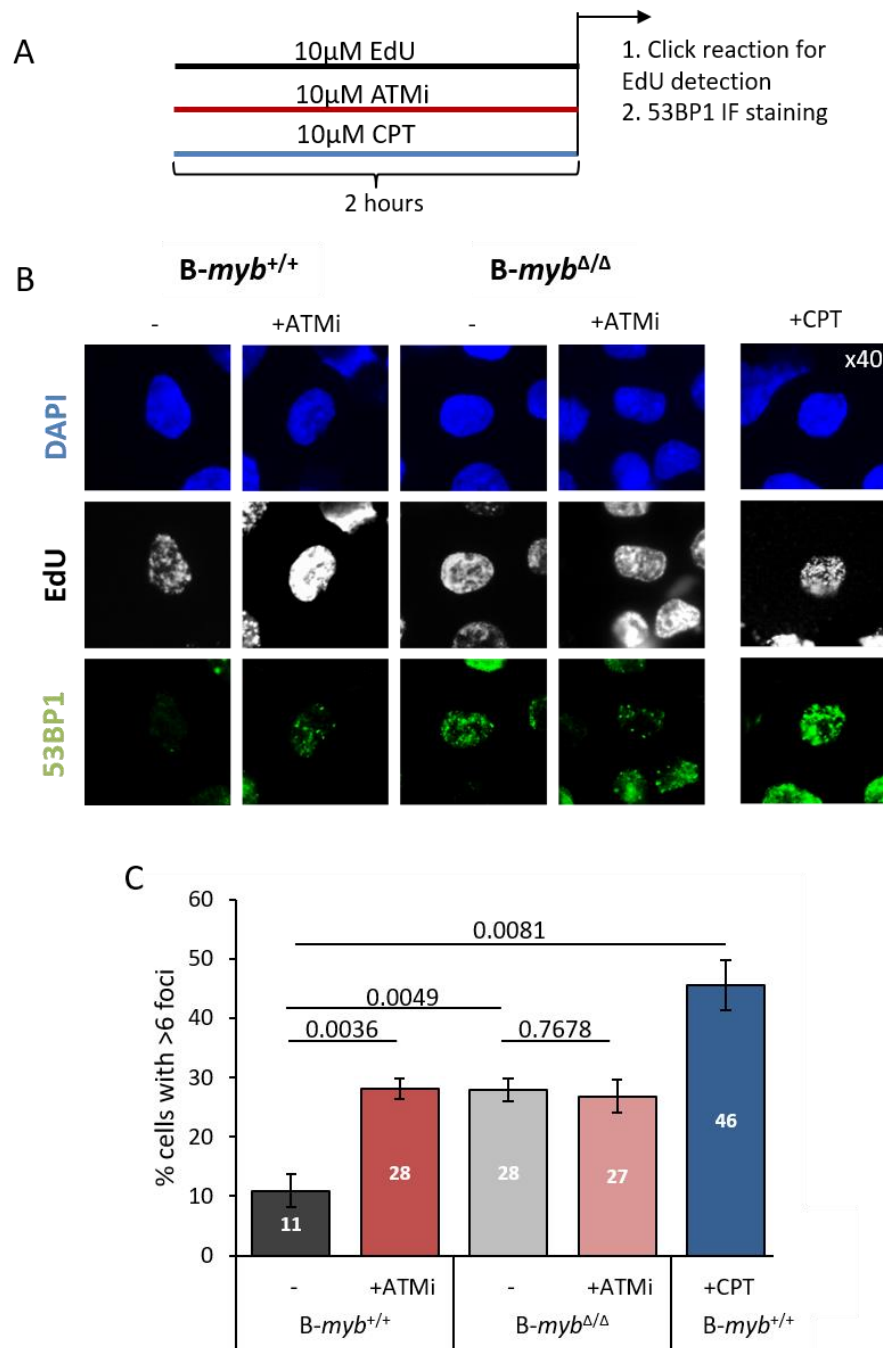


Figure 5.9 Epistasis between ATM inhibition and B-Myb loss in 53BP1 recruitment in ESCs A) B-myb^{+/+} and B-myb^{Δ/Δ} ESCs were treated with or without an inhibitor of ATM (KU60019) for two hours before harvesting. Cells were also treated with EdU to enable labelling of replicating cells and CPT treatment was used as a positive control for stress. B) Immunofluorescent labelling of DNA (blue), EdU (red) and 53BP1 (green) taken at X40 magnification. C) Bar graph presenting the effect of ATM inhibition or CPT treatment upon 53BP1 recruitment in replicating (EdU positive) B-myb^{+/+} and B-myb^{Δ/Δ} ESCs. Cells with over 6 53BP1 foci were considered positive for 53BP1. At least 150 cells were counted per group from 3 separate repeats. Error bars represent SEM. Statistical analysis using two-tailed unpaired student t-test.

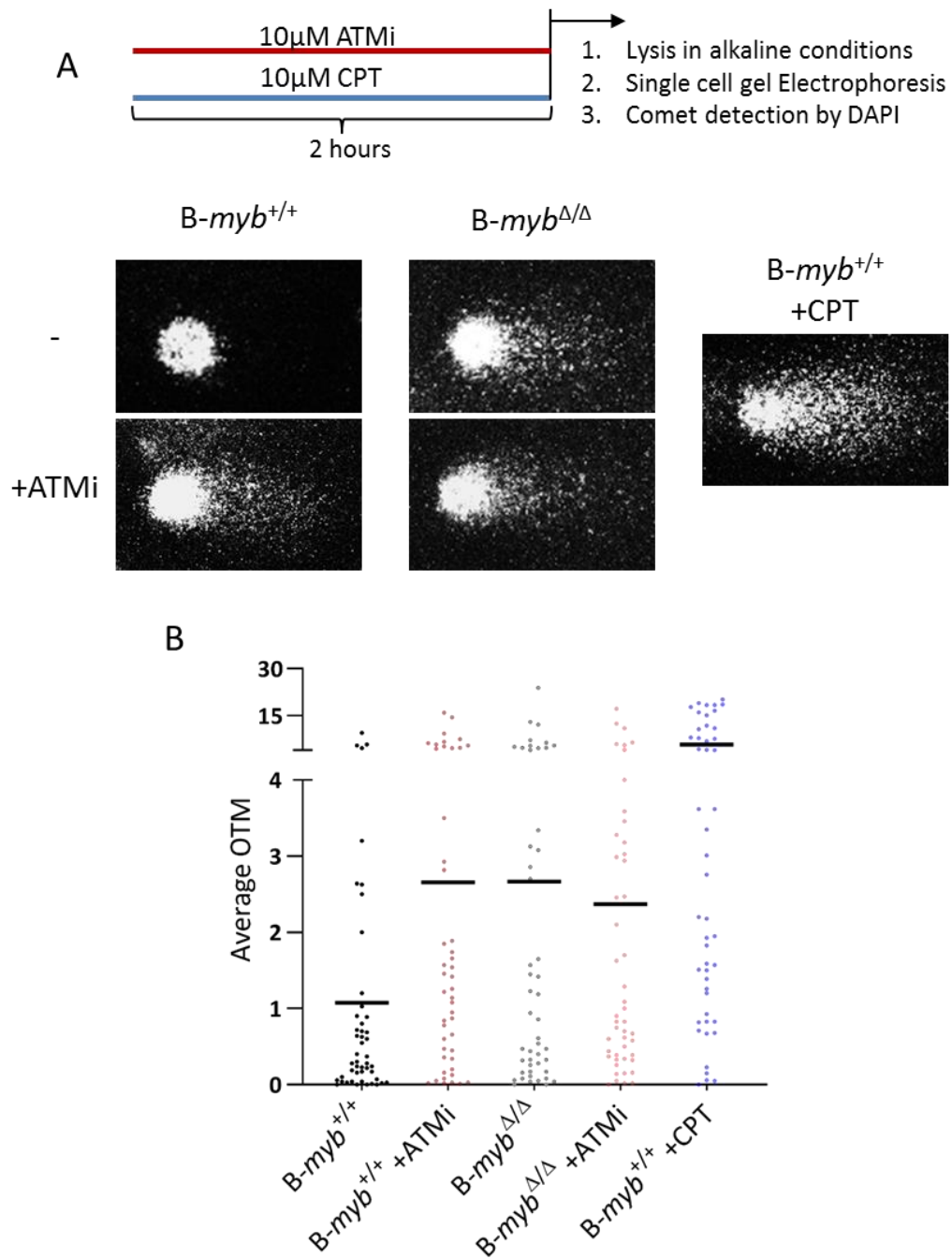


Figure 5.10 Epistasis between ATM inhibition and B-Myb loss in DNA damage accumulation in ESCs A) B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs were treated with or without an inhibitor of ATM (KU60019) for two hours, CPT treatment was used as a positive control for stress. Alkaline comet assay was performed as previously described and comets were visualised using DAPI staining. Shown are example comets from the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs untreated or treated with ATM inhibitor or CPT B) CASP analysis was used to measure the average olive tail moment (OTM). Dot plot presents the average OTMs in the B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs untreated or treated with ATM inhibitor or CPT. Average OTMs were 1.08, 2.66, 2.67, 2.37 and 5.83 in the order presented in the graph. 48 comets were analysed for each group from 1 experimental repeat.

5.2.6 Does loss of B-Myb influence ATM dependent processing of DNA damage?

KRAB-associated protein 1 (KAP1) is associated with signalling the repair of DNA damage in heterochromatin regions; moreover, its activation is induced through ATM dependent serine 824 phosphorylation (Goodarzi et al., 2008, Noon et al., 2010). Due to the epistatic relationship observed between ATM and B-Myb activity, it is possible that an inability to activate KAP1 could be a potential mechanism for the accumulating DNA damage observed in the *B-myb*^{Δ/Δ} ESCs. To test this, *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs were treated with or without 10μM CPT for 30 minutes or 4 hours and in combination with 10μM ATM inhibitor, before processing and performing immunofluorescence staining for P-KAP1 (Ser824) (Figure 5.11A).

In unperturbed conditions, both the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs presented almost undetectable P-KAP1 staining. However, 30 minutes of CPT treatment significantly increased the levels of P-KAP1 to 65.5% in the *B-myb*^{+/+} ESCs (two-tailed unpaired t-test, $p=0.0050$) (Figure 5.11B). CPT treatment also increased the levels of P-KAP1 positive cells up to 62.2% in the *B-myb*^{Δ/Δ} ESCs, although, there was no statistically significant difference in the response to CPT treatment between the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs ($p=0.5741$) (Figure 5.11B). A slight increase in P-KAP1 activation was observed after 4 hours of CPT treatment with 76.9% and 68% of cells positive for P-KAP1 in the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs, respectively. Again, there was no statistically significant difference in the response to this treatment between the two groups ($P=0.6242$) (Figure 5.11B). As expected, treatment with an inhibitor of ATM (KU60019) in combination with CPT resulted in a significant reduction in P-KAP1 ($P=0.0493$) (Figure 5.11B). Overall, this data suggests that the increased damage after loss of B-Myb is not likely attributed to a defect in the recruitment and activation of KAP1.

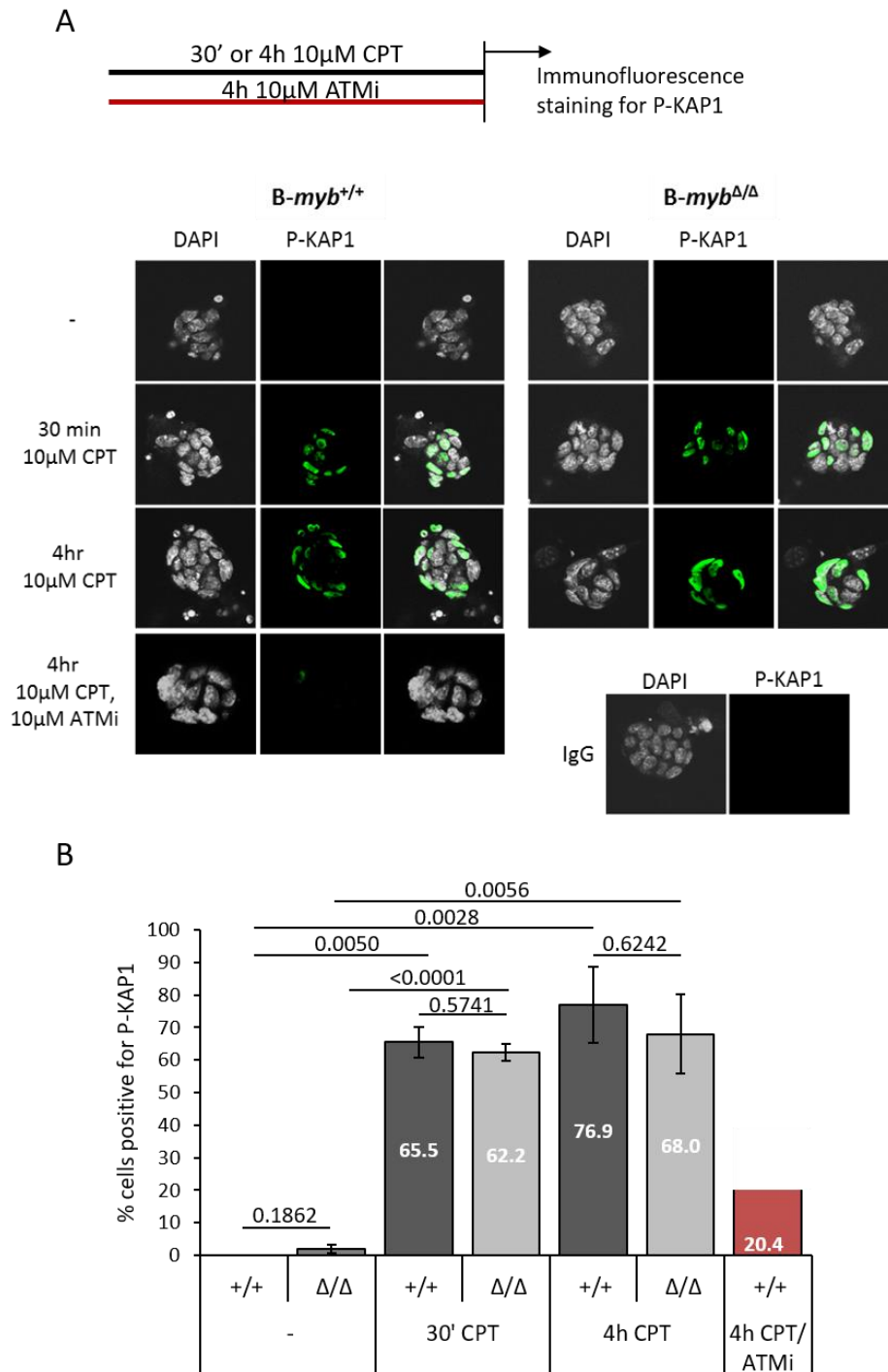


Figure 5.11 ATM dependent KAP1 phosphorylation after CPT treatment in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs A) Cells were untreated or treated with CPT for 30 minutes or 4 hours before harvesting and performing immunofluorescence staining for P-KAP1. Cells were also treated with an ATM inhibitor (KU60019) in combination with 4 hours CPT. B) Representative immunofluorescence images demonstrating staining for P-KAP1 (green) in ESC colonies. Images were taken at x100 magnification. C) Bar graph presenting the percentage of P-KAP1 positive cells after the various treatments in B-*myb*^{+/+} and B-*myb*^{Δ/Δ} ESCs. At least 150 cells were counted per group from 3 independent experiments, apart from the combined CPT and ATM inhibition which was repeated only 2 times. Statistical analysis was carried out using two-tailed unpaired t-tests.

5.2.7 Is the defect observed after ATM inhibition due to deregulation of replication initiation control?

The replication slowdown in the B-*myb*^{Δ/Δ} ESCs can be partly attributed to changes in regulation of new origin firing. Moreover, ATM inhibition does also result in an increase in new origin firing in ESCs determined by DNA fibre structure analysis. Therefore, it was of interest to determine whether inhibition of CDK1 and Cdc7 could rescue the replication defect after ATM inhibition like observed in the B-*myb*^{Δ/Δ} ESCs. Wildtype ESCs were treated with an ATM inhibitor (KU60019) in combination with either a CDK1 inhibitor (RO3306) or Cdc7 inhibitor (PHA-767491) for 1.5 hours before labelling active replication (Figure 5.12A). Interestingly, inhibition of CDK1 resulted in a significant rescue of the replication progression defect observed after ATM inhibition from 0.99 ±0.07 to 1.21 ±0.03 kb/min (Mann Whitney U test, p<0.0001) (Figure 5.12B+D). Inhibition of Cdc7 also resulted in a considerable rescue of the ATM inhibition induced replication defect to levels above untreated Wildtype ESCs from 0.80 to 1.59 kb/min (Figure 5.12C+D).

Analysis of replication structures revealed that inhibition of CDK1 also partially rescued the elevated origin firing observed after ATM inhibition, from 33% to 25% (Figure 5.13A). Interestingly, the increased elongating fork asymmetry after ATM inhibition was significantly rescued after CDK1 inhibition from an average ratio of 2.17 to 1.69 (Mann Whitney U, p=<0.0001) (Figure 5.13C). Similar to the CDK1 inhibition, treatment with the Cdc7 inhibitor resulted in a rescue of the new origin firing from 29% to 18% (Figure 5.13B), plus a rescue of fork ratio was also observed decreasing from 1.79 to 1.44 (Figure 5.13D). Overall, this data suggests that replication speed and fork stability defect observed after ATM inhibition in ESCs is likely attributed to changes in replication control with an influence upon origin firing.

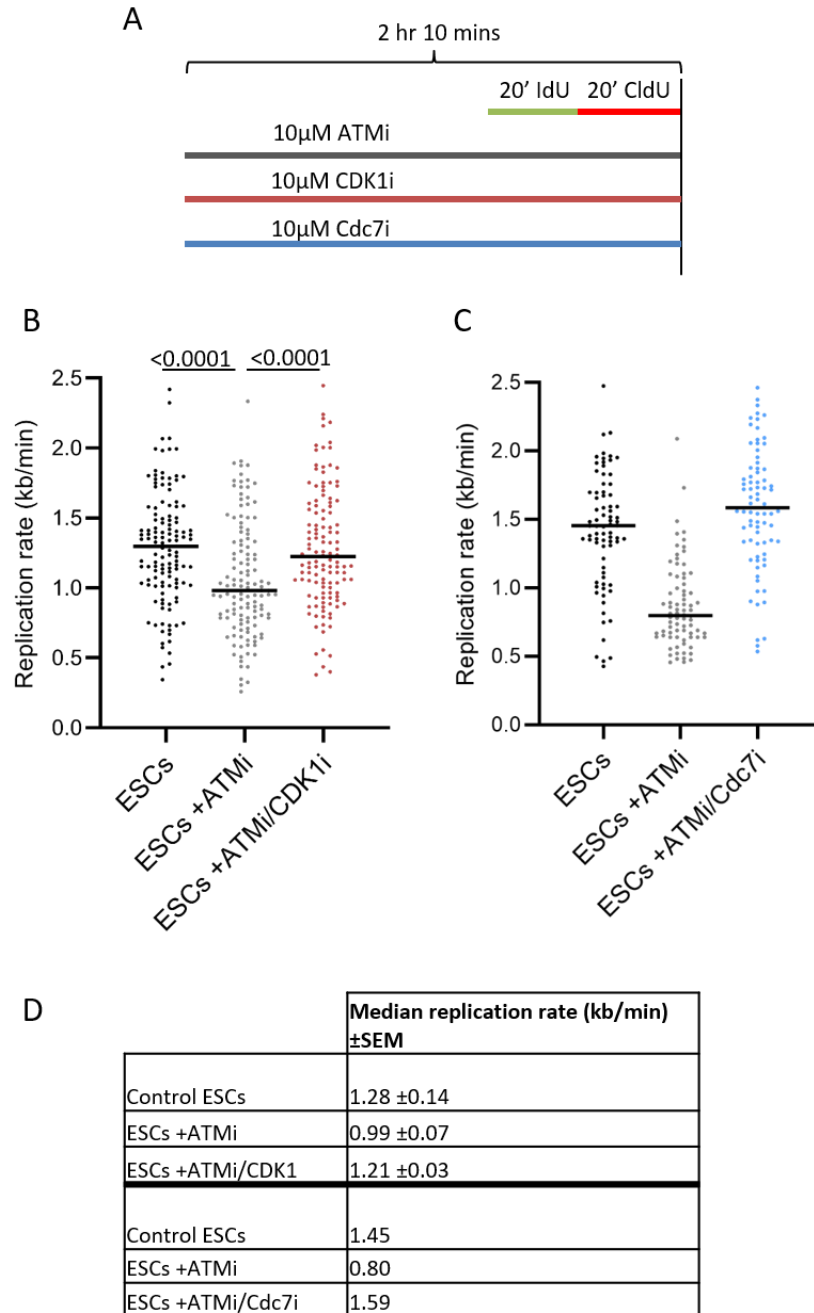


Figure 5.12 Inhibition of CDK1 or Cdc7 results in recovery of the replication rate defect observed after inhibition of ATM kinase A) Wildtype ESCs were treated 1.5 hours with 10µM KU60019, an inhibitor of ATM, in combination with inhibitors of CDK1 (RO3306) or Cdc7 (PHA-767491), before sequential addition of deoxynucleotide analogues IdU and CldU for 20 minutes each. DNA was treated and visualised as previously described. B) Dot plot demonstrating the effect of ATM inhibitor alone and in combination with CDK1 inhibitor upon replication rate (kb/min). Results are representative of four slides from two independent repeats. Statistical analysis was carried out using Mann Whitney U test. C) Dot plot demonstrating the effect of ATM inhibitor alone and in combination with Cdc7 inhibitor upon replication rate (kb/min). Results are from two slides from one independent experiment. D) Table presents the median replication rates (kb/min) ±SEM in ESCs untreated or treated with ATM inhibitor in combination with CDK1 or Cdc7 inhibition.

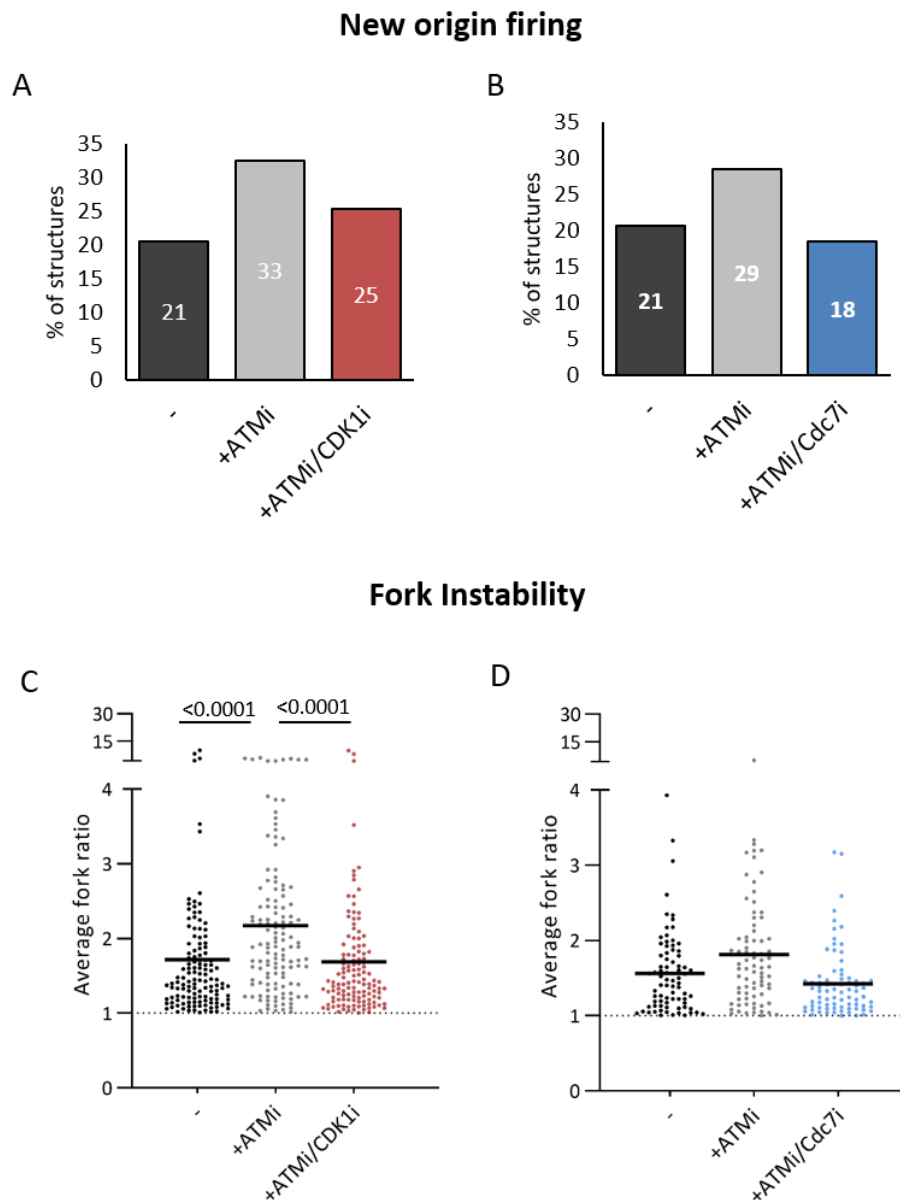


Figure 5.13 Inhibition of CDK1 or Cdc7 results in recovery of the increased origin firing and fork instability observed after inhibition of ATM kinase Wildtype ESCs were treated 1.5 hours with 10 μ M KU60019, an inhibitor of ATM, in combination with CDK1 inhibitor (RO3306) or Cdc7 inhibitor (PHA-767491), before labelling replication. The percentage of new fired origins was quantified relative to total structures. Bar graphs represent the average percentage of new fired origins for A) ATM inhibitor in combination with CDK1 inhibitor and B) ATM inhibitor in combination with Cdc7 inhibitor. For a measure of fork stability, the length of both incorporated labels for each elongating fork was measured in μ m and the positive ratio was calculated. Dot plots present the average fork ratio for C) ATM inhibitor in combination with CDK1 inhibitor with average ratios of 1.29, 1.07 and 1.29, and for B) ATM inhibitor in combination with Cdc7 inhibitor with average ratios of 1.44, 0.87, 1.60 in the order presented. Statistical analysis was carried out using the Mann Whitney U test. 128 ratios per treatment group from 2 repeats were recorded in the CDK1 inhibitor experiment, and 78 ratios per treatment group from 1 repeat were calculated in the Cdc7 inhibition experiment.

It was uncovered in chapter 1 that the B-*myb*^{Δ/Δ} present increased accumulation of UFBs which represent under-replicated DNA in mitosis. Therefore, it was of interest whether loss of ATM activity also results in the formation of UFBs during mitosis. Synchronous cell populations grown on cover slips were untreated or treated with 10μM KU60019 (ATM inhibitor) for 2 hours before fixation and fluorescence staining for ERCC6/PICH to detect UFBs (Baumann et al., 2007, Chan and Hickson, 2011). Using fluorescence microscopy, anaphase cells were located before scoring them for UFB positivity (Figure 5.14A). As observed in chapter 3, there was a highly significant increase (61%) in the percentage of UFB positive anaphases in the B-*myb*^{Δ/Δ} compared to B-*myb*^{+/+} (20%) (Figure 5.14B). Interestingly, ATM inhibition also resulted in a substantial increase in the percentage of UFB positive anaphases with levels greater than in the B-*myb*^{Δ/Δ} ESCs at 73% (Figure 5.14B). Therefore, this data suggests that ATM activity is also vital to prevent the formation of UFBs during mitosis in ESCs.

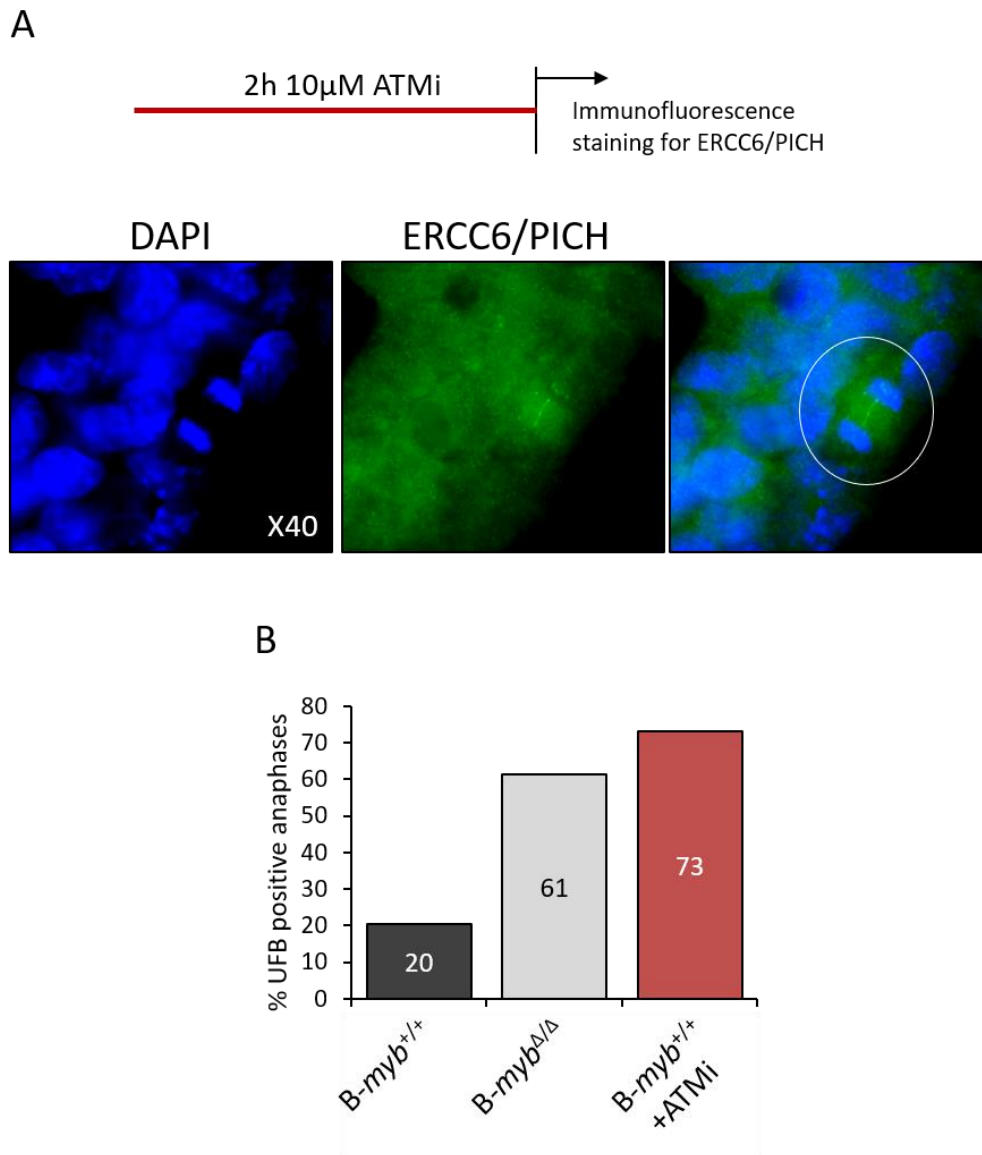


Figure 5.14 Inhibition of ATM activity results in formation of UFBs during mitosis A) Colonies of the B-myb^{+/+}, the B-myb Δ/Δ and the B-myb^{+/+} treated with 2 hours ATM inhibitor (KU60019) were fixed before performing immunofluorescence staining for ERCC6/PICH (green) in combination with DAPI staining (blue). Using fluorescence microscopy, the anaphases were located before scoring for UFB positivity. Images show an example of a UFB positive anaphase cell. B) Bar graph shows the average percentage of anaphases positive for UFBs in the B-myb^{+/+}, B-myb Δ/Δ and the B-myb^{+/+} after ATM inhibition. Data represents at least 30 anaphases from each group from 2 experimental repeats.

5.2.8 Increased reliance upon ATM activity in pluripotent stem cell types

The activity of ATM is predominantly associated with the response to DSBs, thus, it is surprising that inhibition of ATM activity resulted in replication stress under unperturbed conditions in mouse ESCs. Recent evidence suggests that stem cells rely on specialised DDR signalling to maintain a highly stable genome (Ahuja et al., 2016, Kapinas et al., 2013, Swahari et al., 2016, Zhao et al., 2015). Therefore, to test whether this phenotype was specific to stem cells, the role of ATM activity during replication was investigated in somatic cells.

Primary mouse embryonic fibroblasts (MEFs) were used as a model for investigating the DDR in somatic cells. The MEFs were cultured in the presence of 5 μ M ATR inhibitor (AZ20) or 10 μ M ATM inhibitor (KU-60019) for 2 hours and 10 minutes and DNA replication was labelled through addition of IdU and CldU sequentially for the final 40 minutes of treatment (Figure 5.15A). DNA fibres were prepared and visualised as previously described. In response to ATR inhibition, the replication rate was reduced significantly, with the median speed decreasing from 1.67 $\pm$ 0.10 to 0.48 $\pm$ 0.14 kb per minute (Mann Whitney U test, $p < 0.0001$) (Figure 5.15B+D). Moreover, this was similar to the phenotype observed after the same treatment in ESCs, in which median replication speed was reduced from 1.29 $\pm$ 0.02 to 0.44 $\pm$ 0.00 kb per minute (Figure 5.2B+D).

Inhibition of ATM activity in MEFs had little effect upon replication rate with median rate remaining at 1.67 kb/min. Analysis of the distribution of replication rates, revealed there was a slight increase in the spread around the median with an increase in higher and lower rates, however, the changes in distribution were not statistically significant (Mann Whitey U, $p = 0.6225$). The frequency of new origin firing increased slightly after ATM inhibition from 29% to 33% although this difference was not statistically significant (unpaired student t-test $p = 0.3453$) (Figure 5.16B). Moreover, this increase was minimal compared to the 11% increase in new origin firing observed after ATM inhibition in ESCs (Figure 5.5B).

Fork instability (fork asymmetry) was also examined through measuring the length ratio of elongating forks. Treatment with the ATM inhibitor in MEFs resulted in a slight increase in average fork ratio from 1.38 to 1.47, although this increase was determined to be non-statistically significant producing a p-value of 0.3534 (unpaired Mann Whitney U test). In addition, the percentage of forks with a ratio greater than 2 was increased from 3.2% to 11.6% after ATM inhibition (Figure 5.16C). However, this increase was much weaker than the strong increase in global fork asymmetry observed after ATM inhibition in ESCs (Figure 5.5B). Overall, ATM inhibition appears to exert some level of stress during replication in MEFs, but its influence is not comparable to the detrimental effects observed after loss of ATM activity in ESCs.

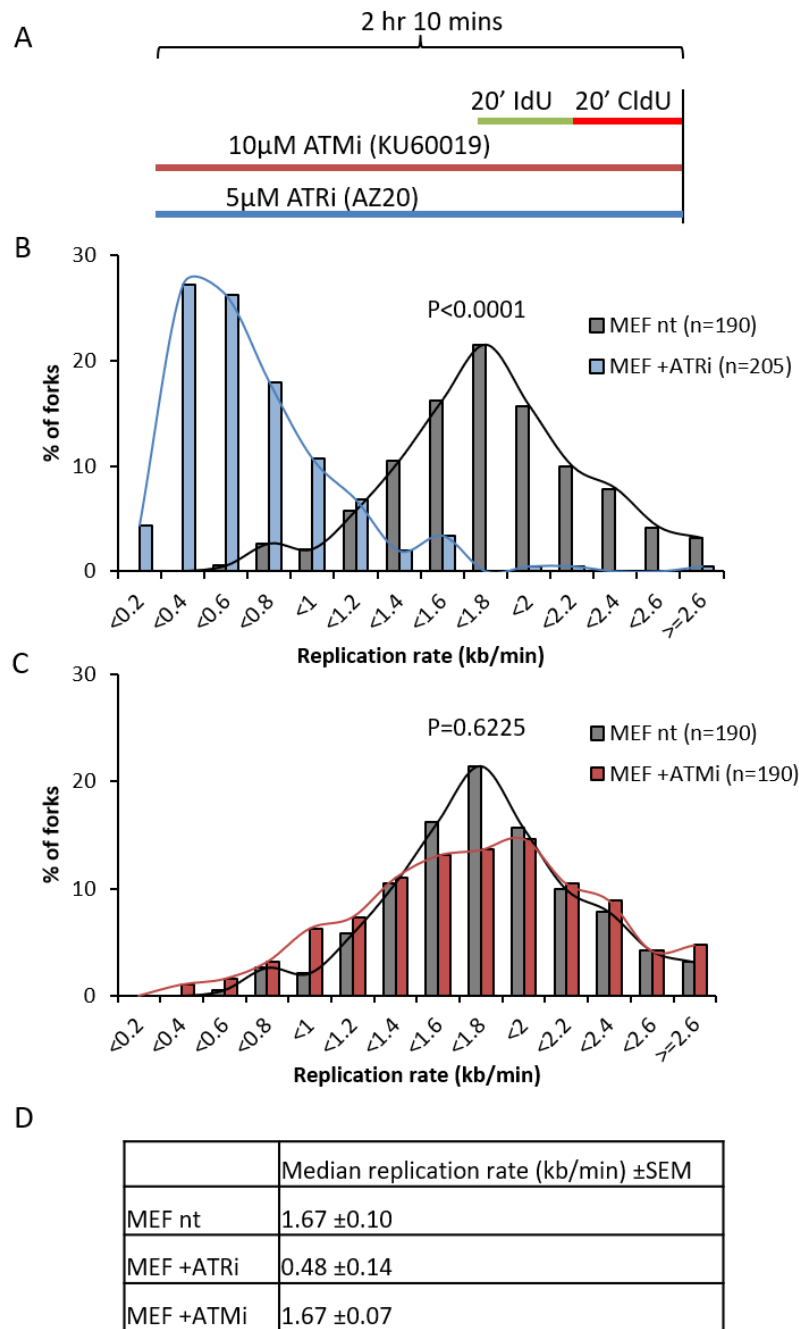


Figure 5.15 ATR but not ATM activity is vital for normal replication progression in MEFs A) MEFs were treated for 2h and 10 minutes with 5µM AZ20 (ATRi) or 10µM KU60019 (ATMi), before sequential addition of deoxynucleoside analogues IdU and CldU for the final 40 minutes of treatment. DNA was treated and visualised as previously described. B) Distribution curve demonstrating the influence of ATR inhibitor treatment upon replication speed in MEFs. C) Distribution curve for the effect of ATM inhibitor treatment in MEFs. Statistical analysis of distributions was carried out using an unpaired Mann Whitney U test. D) Table showing median replication speed in MEFs untreated or treated with ATR or ATM inhibitor. n=3. The number of forks measured for each condition is shown on the respective distribution curves.

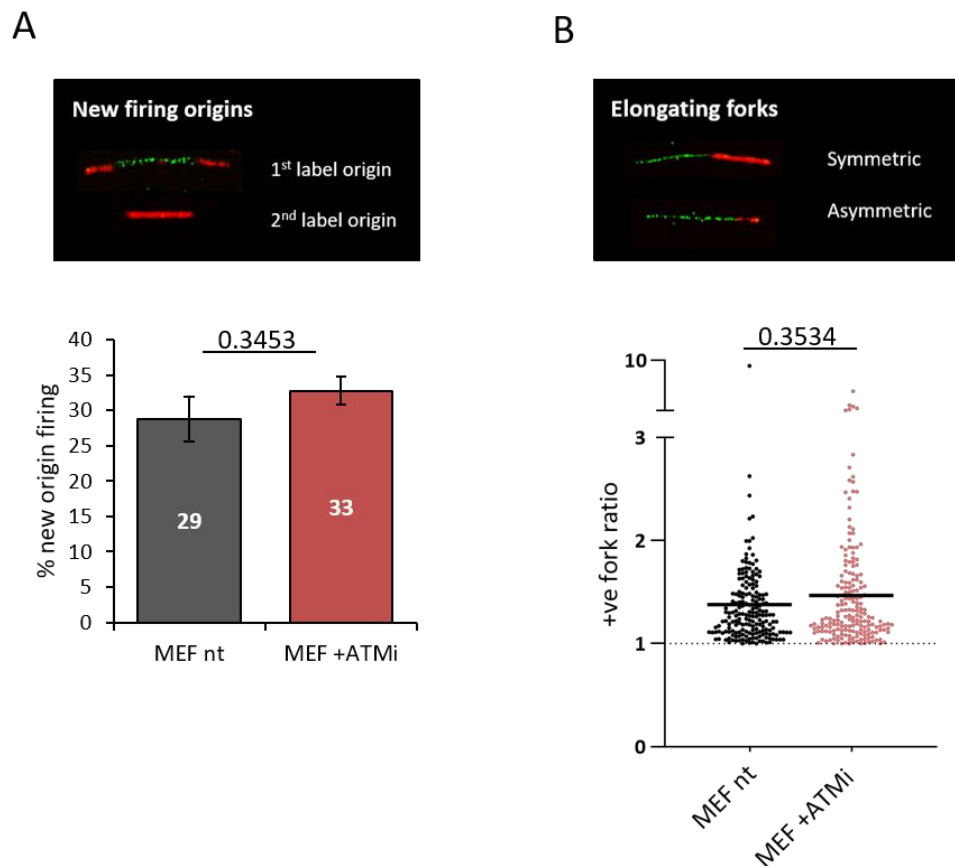


Figure 5.16 Inhibition of ATM activity in MEFs has minimal effect upon replication dynamics MEFs were treated for 2h and 10 minutes with 10 μ M KU60019 (ATMi) before addition of IdU and CldU for the final 40 minutes of treatment. DNA fibres were prepared as previously described. A) The staining patterns were used to identify elongating forks (symmetric and non-symmetric) and newly fired origins as shown B) The percentage of new fired origins was quantified relative to total structures. Bar graph representing the average percentage of new fired origins in untreated MEFs and MEFs treated with ATM inhibitor, error bars represent SEM. Statistical analysis was performed using two-tailed unpaired t-tests. At least 600 replication structures were counted per treatment group from 3 separate repeats. C) For a measure of fork stability, the length of both incorporated labels for each elongating fork was measured in μ m and the positive ratio was calculated. Dot plot demonstrating the average fork ratio in untreated MEFs and MEFs treated with ATM inhibitor. Average ratios were 1.38 and 1.47. Statistical analysis was carried out using the Mann Whitney U test. 190 ratios were calculated per treatment group from 3 separate experiments.

5.3 Discussion

5.3.1 The potential role of B-Myb in ATR dependent DDR signalling

The ATR dependent arm of the DDR has been shown to be instrumental for maintaining normal replication behaviour (Ge and Blow, 2010, Petermann et al., 2010, Syljuasen et al., 2005), therefore, to investigate whether loss of B-Myb had an influence upon this pathway, the activation of Chk1 was investigated. It was first demonstrated that that levels of unmodified Chk1 protein were unchanged upon loss of B-Myb, ruling out a possible role in Chk1 transcription. However, the *B-myb*^{Δ/Δ} presented increased levels of Chk1 phosphorylated at serine 345 compared to the *B-myb*^{+/+}, likely reflecting a response to the increased replication stress observed in the *B-myb*^{Δ/Δ}. Cells were treated with either CPT or ionising radiation to promote increased replication stress and DNA damage, moreover, in both the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs, the level of active Chk1 was increased substantially by both treatments, but there was no difference in the response between the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ruling out a potential role for B-Myb in activation of the ATR-Chk1 pathway.

DDR inhibitors were also used to investigate the dynamics of Chk1 activation in ESCs. There is evidence in the literature to suggests that there is crosstalk between the activities of the ATR and ATM dependent arms of the DDR (Palii et al., 2013, Gatei et al., 2003, Adams et al., 2006, Stiff et al., 2006, Rhind, 2009). It was demonstrated that inhibition of Chk1 activity results in considerably more DNA damage accumulating compared to inhibition of ATR alone, therefore suggesting Chk1 may be activated by an alternative mechanism in the absence of ATR activity (Toledo et al., 2011). Here, as expected, dual inhibition of ATR and ATM resulted in complete loss of P-Chk1 after 5Gy irradiation. Interestingly, ATM inhibition alone was also able to reduce the levels of P-Chk1 after 5Gy IR, albeit not as much as treatment with an ATR inhibitor alone. Therefore, this data supports the theory of crosstalk between ATR and ATM in activation of Chk1 in ESCs.

Chk1 activation has been demonstrated to be vital for arrest of cell cycle and replication origin firing in response to stress (Paulsen and Cimprich, 2007, Petermann et al., 2010, Syljuasen et al., 2005), therefore, it is surprising that the *B-myb*^{ΔΔ} ESCs, which present increased Chk1 activation in unperturbed conditions, also present a contrasting increase in origin firing and are able to pass through the cell cycle. It may be that B-Myb functions further downstream of the ATR-Chk1 pathway controlling replication initiation. Furthermore, these findings suggest that loss of B-Myb allows the cell to ignore anti-proliferation signals, similar to behaviour seen in cancer cells (Jackson and Bartek, 2009).

From the DNA fibre analysis after ATR inhibition, it was clear that loss of ATR activity is highly disruptive to replication dynamics, highlighted by a striking decrease in replication progression speeds, a substantial increase in new origin firing and an increase in fork instability. This data supports the reported roles of ATR activity in regulating DNA replication, specifically with an influence on replication initiation (Paulsen and Cimprich, 2007, Petermann et al., 2010, Syljuasen et al., 2005). Interestingly, ATR inhibition was more severe in the *B-myb*^{ΔΔ} compared to the *B-myb*^{+/+}, with fork rate reducing to levels almost reminiscent of complete fork arrest, and with the already elevated new origin firing increasing even further. The effect of ATR inhibition on fork instability in the *B-myb*^{ΔΔ} appeared to be less compared to the *B-myb*^{+/+}, however, this can be attributed to the extremely short fibres produced in the *B-myb*^{ΔΔ} ESCs in which changes in fork ratio are beyond detection using this technique.

The replication stress phenotype after inhibition of ATR, although extremely amplified, was not too dissimilar from that seen in the *B-myb*^{ΔΔ} ESCs. The DDR has been demonstrated to influence replication by targeting CDK1 activity, through influencing its modifiers such as WEE1 (Beck et al., 2012, Petermann et al., 2010). Consistently, the replication phenotype after ATR inhibition was similar to that seen after WEE1 inhibition (Chapter 4), in which complete loss of CDK1 inhibitory phosphorylation was the cause. Therefore, it cannot be ruled out that

loss of B-Myb and inhibition of ATR do not cause replication stress through the same mechanism, with a potential influence on the activity of CDKs.

Another important point is that ATR activity is vital for various other processes, for example, its roles in maintaining fork stability (Cimprich and Cortez, 2008, Paulsen and Cimprich, 2007). Moreover, if B-Myb was functioning as a downstream component of ATR with a more specific role in replication initiation, this could explain why the effect of inhibition of ATR upon replication is more severe in comparison. Alternatively, B-Myb could influence replication through a completely separate mechanism and loss of B-Myb in combination with inhibition of ATR activity results in an amalgamation of multiple replication stress inducing mechanisms. Therefore, further study is required to pinpoint how B-Myb may be interacting with this pathway.

5.3.2 An epistatic relationship between B-Myb and ATM activity for replication control

Recent studies have highlighted a potential direct interaction of B-Myb with the ATM dependent DDR response (Bayley et al., 2018a, Henrich et al., 2017). Therefore, an ATM dependent mechanism for the replication defect in the *B-myb*^{Δ/Δ} ESCs was investigated. Firstly, it was shown that ATM inhibition resulted in a strong replication slow-down, increased fork instability and increased replication initiation in unperturbed ESCs. In contrast, inhibition of ATM activity in the *B-myb*^{Δ/Δ} ESCs had no additional influence on the pre-existing replication stress, with the elevated new origin firing as well as the fork instability remaining. Furthermore, the replication stress produced through ATM inhibition was almost identical to that seen in the *B-myb*^{Δ/Δ} ESCs. This finding proposes a potential epistatic relationship between ATM and B-Myb activity for their influence upon the replication programme. This data builds upon previous findings from the Klempnauer lab who provided evidence for an ATM dependent role in B-

Myb activation (Henrich et al., 2017). However, this study suggested that B-Myb is activated by ATM in response to DNA damage specifically (Henrich et al., 2017), whereas, data here suggests that in ESCs, both ATM and B-Myb are vital for unperturbed DNA replication, and their potential interaction takes place in unperturbed conditions.

Interestingly, inhibition of ATM in wildtype ESCs also resulted in elevated levels of replication factories. However, this increase was not comparable to the further elevated levels of factories observed in the *B-myb*^{Δ/Δ} ESCs. A possible explanation for this is the ATM inhibitor treatment time may be too short to produce the phenotype observed in the permanent B-Myb knockout cells. Alternatively, it could be that B-Myb has a more direct influence upon replication factories compared to ATM activity. Moreover, it is the ATR-Chk1 pathway which has been primarily associated with replication factory control in the literature (Ge and Blow, 2010, Petermann et al., 2010), therefore, the role of B-Myb in replication factory control could also be linked to this pathway.

5.3.3 Alternative roles for ATM activity in regulating replication initiation in ESCs

Origin activation and suppression in response to stress has been shown to be controlled through the activity of the ATR-Chk1 pathway (Patil et al., 2013, Syljuasen et al., 2005, Costanzo et al., 2003, Zeman and Cimprich, 2014). The data presented here agrees that inhibition of ATR activity results in elevated origin firing in ESCs (Syljuasen et al., 2005). However, it also suggests that inhibition of the ATM dependent DDR specifically, which is normally exclusively associated with the response to DSBs (Shiloh, 2003, Bakkenist and Kastan, 2003, Lee and Paull, 2004), results in a similar change in replication dynamics in unperturbed conditions, including an increase in replication initiation. Inhibition of ATM activity in wildtype ESCs also resulted in elevated levels of replication factories globally, for which control is also normally associated

with the activity of ATR-Chk1 pathway (Ge and Blow, 2010, Petermann et al., 2010). Supporting these concepts, suppression of origin firing through inhibition of CDK1 or Cdc7 was able to rescue the replication rate defect, increased origin firing, and the fork instability observed after loss of ATM activity in ESCs.

Current understanding denotes that ATM is inactive under unperturbed conditions and its activation relies upon MRN dependent recruitment to sites of DSBs (Bakkenist and Kastan, 2003, Lee and Paull, 2004). However, various studies have challenged this canonical pathway for ATM activation through demonstrating that its recruitment and activity is dependent upon chromatin context, and ATM may be activated in the absence of DSBs (Bakkenist and Kastan, 2003, Bencokova et al., 2009, Cam et al., 2010, Ewald et al., 2008, Iwahori et al., 2014, Olcina et al., 2010, Olcina et al., 2013). It is also suggested that the activity of the DDR in stem cells is highly efficient compared to somatic cell types making up for a lack of cell cycle checkpoint control and to maintain a highly stable genome (Ahuja et al., 2016, Kapinas et al., 2013, Murga et al., 2007, Zhao et al., 2015). In addition, studies in *xenopus laevis* embryos, which represent a premature stem cell type, revealed a specific role for ATM in the timing of replication (Shechter et al., 2004). Therefore, it was hypothesised that ATM could have additional roles in replication control, specifically in stem cell types.

To investigate this hypothesis, MEFs (somatic cells) were subjected to the same experimental conditions used to analyse the influence of ATM inhibition in ESCs. Although there was some evidence of stress, this was negligible compared to the global shift in replication dynamics observed in the ESCs after ATM inhibition. Thus, these findings support the theory that ATM is important for controlling replication dynamics in unperturbed conditions specifically in ESCs, which could extend to other stem cell types. The mechanism for how stem cells may have the capacity for increased usage of ATM dependent DDR signalling is not known. A potential explanation is a difference in recruitment capacity due to variances in chromatin

architecture. ESCs possess high levels of γ H2AX throughout the nucleus under untreated conditions, shown to be a result of their relaxed chromatin state (Chuykin et al., 2008, Banath et al., 2009, Zhou et al., 2011, Gaspar-Maia et al., 2011, Hawkins et al., 2010). Moreover, a study in which chromatin accessibility was manipulated in ESCs demonstrated that the strength of the DDR response was dependent upon the level of chromatin compaction (Murga et al., 2007). Therefore, it is possible that the increased chromatin accessibility in stem cells allows for increased capacity for ATM activity which may contribute to their increased repair efficiency and their ability to maintain a highly stable genome (Ahuja et al., 2016).

It is also unclear how ATM is influencing DNA replication in this model. One possibility is that ATM has an increased influence upon Chk1 activity in ESCs through crosstalk with the ATR-Chk1 pathway. Alternatively, ATM could influence origin firing through a completely different mechanism, for example, through the activity of its downstream target Chk2 (Chaturvedi et al., 1999). It is also possible that the mechanism for the role of ATM in replication is reliant on the activity of B-Myb, for which loss results in the same detrimental effect upon replication. To answer these unresolved uncertainties, further work looking at the interactions of these pathways in control of replication dynamics would be necessary.

5.3.4 The potential role of nucleolytic processing in the replication rate defect in the B-*myb*^{Δ/Δ} ESCs

Recent work revealed an epistatic relationship between ATM and RNF168 ubiquitin ligase activity for their influence upon replication progression control (Schmid et al., 2018). Moreover, inhibition of MRE11 activity rescued the replication rate defect after both inhibition of RNF168 and ATM activity, predicted to rescue through inhibition of MRE11 dependent nucleolytic processing of stalled and reversed replication forks (Schmid et al., 2018). In the B-

myb^{Δ/Δ} ESCs, mirin treatment did not rescue the replication stress phenotype, but had little to no influence on the replication rate and levels of new origin firing. In fact, mirin treatment increased the already elevated fork instability in B-*myb*^{Δ/Δ} ESCs. This data suggests that the replication stress observed upon loss B-Myb is not due to MRE11 dependent nucleolytic processing.

The replication phenotype observed after mirin was more akin to that seen upon inhibition of ATM. Mirin not only inhibits MRE11 exonuclease activity, but it was also shown to prevent its activation of ATM, as well as abolishing G2/M checkpoint control and homologous recombination (Dupre et al., 2008). Therefore, it is more likely that the phenotype observed in the B-*myb*^{Δ/Δ} ESCs after mirin treatment is related to ATM dependent activity. An alternative explanation for a lack of rescue after mirin treatment, is that mirin could be more toxic in ESCs compared to other cell types. Mirin treatment in wildtype ESCs resulted in reduced replication rates and substantial fork instability, which was not observed after mirin treatment in U2OS cells (Schmid et al., 2018). Moreover, a potential rescue by mirin may be outweighed by an increase in replication toxicity by other mechanisms. ESCs display an increased accumulation of ssDNA gaps and fork reversal which could be one way to explain why ESCs may have an increased requirement for MRE11 activity (Ahuja et al., 2016).

5.3.5 A potential epistatic relationship between ATM and B-Myb activity in the occurrence of DNA damage

The B-*myb*^{Δ/Δ} ESCs present elevated levels of DNA damage in unperturbed conditions, measured by the percentage of 53BP1 positive cells and by alkaline comet assays. Interestingly, inhibition of ATM activity in the B-*myb*^{+/+} resulted in similar phenotype indicating that ATM activity is important in ESCs to prevent DNA damage accumulation during replication in ESCs.

Moreover, the already elevated levels of DNA damage observed in the B-*myb*^{ΔΔ} ESCs was not increased further by the ATM inhibitor treatment. This data provides some support for the theory of an epistatic relationship between ATM and B-Myb activity during replication, with loss of either resulting in increased genome instability. Although, further repeats of the alkaline comet assay after ATM inhibition would be necessary to validate these findings further.

It has been reported that un-replicated DNA can pass from S phase into mitosis, leading to the formation of UFBs (Chan et al., 2007). There are many different causes of UFB formation including deregulation of DDR signalling (Saldivar et al., 2018, Chan et al., 2018, Naim et al., 2013, Nera et al., 2015). The B-*myb*^{ΔΔ} ESCs present a highly significant increase in the formation of UFBs reflecting their chronic replication stress and genome instability. Interestingly, inhibition of ATM was sufficient to induce a strong increase in UFBs formation during mitosis, moreover, the levels surpassed those seen in the B-*myb*^{ΔΔ} ESCs. This suggests that ATM activity is also important for preventing the passage of un-replicated DNA into mitosis, which also strengthens the roles of ATM activity during unperturbed S phase in ESCs.

5.3.6 The role of B-Myb in ATM activity is not dependent upon activation of KAP1

ATM has been shown to be required for approximately 10-15% of DSBs after irradiation (Deckbar et al., 2007, Riballo et al., 2004), moreover, ATM is required specifically for the repair of DNA damage in heterochromatin regions through its activation by phosphorylation of KAP1 (Ziv et al., 2006). Therefore, a potential mechanism for the epistatic relationship between ATM and B-Myb, was a role for B-Myb in ATM dependent activation of KAP1 important for DNA repair. By measuring the recruitment of KAP1 after CPT treatment, it was demonstrated that loss of B-Myb had no influence on the activation of KAP1, providing evidence against this

potential mechanism. In addition, treatment with an ATM inhibitor in combination with CPT completely diminished P-KAP1 staining, confirming the requirement for ATM in its activation.

In the literature, P-KAP1 has been shown to form distinct nuclear foci in response to irradiation (White et al., 2012, Bayley et al., 2018a). Here, KAP1 presented pan-nuclear staining and did not appear to form distinct nuclear foci. This could be attributed to differences in the DNA damaging treatments used in these studies, with CPT known to produce damage specifically at replication sites (Berniak et al., 2013). Alternatively, this response could be specific to ESCs and to the changes in chromatin context associated with pluripotency, resulting in changes to P-KAP1 activity (Chuykin et al., 2008, Banath et al., 2009, Zhou et al., 2011, Gaspar-Maia et al., 2011, Hawkins et al., 2010).

5.3.7 Does B-Myb function as a component of the DDR?

The findings presented in this chapter suggest that B-Myb is implicated in DDR signalling, although, from this data it is not possible to determine how B-Myb interacts with these pathways. There could be a direct protein interaction with components of the DDR, such as the MRN complex, for which evidence in the literature suggests (Henrich et al., 2017). Alternatively, B-Myb could have a transcriptional role which is dependent on DDR signalling, which has also been proposed (Saldivar et al., 2018). It is also possible that B-Myb and the DDR influence the replication programme as completely separate entities. Therefore, to accurately determine whether B-Myb actually cooperates with the DDR, potential interactions between them would have to be investigated. One way to do this would be to uncover B-Myb binding proteins through proteomic analysis. An additional caveat of the experiments presented is that they compare short-term inhibition of the DDR with long-term loss of B-Myb. Moreover, it would be valuable to produce ATM knockout ESCs, to properly determine the effect of ATM signalling loss in ESCs.

**Chapter 6 Investigating the potential transcriptional
roles of B-Myb in regulating cell cycle and replication
progression in ESCs**

6.1 Introduction

It has long been reported that B-Myb performs essential roles as a transcription factor in a cell cycle dependent manner (Georlette et al., 2007, Sala, 2005, Sala et al., 1997, Sadasivam et al., 2012, Lam and Watson, 1993), exerting an influence on a wide range of cell cycle progression associated genes (Okada et al., 2002, Henrich et al., 2017, Lorvellec et al., 2010, Wolter et al., 2017, Down et al., 2012, Saldivar et al., 2018). However, recent evidence suggests that the transcriptional roles of B-Myb are just one aspect of its activity. Firstly, B-Myb was shown to promote cell cycle transitions independently of its DNA binding activity (Werwein et al., 2012). In addition, recent studies demonstrate its direct interaction with protein complexes and its transcription independent roles important for normal cellular function and genome stability maintenance (Ahlbory et al., 2005, Yamauchi et al., 2008, Henrich et al., 2017, Shepard et al., 2005, Seong et al., 2011). It is unclear whether the changes in the replication programme upon loss of B-Myb in ESCs are due to a transcriptional or non-transcriptional influence, although, the changes in cell cycle and replication control are reminiscent of that seen in cancer cells which frequently present abnormal activation of transcription (Kotsantis et al., 2016). In this chapter, through global transcriptome analysis of the *B-myb*^{Δ/Δ} ESCs, I aimed to uncover potential alterations which could be linked to the role of B-Myb in cell cycle signalling and replication control.

6.2 Results

6.2.1 The localisation of B-Myb in unperturbed and stressed conditions

Firstly, to gain more insight into the behaviour of B-Myb, the capacity of B-Myb to bind to chromatin was assessed. The soluble cytoplasmic and nuclear protein was isolated before extraction of the non-soluble chromatin bound protein. Protein samples were subjected to SDS-PAGE before immunoblotting for B-Myb protein. Blotting for tubulin and histone H3 was also performed to ensure separation of chromatin bound proteins was carried out successfully. Interestingly, in unperturbed ESCs, B-Myb was almost entirely bound to chromatin with only a very weak band appearing in the soluble protein fraction (Figure 6.1). In addition, generation of cellular stress through treatment with CPT had no effect upon the localisation of B-Myb (Figure 6.1).

B-Myb activation has been proposed to be dependent upon ATM activity (Henrich et al., 2017). Therefore, it was of interest to determine whether B-Myb localisation to chromatin is influenced by ATM activity. Cells were treated with an inhibitor of ATM (KU60019) with or without combined 1 hour CPT treatment, before analysing the localisation of B-Myb. In both the untreated and CPT treated cells, ATM inhibition had no influence on the localisation of B-Myb to chromatin (Figure 6.1). This finding suggests that ATM is not responsible for B-Myb recruitment to chromatin.

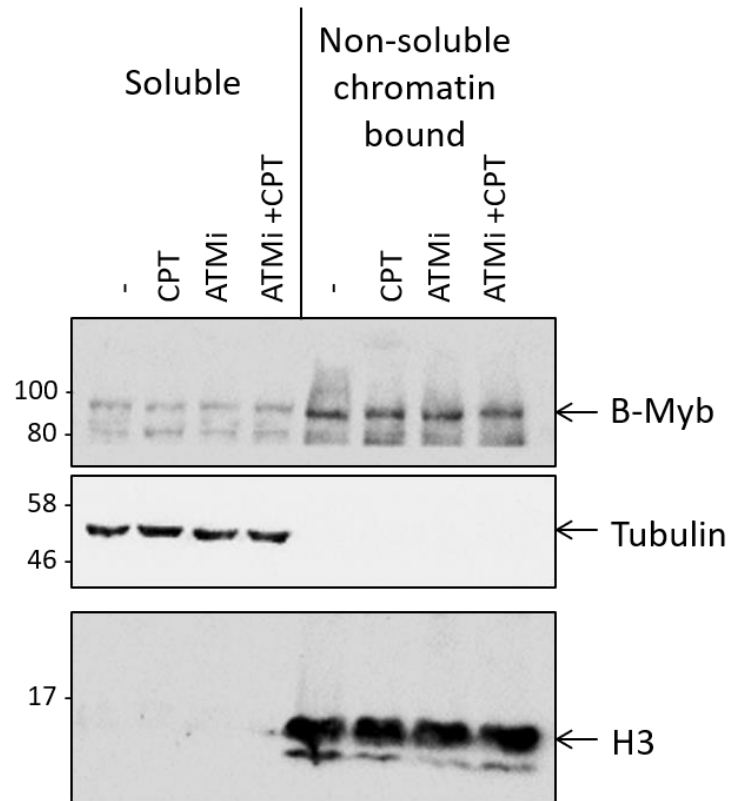


Figure 6.1 Neither CPT treatment nor inhibition of ATM kinase influences the association of B-Myb protein with chromatin in ESCs Wildtype ESCs were either untreated or treated with 1hr 10 μ M CPT, 1.5hr 10 μ M ATM inhibitor (KU60019) or both drugs in combination. Cells were harvested and subjected to protein fractionation of soluble and insoluble chromatin bound proteins through various lysis buffer treatments. Samples were subjected to SDS-PAGE before performing western blot analysis. Image shows Immunoblotting for B-Myb plus tubulin and Histone H3 as loading controls. Molecular weight (kDa) is indicated on the left determined through a protein ladder and arrows point to target protein bands.

6.2.2 Whole transcriptome sequencing in *B-myb*^{Δ/Δ} versus *B-myb*^{+/+} ESCs

The persistent localisation of B-Myb to chromatin may support the idea that B-Myb is exerting its influence directly upon transcription. To investigate whether B-Myb ablation leads to transcriptional changes, the transcriptome was analysed globally through RNA sequencing. RNA was extracted from the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs before ensuring the RNA was of high quality. The RNA sequencing was performed through the transcriptome analysis service provided by ThermoFisher (see chapter 2.13.1). Bioinformatic analysis of the raw output data and generation of plots was performed by Dr Carl Ward. A more detailed description of how the data was processed can be found in the materials and methods, section 2.13.2. Firstly, Principal component analysis (PCA) was performed demonstrating that the three repeats from both the *B-myb*^{+/+} and the *B-myb*^{Δ/Δ} groups clustered together (Figure 6.2). Next, differential gene expression analysis was performed using the DESeq2 analysis method (Love et al., 2014), revealing 802 upregulated and 1034 downregulated genes (adjusted p-value = 0.1). This data highlighted significant changes in global gene expression in the *B-myb*^{Δ/Δ} compared to the *B-myb*^{+/+} ESCs (Figure 6.3).

To analyse in more detail the changes in transcription upon loss of B-Myb, the top 50 genes with the most significant changes in expression were analysed (Figure 6.4). In the top 50 genes, 14 genes were upregulated; amongst them was *Pnp2* (Purine nucleoside phosphorylase 2), (fold change=5.2, q=8.82E-17) which was of particular interest due to its involvement in nucleotide synthesis (Borgers et al., 1978). Further analysis of *Pnp2* using the UCSC genome browser revealed increased expression of all exons 1-7 (Figure 6.5). There were also 35 downregulated genes present in the top 50, these included *Kdm5d* (Lysine demethylase 5D) (fold change=-30, q=2.55E-34) and *H2afx* (Histone variant H2A) (fold change=-1.82, q=2.48E-17) which were interesting due to their association with post-translational modification of chromatin structure and accessibility (Sharma et al., 2012, Shen et al., 2019). Further analysis of expression by

UCSC revealed that the expression levels were reduced in all exons in the B-*myb*^{Δ/Δ} compared to the B-*myb*^{+/+} (Figure 6.5). There were also multiple downregulated genes associated with chromosome segregation and cytokinesis for which B-*myb* has been associated with in the literature (Musa et al., 2017). These genes included *Prc1* (Protein Regulator of cytokinesis 1) (fold change=-2.19, q= 7.35E-36) and *Aurka* (aurora kinase A) (fold change=-3.20, q=5.56E-53) (Li et al., 2018, Goldenson and Crispino, 2015). Both *Prc1* and *Aurka* also demonstrated strong downregulation of expression of all exons (Figure 6.5).

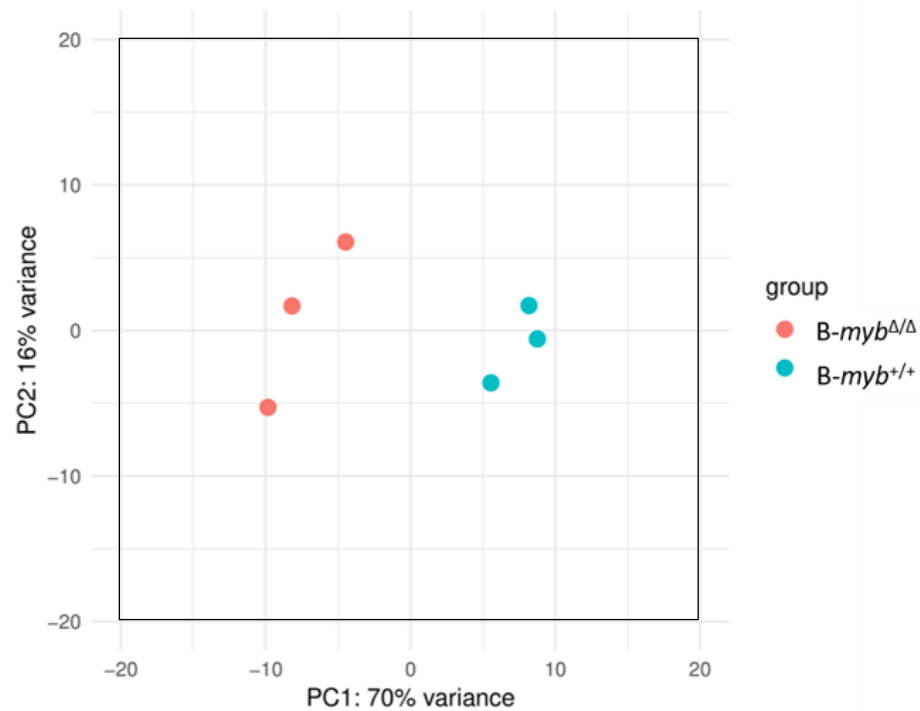


Figure 6.2 Principal component analysis (PCA) in the B-myb^{+/+} versus the B-myb^{Δ/Δ} groups PCA analysis was performed and plot generated by Dr Carl Ward using the DeSeq2 Bioconductor/R package. PCA plot demonstrating the clustering of individual samples from the B-myb^{+/+} and the B-myb^{Δ/Δ} groups analysed by RNA sequencing. The x and y axis values represent the variation between the sample groups. Blue dots represent the B-myb^{+/+} ESC sample group and red dots represent the B-myb^{Δ/Δ} ESC sample group.

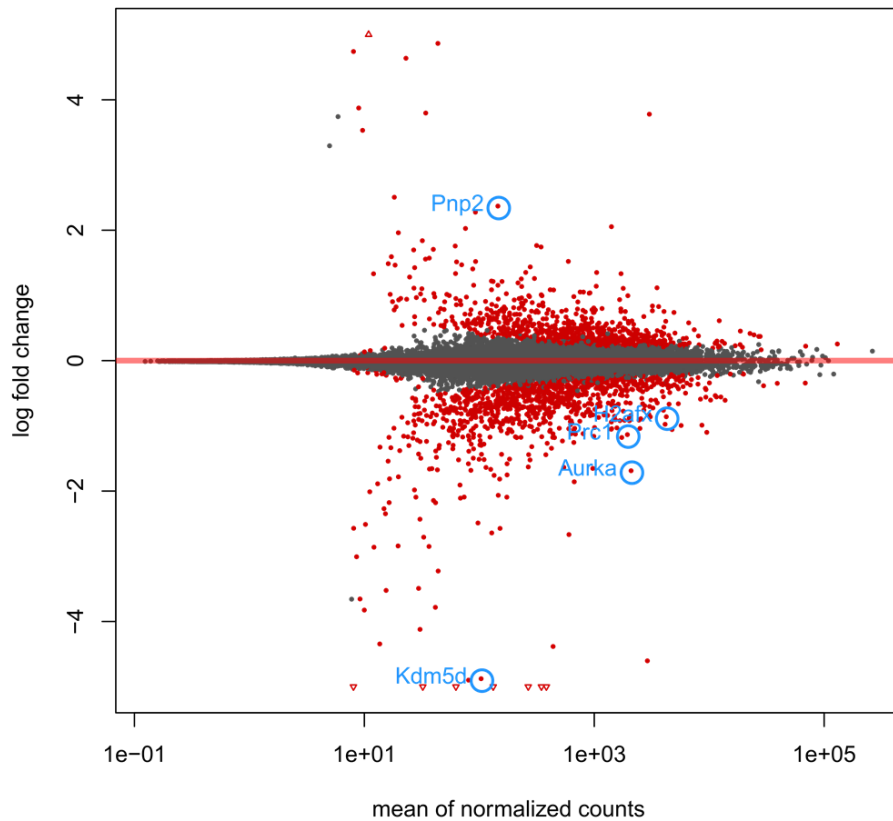


Figure 6.3 Differential gene expression analysis in the B-myb^{+/+} versus the B-myb^{Δ/Δ} groups
Differential gene expression analysis was performed using the DeSeq2 method (Love et al., 2014). Presented is an MA plot demonstrating the differentially expressed genes in the B-myb^{Δ/Δ} versus the B-myb^{+/+} ESCs. The x-axis represents the mean value acquired for the count for each gene, and the y-axis represents the Log₂ fold change in expression. Highlighted on the graph are specific genes: *Pnp2*, *H2afx*, *Prc1*, *Aurka* and *Kdm5d* which were amongst the top 50 most altered genes and were of particular interest due to their roles in processes previously associated with B-Myb activity. MA plot was generated by Dr Carl Ward using the DeSeq2 Bioconductor/R package.

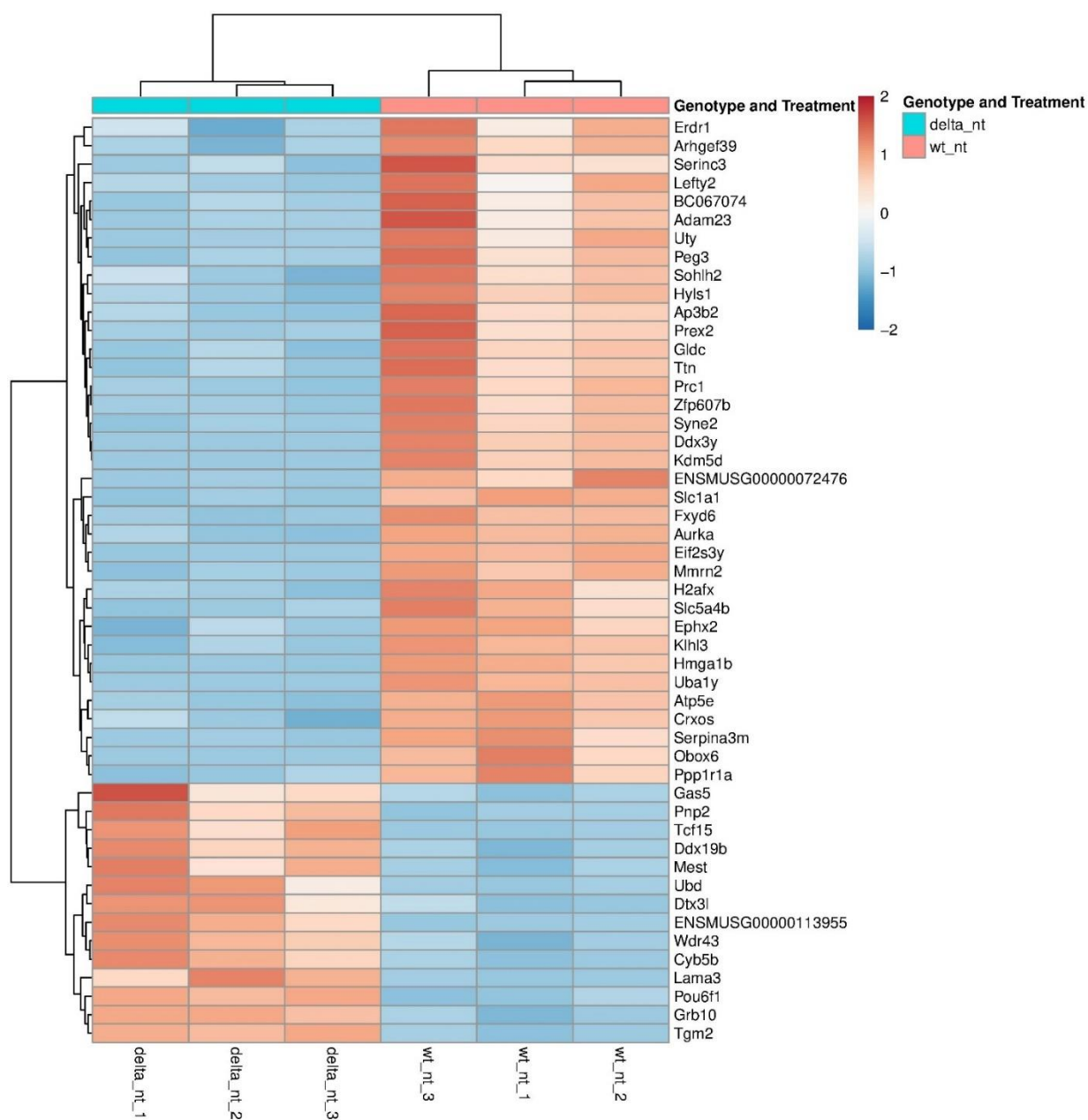


Figure 6.4 Top 50 genes with the most significantly altered expression in B-myb^{Δ/Δ} ESCs Heatmap presents genes downregulated in blue and upregulated in red; scale represents z-score. Each lane represents one of three samples from the B-myb^{+/+} (wt_nt_) and B-myb^{Δ/Δ} (Delta_nt_) ESCs. In the top 50 most significantly changed genes, 36 are downregulated, and 14 genes are upregulated in the B-myb^{Δ/Δ}. Heatmap was generated by Dr Carl Ward using the DeSeq2 Bioconductor/R package.

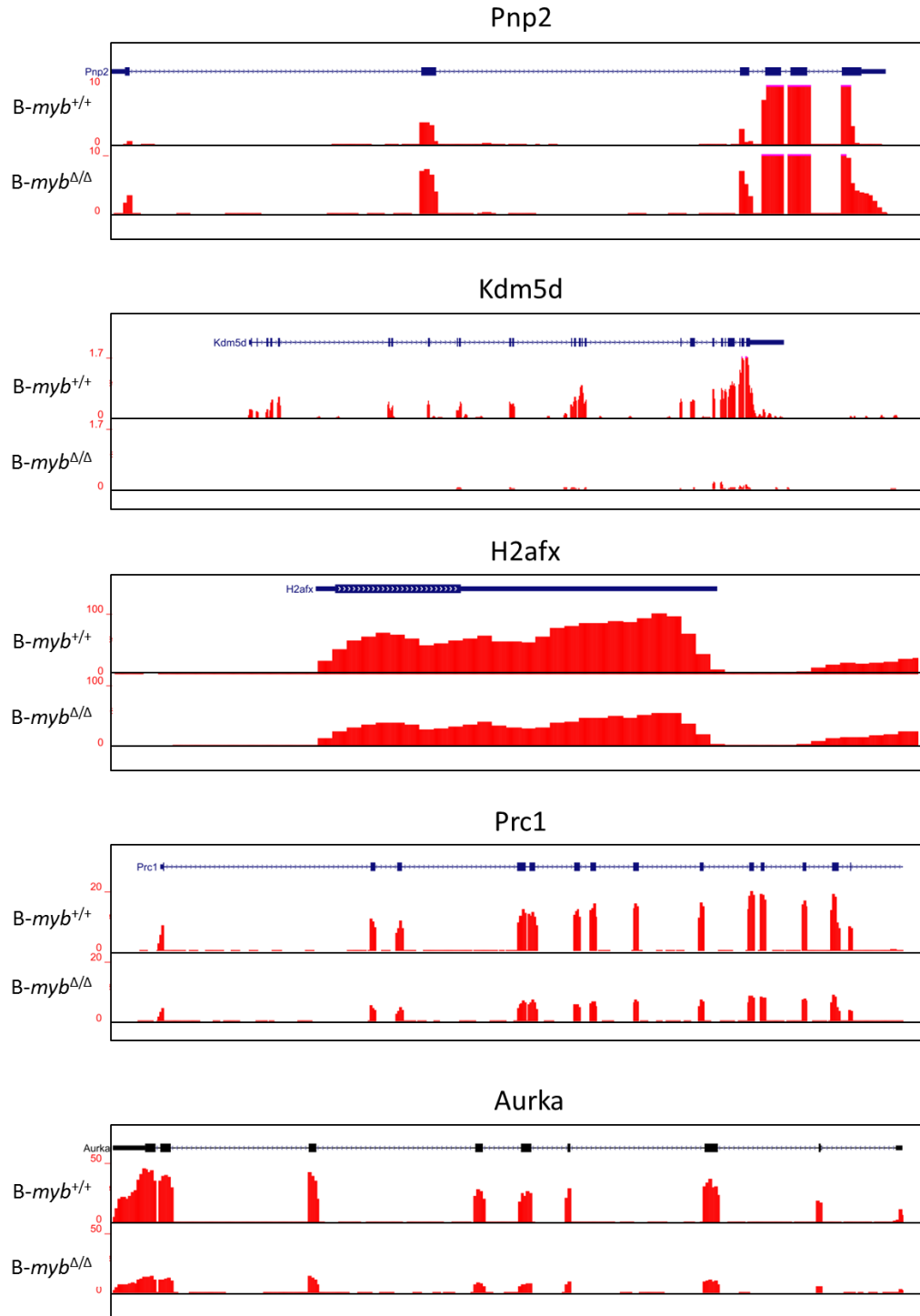
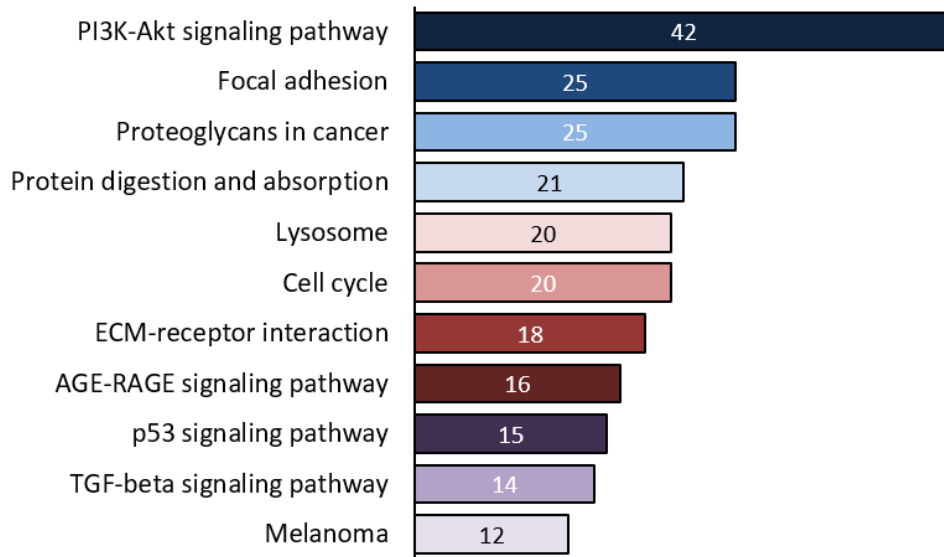


Figure 6.5 The gene expression profile of specific upregulated and downregulated genes in *B-myb*^{+/+} versus *B-myb*^{Δ/Δ} ESCs Using UCSC genome browser, the gene expression profile of specific genes was analysed. The generated gene expression tracks are presented for *Pnp2*, *Kdm5d*, *H2afx*, *Prc1* and *Aurka*. Shown in red are the different levels of expression within each exon/intron between the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs. The scale used is presented on the left representing arbitrary units.

6.2.3 Gene ontology analysis in the B-myb^{Δ/Δ} ESCs

KEGG pathway enrichment analysis using ClueGo was performed upon the upregulated and downregulated gene sets acquired from the DeSeq2 analysis with an adjusted p-value of less than 0.1. Firstly, the upregulated gene set containing 802 genes was evaluated, however, there were no terms associated with this dataset. The downregulated gene list, consisting of 1034 genes, was associated with 11 GO terms (Figure 6.6). Interestingly, these terms included ‘cell cycle’ (P=5.26E-04) comprising of 20 genes [*Abl1*, *Anapc13*, *Bub1b*, *Bub3*, *Ccnd1*, *Ccnd2*, *Cdc20*, *Cdc26*, *Cdkn1a*, *Cdkn2a*, *Cdkn2b*, *Dbf4*, *Gadd45g*, *Plk1*, *Pttg1*, *Smad3*, *Tgfb2*, *Tgfb3*, *Trp53*, *Wee1*]. Also included were gene ontology groups encompassing well known cancer associated pathways; ‘p53 signalling pathway’ (P=3.24E-04) with 15 genes included [*Apaf1*, *Ccnd1*, *Ccnd2*, *Ccng1*, *Cdkn1a*, *Cdkn2a*, *Cyct*, *Ei24*, *Fas*, *Gadd45g*, *Serpine1*, *Sesn2*, *Steap3*, *Thbs1*, *Trp53*], and ‘PI3K-AKT signalling pathway’ (P=5.94E-05), containing 42 genes [*Bdnf*, *Ccnd1*, *Ccnd2*, *Cdkn1a*, *Col1a1*, *Col1a2*, *Col2a1*, *Col4a1*, *Col4a2*, *Col4a5*, *Col6a1*, *Col6a2*, *Col6a3*, *Creb3l1*, *Csf1*, *Ereg*, *F2r*, *Fgf10*, *Fgf2*, *Fgf7*, *Ghr*, *Gnb5*, *Gng12*, *Il4ra*, *Itga1*, *Itgb5*, *Lama4*, *Lamb1*, *Met*, *Osm*, *Osmr*, *Pdgfc*, *Pdgfra*, *Pdgfrb*, *Ppp2r3d*, *Sgk3*, *Tcl1*, *Tek*, *Thbs1*, *Thbs2*, *Trp53*, *Vegfd*] (Fruman et al., 2017, Herrero et al., 2016). Another interesting term was ‘Proteoglycans in cancer’ (P=8.95E-03), containing 25 genes [*Ank2*, *Cav1*, *Ccnd1*, *Cd44*, *Cdkn1a*, *Col1a1*, *Col1a2*, *Fas*, *Fgf2*, *Fzd1*, *Fzd8*, *Gpc1*, *Gpc3*, *Hspg2*, *Itgb5*, *Lum*, *Met*, *Pdcd4*, *Plce1*, *Sdc2*, *Sdc4*, *Tgfb2*, *Thbs1*, *Trp53*, *Twist2*]. Moreover, changes in proteoglycan expression are found in cancer and has been associated with promoting metastasis (Theocharis et al., 2015).

KEGG pathway analysis (Nr of genes)



GO term	P-value Corrected with Bonferroni step down	% Associated Genes
PI3K-Akt signaling pathway	5.94E-05	11.73
Proteoglycans in cancer	8.95E-03	12.25
Focal adhesion	3.71E-03	12.56
Protein digestion and absorption	4.38E-07	23.33
Cell cycle	5.26E-04	16.26
Lysosome	5.95E-04	16.13
ECM-receptor interaction	2.03E-05	21.69
AGE-RAGE signaling pathway	6.92E-03	15.84
p53 signaling pathway	3.24E-04	21.13
TGF-beta signaling pathway	2.81E-02	15.38
Melanoma	3.74E-02	16.67

Figure 6.6 KEGG pathway analysis of genes downregulated upon loss of B-Myb in ESCs
Downregulated genes with a p-value adjusted of less than 0.1 were subjected to gene ontology analysis using the KEGG pathway ontology source through ClueGo. Bar chart presents the 11 ontology groups resulting from the analysis with the respective number of genes per group. Table presents the p-value corrected with Bonferroni step down for each GO term, along with the percentage of genes from each term that were found in the data set.

6.3 Discussion

6.3.1 B-Myb is bound to chromatin in unperturbed and stressed conditions

A direct interaction between B-Myb and the MRN complex in unperturbed conditions was recently demonstrated. Moreover, in response to UV induced DNA damage, B-Myb was phosphorylated by ATM before dissociating away from the damage site to partake in transcriptional activity in somatic cells (Henrich et al., 2017). Data presented here supports that B-Myb is bound to chromatin in unperturbed conditions. However, treatment with CPT did not seem to influence the localisation of B-Myb to chromatin at all, which argues against the dissociation of B-Myb observed in the mentioned study (Henrich et al., 2017). A possible explanation could be that the dissociation of B-Myb from sites of DNA damage started within 5 minutes and by 30 minutes, B-Myb was completely dissociated (Henrich et al., 2017). Data presented here represents the effect of one hour of continuous CPT treatment; therefore, the cellular response could differ. Inhibition of ATM also had no influence upon the localisation of B-Myb to chromatin in unperturbed nor CPT treated conditions suggesting ATM is not important for B-Myb recruitment in ESCs. However, this does not rule out a potential role for ATM in the activation of B-Myb which would require further investigation. The transcriptional influence of B-Myb upon chromatin accessibility and cell invasiveness

Global transcriptome analysis was performed to explore transcriptional changes upon loss of B-Myb in ESCs. *H2afx*, which encodes a known tumour suppressor, was found downregulated in the *B-myb*^{Δ/Δ} ESCs (Bonner et al., 2008, Redon et al., 2012). Phosphorylation of H2AX (Ser139) influences chromatin accessibility with roles in DDR activation and DSB repair (Sharma et al., 2012). Amongst the most strikingly changed genes was *Kdm5d* (Lysine demethylase 5D), which presented complete loss of expression in the *B-myb*^{Δ/Δ} ESCs (Figure 6.5). Moreover, this expression profile could suggest that *Kdm5d* is a direct transcriptional target of B-Myb. KDM5D is a histone demethylase which has been shown to specifically target

Histone 3 lysine 4 trimethylation (H3K4me3) (Blair et al., 2011). High levels of H3K4me3 are associated with transcriptionally active chromatin (Nightingale et al., 2006), and is associated with bivalent domains in pluripotent cells, marking genes which are poised for rapid activation upon initiation of differentiation (Bernstein et al., 2006). Therefore, loss of B-Myb potentially has an influence upon chromatin accessibility which could help to explain the phenotypes observed during DNA replication.

One theory is that a decrease in KDM5D may result in increased transcriptional activity which could interfere with DNA replication resulting in replication stress. Moreover, changes in transcription have been shown to influence replication progression through forming a physical barrier to fork progression (Kotsantis et al., 2016). It would be of interest to observe whether global levels of transcription are elevated upon loss of B-Myb. It has also been described that sites of DNA damage are marked by H3K4 methylation to promote DNA repair (Gong et al., 2017). Thus, the decrease in demethylase activity could also be a response to the increased levels of DNA damage observed in the *B-myb*^{Δ/Δ} ESCs.

As loss of KDM5D would result in a more open chromatin state, an alternative theory is that the decrease in KDM5D expression could be responsible for the abnormal levels of origin firing observed in the *B-myb*^{Δ/Δ}. Chromatin is constantly manipulated during S phase to ensure efficient DNA replication progression (Groth et al., 2007, Jones and Petermann, 2012) There is evidence that origin initiation is partly determined by chromatin accessibility, plus, KDM5 demethylases have been implicated in control of replication behaviour with a direct influence upon replication origins (Rondinelli et al., 2015). A useful experiment would be to overexpress or activate KDM5D possible through CRISPRa, to determine whether loss of expression of this gene contributes to the replication programme deregulation observed upon loss of B-Myb.

Both KDM5D and H2AX have been implicated in a role in the epithelial to mesenchymal transition through their influence upon chromatin accessibility (Weyemi et al., 2016, Shen et al., 2019). Recent work has revealed a role for B-Myb in regulating the expression of mesenchymal to epithelial transition (MET) associated genes through its influence upon chromatin accessibility (Ward et al., 2018). Also, supporting a role for B-Myb in the epithelial to mesenchymal transition (EMT) or MET, knockout of B-Myb in breast cancer cells restores the expression of E-cadherin and suppresses cell invasiveness. In contrast, its overexpression resulted in loss of E-cadherin and gain of mesenchymal associated markers (Tao et al., 2015). Therefore, these findings further support a potential role for B-Myb in regulating chromatin accessibility which has the potential to influence EMT/MET gene expression as described in the literature (Ward et al., 2018).

6.3.2 The role of B-Myb in regulation of genes associated with cell cycle progression

Previous published work demonstrates that B-Myb is involved in the transcription of an array of genes associated with cell cycle transitions. Initial studies described a transcriptional role for B-Myb regulating the expression of genes involved in governing the transition into S phase (Iwai et al., 2001, Musa et al., 2017). However, more recent studies have found that B-Myb is more associated with transcription of G2-M transition associated genes, including *Ccnb1*, *Ccnb2*, *Aurka*, *Prc1*, *Cenpf*, *Plk1* and many more (Iltzsche et al., 2017, Osterloh et al., 2007, Sadasivam et al., 2012, Wolter et al., 2017, Musa et al., 2017).

In this study, both *Prc1* and *Aurka* were found downregulated in the *B-myb*^{Δ/Δ} ESCs in the RNA sequencing analysis amongst the top 50 most altered genes, supporting these previous studies and a role for B-Myb in regulating some genes associated with G2-M transition. Recently, a role for FOXM1 in regulation of *Aurka* expression was revealed. Overactivation of this axis was found in RAF or RAS mutated cancers and inhibition of this axis was represented as a suitable therapeutic target (Puig-Butille et al., 2017). Moreover, this study, plus the described

involvement of B-Myb in FOXM1 activity, highlights a potential signalling axis involving FOXM1, B-Myb and AURKA important for maintaining normal cellular growth (Down et al., 2012).

In this experiment, the *B-myb*^{Δ/Δ} ESCs did not present significant changes in expression of a whole range of genes which have been described in previous studies. A possible explanation is that although B-Myb transcriptional activity has been described as quite widespread through binding to the relatively abundant myb binding sites found throughout the genome, B-Myb transcriptional activity has also been described as weaker than the other myb family transcription factors due to its suppression by cell cycle associated post translational modifiers (Sala, 2005). In addition, this experiment was performed in stem cells in which cell cycle associated signalling is significantly altered compared to somatic cells which may have an influence upon the transcriptional activity of B-Myb (Ahuja et al., 2016, Coronado et al., 2013). It could also be that the conditions used in the RNA sequencing analysis to sort the significantly altered genes, could be too stringent to pick up the subtle changes in expression exerted by B-Myb. It is also possible that previous studies reporting transcriptional changes exerted by B-Myb look at the effect immediately after B-Myb knockout, and therefore may be pulling out changes associated with an acute stress response, whereas, the data presented here represents the expression after more long-term B-Myb loss.

Gene ontology analysis revealed various ontology groups including the ‘cell cycle’ group revealing genes such as *Cdkn1b*, *Cdkn2a* and *Cdkn2b* involved in regulating the production of multiple proteins including tumour suppressor proteins such as P16 and P27 involved in regulating cell cycle progression (Wiesner et al., 2010). ‘p53 signalling’ and ‘PI3K-AKT signalling’ were also significant GO terms. Moreover, both of these pathways have well characterised roles in regulation of cell cycle and growth behaviour, with their deregulation commonly associated with cancer cell growth (Herrero et al., 2016, Fruman et al., 2017). Also

of interest was the ‘Proteoglycans in cancer’ GO term. Changes in expression of genes associated with proteoglycans, such as *Sdc2* and *Fgf2*, have been linked to processes such as cellular growth and angiogenesis important for tumour growth and metastasis (Theocharis et al., 2015, Li et al., 2019, Nikitovic et al., 2018, Sayyad et al., 2019, Zaravinos et al., 2012). Data here partly supports the proposed role of B-Myb in changes in expression of genes important for growth and cell cycle control. B-Myb has been shown to function as both an oncogene and tumour suppressor protein (Musa et al., 2017, Garcia and Frampton, 2006, Lorvellec et al., 2010, Martinez and Dimaio, 2011). Moreover, loss of tumour suppressor activity or expression of oncogenes in cancer has been related to the development of replication stress through deregulation of the cell cycle and replication control (Dominguez-Sola et al., 2007, Dereli-Oz et al., 2011, Neelsen et al., 2013). Therefore, it may be that the observed changes in gene expression could have some influence upon the genome instability observed in the B-*myb*^{ΔΔ} ESCs.

6.3.3 Limitations of the RNA sequencing

Although the RNA sequencing revealed useful information concerning the role of B-Myb in transcriptional regulation, there are also some key limitations. A potential issue is that certain genes may present a small fold-change, but this could also represent complete loss of just one or a few exons, which may be vital for normal production of the gene protein product. An example of this is B-*myb* itself. Although B-*myb* expression was highly significantly changed ($p=3.04 \times 10^{-8}$) in the B-*myb*^{ΔΔ} ESCs, it only presented a fold change of 1.48. This was surprising as the B-*myb*^{ΔΔ} ESCs present complete loss of B-Myb protein. Further analysis of B-*myb* expression using the UCSC genome browser revealed that the expression of exons 2-5 were completely ablated and exons 1 and 5-17 presented normal expression levels (Figure 6.7). Deletion of these exons must be sufficient to impair B-Myb protein production and activity.

Therefore, it may be that other genes present a similar outcome, but these genes would be lost in the analysis performed.

Overall, the RNA sequencing revealed that loss of B-Myb results in global changes to the transcription programme which could contribute to the cellular changes observed in the B-*myb*^{Δ/Δ} ESCs. It was demonstrated in previous chapters that the B-*myb*^{Δ/Δ} ESCs present G2-M checkpoint deficiency and also continue proliferating under conditions of chronic replication stress. Data here demonstrates deregulation of key cell cycle genes in the B-*myb*^{Δ/Δ} ESCs, proposing a potential mechanism for this unusual behaviour. Interestingly, there were no obvious changes in expression of genes involved directly in DNA replication, DNA damage response or DNA repair. It could be that the changes in transcription observed alter replication behaviour indirectly. Alternatively, B-Myb may influence replication independently of its role in transcription. Considering the pleiotropic roles for B-Myb identified in the literature, it is plausible that a combination of different mechanisms are responsible for the genome instability observed.

B-myb (*Mybl2*)

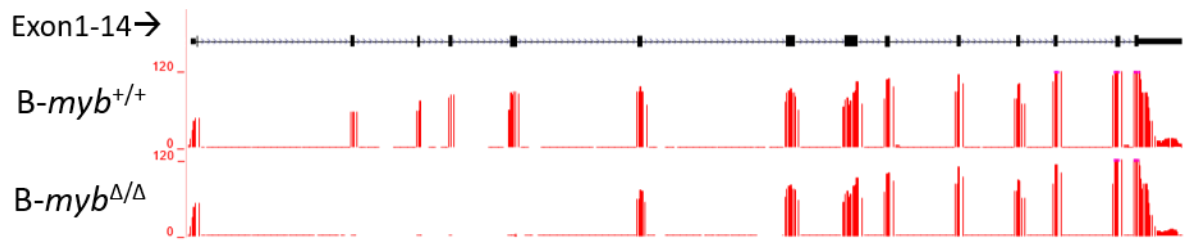


Figure 6.7 Gene expression profile of *B-myb* (*Mybl2*) in *B-myb*^{+/+} versus *B-myb*^{Δ/Δ} ESCs Using UCSC genome browser, the gene expression profile of *B-myb* (*Mybl2*) was analysed. Shown in red are the different levels of expression of exons 1-14 between the *B-myb*^{+/+} and *B-myb*^{Δ/Δ} ESCs. The scale used is 0 – 120 arbitrary units.

Chapter 7 Final Discussion

7.1 An ATM dependant role for B-Myb in control of the replication programme in ESCs

The main aim of this study was to understand the mechanisms behind the replication stress and genome stability observed upon loss of B-Myb in ESCs. Recent findings support a potential role of B-Myb implicated in the DNA damage response (Ahlbory et al., 2005, Henrich et al., 2017). In this study, inhibition of ATM signalling was epistatic with B-Myb loss for both their influence upon DNA replication progression and also accumulation of DNA damage. Despite this, ATM dependent processes such as phosphorylation of KAP1 were unaffected upon B-Myb loss. Therefore, this study proposes that B-Myb functions as downstream target of the ATM dependent DDR with an influence upon control of the replication programme.

Due to the exclusive role of ATM in the response to DSBs, it was surprising to observe a requirement for ATM activity under unperturbed conditions in this system. One possible explanation is that ESCs could accumulate high levels of DNA damage during normal replication and ATM activity may be required to alleviate these intermediates. Alternatively, it could be that ATM activity is atypical in ESCs. In support of this theory, ESCs are shown to possess a highly stable genome (Coronado et al., 2013, Kapinas et al., 2013), plus, ESCs present a highly open chromatin landscape due to changes in the activity of chromatin modifiers, and the activity of ATM has been demonstrated to be dependent upon chromatin accessibility (Kapinas et al., 2013, Murga et al., 2007). The high levels of basal γ H2AX presented by stem cells also supports this (Ahuja et al., 2016). Therefore, more work is required to explore the potential differences in the activity of the DDR and how it contributes to the capacity of ESCs to maintain a highly stable genome.

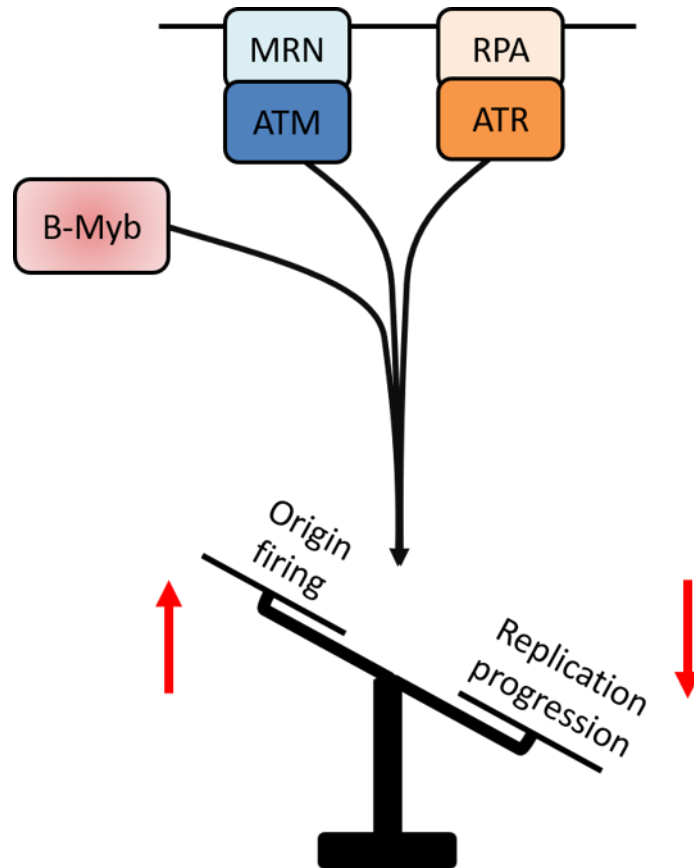


Figure 7.1 An ATM-B-Myb axis in control of DNA replication in ESCs This study suggests that B-Myb is important for control of replication dynamics through an influence upon the activity of the DNA damage response pathways, namely ATM dependent signalling. How B-Myb interacts with this pathway remains unknown. Loss of this pathway results in deregulation of replication control resulting in elevated origin firing, fork instability, DNA damage accumulation plus loss of G2-M checkpoint control. Therefore, this pathway is vital for normal cellular function and maintenance of genome stability in ESCs.

7.1.1 Determining how B-Myb interacts with the DDR

There are still outstanding questions concerning how B-Myb interacts with the identified pathways. Recent literature suggests that B-Myb interacts directly with the MRN complex involved in the ATM dependent DDR in somatic cell types (Henrich et al., 2017). Findings presented here could support this concept due to the identified epistatic relationship between B-Myb and ATM dependent activity. It was also demonstrated that B-Myb is primarily localised to chromatin, which would also agree with this theory. However, it is unclear from the data presented here if the shared role of B-Myb and ATM during replication is due to a direct interaction between them or whether their roles are independent from each other.

To overcome these questions, it would be useful to identify potential direct protein interaction between B-Myb and components of the DDR. This could be performed through proteomic techniques such as protein pulldown assays or mass spectrometry analysis of B-Myb binding partners. It would also be interesting to determine whether B-Myb localisation to chromatin is specific to replication forks, and if so, which proteins it is interacting with at forks. A way to do this would be to perform IPOND, which enables the isolation of proteins associated specifically with nascent DNA incorporated at replication forks (Sirbu et al., 2013). Overall, these experiments would help to identify whether the role of B-Myb is dependent upon direct protein binding.

7.1.2 B-Myb in control of origin firing

In this study, it was demonstrated that loss of B-Myb results in replication slowdown and elevated origin firing, however, from the data presented, it is unclear which of these events comes first. It could be that B-Myb has a direct influence upon replication origins. Alternatively, loss of B-Myb could result in a slowdown to replication potentially by forming a fork barrier; in this case, the increase in origin firing could be an indirect mechanism to compensate for the replication slow down.

Although inhibition of origin firing resulted in a rescue of the fork speed and fork instability observed, it did not rescue the DNA damage, potentially arguing against a direct role for B-Myb in controlling origin firing. However, it could be that the origin firing inhibitors were too toxic to produce a rescue or alternatively the DNA damage in the *B-myb*^{Δ/Δ} ESCs could be acquired through a different mechanism independent of origin firing. Therefore, more work is required to fully elucidate the mechanism for how B-Myb is influencing origin firing. Through performing mass spectrometry analysis, it may be possible to determine whether B-Myb could bind to proteins associated with replication origin or origin initiation. It would also be interesting to perform ChIP-sequencing to determine whether B-Myb binds to sites upon chromatin which are associated with sites of replication initiation.

7.1.3 A role for B-Myb in controlling replication progression through an influence upon CDK1 activity

A finding from this study was changes in the phosphorylation status of CDK1 upon loss of B-Myb in ESCs. In support of this, elevated CDK1 activity has been demonstrated to result in loss of G2-M checkpoint control (Malumbres, 2014), similar to that seen in the *B-myb*^{Δ/Δ} after treatment with CPT. Plus, CDK1 overactivity has also been shown to result in elevated origin firing and DNA damage due to nucleotide depletion, also a phenotype which is similar the that seen in the *B-myb*^{Δ/Δ} (Beck et al., 2012). Therefore, this data suggests that B-Myb, through CDK1 activity, is having an influence upon origin firing.

How B-Myb is influencing CDK1 status is still an outstanding question. From the RNA sequencing analysis, there was no clear expression changes in genes associated with CDK1 control, potentially ruling out a transcription dependant role. Alternatively, B-Myb may be having a direct influence upon a CDK1 activator or suppressor protein via a direct protein-protein interaction. Using mass spectrometry analysis, it would be possible to look at B-Myb protein interaction partners to determine whether this could contribute to changes in CDK1 status.

The data presented also provides no link between ATM and CDK1 activity. A link between ATM activity and CDK activation has already been described through regulating the activity of Artemis, with a role in G2-M checkpoint recovery (Geng et al., 2007). It would be valuable to observe whether inhibition of ATM, like B-Myb loss, also results in disruption of CDK1 activity; this could be achieved through performing further western blot analysis of phosphorylated CDK1. If ATM did influence CDK1 status and was also epistatic with B-Myb loss, this could potentially represent a novel ATM-CDK1-B-Myb axis in control of origin firing and the replication programme.

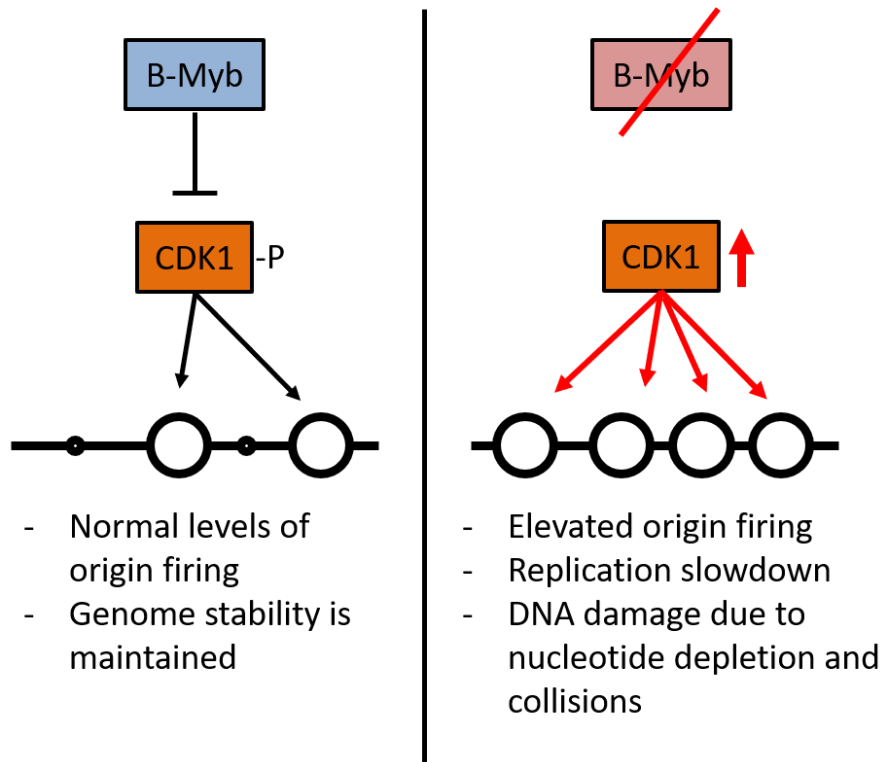


Figure 7.2 A potential role for B-Myb in regulating origin firing through regulating CDK1 activity
 CDK1 activity has been shown to influence the replication programme through an influence upon control of origin firing (Beck et al., 2012). Loss of B-Myb results in increased activation of CDK1 and replication stress. Moreover, this stress can be partially rescued through inhibition of origin firing. Therefore, this model proposes that B-Myb is important for control of the replication programme through an influence upon CDK1 activity.

7.1.4 A potential role for B-Myb in regulating replication through changes in chromatin accessibility

Recent evidence has demonstrated a potential role for B-Myb in regulating chromatin accessibility which relates to its reported role in expression of EMT associated genes implicated in the metastasis of tumours (Ward et al., 2018, Tao et al., 2015). The RNA sequencing analysis presented in this study revealed changes in the expression of genes associated with chromatin accessibility, including complete loss of expression of KDM5D, suggesting that KDM5D could be a direct transcriptional target of B-Myb.

KDM5D is a histone demethylase which has been shown to specifically target Histone 3 lysine 4 trimethylation (H3K4me3). Similar to the role of B-Myb as an oncogene, *Kdm5d* deregulation has been associated with cancer aggressiveness and metastasis through its demethylase activity (Li et al., 2016, Shen et al., 2019). H3K4 methylation has been associated with a role in maintaining normal replication initiation through regulating chromatin accessibility during S phase (Rondinelli et al., 2015, Groth et al., 2007). Also, a recent study demonstrated that loss of KDM5D was responsible for aggressive prostate cancer. Moreover, KDM5D was shown to bind to promoters of genes required for regulation of the cell cycle progression and replication. Loss of KDM5D expression was associated with acceleration of the cell cycle, increased mitotic entry and sensitivity to ATR inhibition, leading to increased DNA-replication stress (Komura et al., 2018). This phenotype is similar to that seen upon loss of B-Myb, strengthening the theory that changes in KDM5D could contribute to the replication stress observed (Figure 7.3).

A role in chromatin accessibility for B-Myb could also potentially explain how B-Myb is implicated in activation of ATM activity. It has been demonstrated that ATM activation is dependent upon chromatin context, moreover, manipulation of chromatin accessibility determines the strength of the DDR response (Goodarzi et al., 2008, Olcina et al., 2010, Murga et al., 2007, Olcina et al., 2013). Therefore, a plausible hypothesis is that B-Myb is required to

promote ATM binding and activation, which could explain why their activities are epistatic in ESCs. Overall, these theories are very preliminary, therefore, more work is required to investigate how B-Myb might be interacting with this gene. It would be interesting to overexpress KDM5D in the B-Myb or ATM mutant ESCs to observe whether there is any rescue of the replication stress observed.

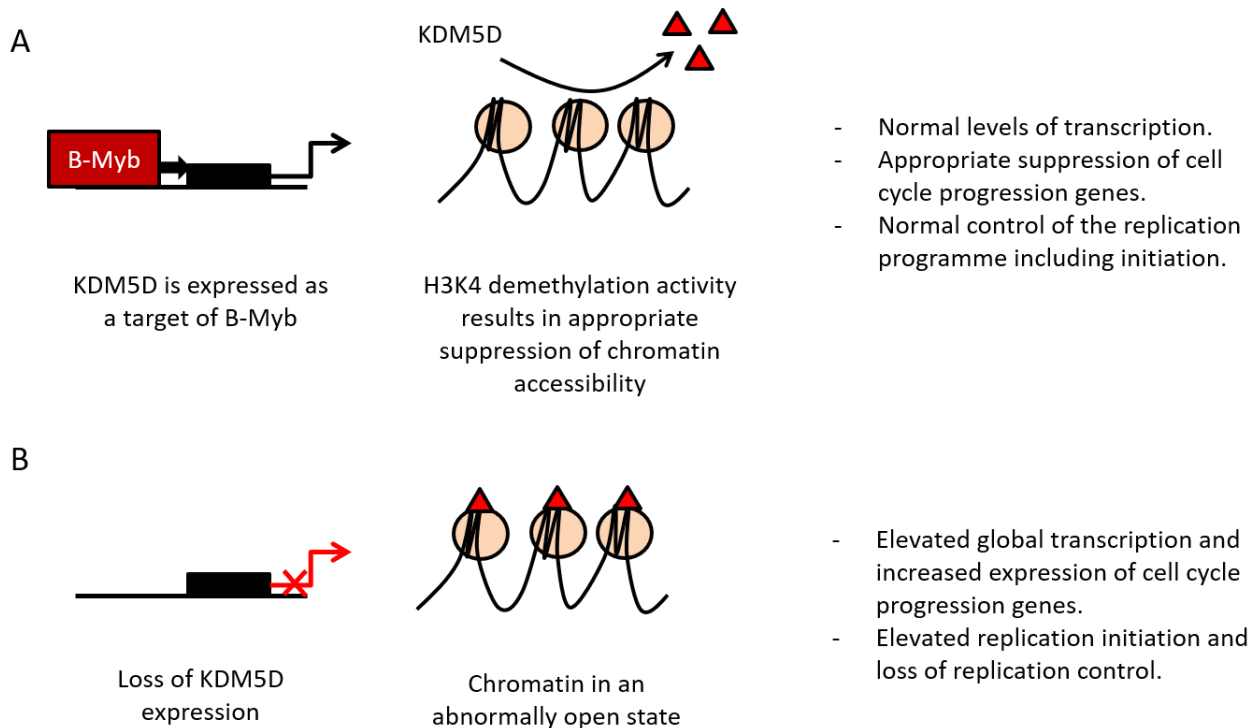


Figure 7.3 A potential role for B-Myb in regulating replication through an influence upon KDM5D expression RNA sequencing analysis revealed loss of KDM5D expression, involved in regulating of H3K4 methylation. A) In a normal situation, demethylation activity is important to control the transcription of cell cycle progression genes and the activity of the replication programme (Komura et al., 2018, Rondinelli et al., 2015). B) In the *B-myb*^{Δ/Δ} ESCs, KDM5D expression is lost. Therefore, it is proposed that loss of demethylation activity results in deregulation of transcription and replication which could contribute the replication stress observed.

7.2 Implications of research findings

7.2.1 B-Myb as a tumour suppressor

The role of B-Myb as an oncogene is well characterised, with B-Myb overexpression linked to increased cancer aggressiveness and poor patient outcome in multiple systems (Inoue and Fry, 2016, Ren et al., 2015, Bayley et al., 2018b, Calvisi et al., 2011, Nord et al., 2010, Sakai et al., 1993). Data presented here instead highlights the roles of loss of B-Myb expression, revealing its detrimental effects upon cellular function and genome stability. Recently, reduced B-Myb expression was demonstrated to result in worse prognosis for patients with myelodysplastic syndrome through defects in the response to DNA damage (Bayley et al., 2018a). Here, loss of B-Myb results in replication dysfunction and genome instability, which over time would accelerate cancer progression through an increased chance for genomic changes. These findings support that B-Myb is not only an oncogene, but also an important tumour suppressor protein.

7.2.2 Potential therapeutic opportunities

Recent work in various models have also revealed a potential function for B-Myb in DDR signalling (Bayley et al., 2018a, Henrich et al., 2017, Saldivar et al., 2018). The findings from this study builds upon this concept and proposes that the role B-Myb in DDR signalling is also implicated in control of DNA replication dynamics. The discovery of novel therapies targeting the DDR is currently sought-after, owed to the recent success of PARP1 inhibition for targeting BRCA1 or BRCA2 mutated cancers (Bryant et al., 2005, Farmer et al., 2005, Ledermann et al., 2015). Therefore, the identified role for B-Myb in DDR signalling highlights its potential, firstly as a biomarker for cancer patient outcome (Bayley et al., 2018b), but also as a possible target for development of anti-cancer therapeutics.

This work was performed in stem cells, representing a good model to investigate cancer cells, as they both possess atypical cell cycle control and elevated growth behaviour (Yu et al., 2012, Ahuja et al., 2016, Hanahan and Weinberg, 2011). Findings from this study provided novel evidence for an atypical requirement for ATM dependent signalling during DNA replication in ESCs. It would be interesting to determine whether similar DDR signalling could be observed in cancer stem cells (CSCs), which are thought to represent a small population of cells in all tumours (Yu et al., 2012, Kelly et al., 2007). CSCs are highly clinically relevant due to evidence suggesting they are responsible for resistance to anti-cancer therapies and may be involved in initiation of metastasis (Li et al., 2008, Meirelles et al., 2012, Phi et al., 2018, Li et al., 2007). In addition, the recently identified roles for B-Myb in both DDR signalling (Bayley et al., 2018a, Henrich et al., 2017) and in the EMT/MET control (Tao et al., 2015, Ward et al., 2018) suggests that B-Myb could be a key player in the behaviour of these cells. Therefore, although this concept is preliminary, it represents a potentially interesting avenue for identifying novel methods for targeting cancer stem cells to improve anti-cancer therapeutics and patient outcome.

Another interesting finding was an increased sensitivity to CPT observed after B-Myb loss. There has been ample work investigating the potential use of topoisomerase inhibitors for their use as anti-cancer therapeutics (Delgado et al., 2018). The loss of cell cycle and unscheduled replication behaviour, through increased collisions with TOPO1cc's, could contribute to the sensitivity to CPT seen in the B-*myb*^{Δ/Δ} ESCs (Shao et al., 1997, Furuta et al., 2006, Pommier, 2006). Alternatively, it could result from an inability to remove TOPO1cc's and to appropriately repair the resulting DNA damage (Regairaz et al., 2011, van Waardenburg et al., 2004). Therefore, more work is required to pinpoint the exact mechanisms for the sensitivity observed. It could be that B-Myb deficiency may serve as a marker for sensitivity to CPT for its use in cancer therapy.

7.2.3 Implications for somatic reprogramming

It is also important to mention the potential application of these findings with understanding the roles of B-Myb during somatic cell reprogramming. Recently, the correct levels of B-Myb were shown to be vital for successful reprogramming due to its deregulation resulting in abnormal changes to the chromatin landscape and accessibility of key pluripotency transcription factors to their target sites (Ward et al., 2018). Somatic cell reprogramming offers massive potential in the field of regenerative medicine and disease modelling; however, its potential is limited by several issues with the reprogramming process. This includes the presence of an epigenetic memory of the original somatic cell which limits their identity as stem cells (Halley-Stott and Gurdon, 2013). In addition, although iPSCs have been shown to share DDR properties with ESCs (Momcilovic et al., 2010), reprogramming induces widespread DNA damage due to the rearrangement of chromatin for which ATM dependent DDR signalling plays an important role (Kinoshita et al., 2011, Gonzalez et al., 2013). Moreover, the DDR has been shown to be involved in promoting cellular differentiation in response to stress

highlighting its influence upon these mechanisms (Inomata et al., 2009, Lin and Lin, 2017). The work presented here highlights important additional roles for both ATM and B-Myb activity in maintaining replication control and genome stability in stem cells (ESCs). Therefore, it would be of interest to determine whether manipulation of the DDR could improve efficacy of the reprogramming process improving understanding of the barriers to reprogramming.

7.3 Further questions and future perspectives

7.3.4 Limitations of the model used in this study

The model used in this study examined the effect of permanent B-Myb loss upon ESC function after several passages. This allowed proper investigation of the effect's loss of B-Myb has upon all aspects of ESC growth, rather than potentially acquiring false positives through recognising the immediate effects of B-Myb loss. In addition, this study used primary cells which are a better representation of normal cell types compared to the use of cancer cell lines (Pan et al., 2009). Despite this, there are also limitations with using this system. As observed in this study, loss of B-Myb has detrimental effects upon genome instability, over time this would result in elevated mutation rates and chromosome aberrations. Therefore, it is not certain whether the phenotypes observed are a direct cause of B-Myb loss, or whether they are due to changes induced by the progressive acquisition of mutations. However, this possibility was reduced through limiting the number of passages permitted before performing experiments. Another limitation to the model used is that the knockout was targeted to the DNA binding domain exclusively. Therefore, there is a chance that a truncated version of B-Myb could still be produced after the knockout. To overcome this limitation, western blot analysis using an antibody against the C-terminal region could be performed.

An additional limitation to the experiments performed in this study is the use of chemical inhibitors, for example, the ATM inhibitor. Confirmation of the ATM inhibitors efficacy was performed through staining for ATM dependent P-KAP1, with staining dissipating after the inhibitor treatment. However, there are limitations with using inhibitors as opposed to knockout systems. Firstly, using an inhibitor often only targets a specific aspect of the protein's activity, compared to gene knockout systems which abolish the protein completely. Different drugs also present different half-lives which may affect the results acquired depending on time of drug treatment. Plus, using the correct concentration is vital to ensure that the drug is working whilst also avoiding off-target effects. For these reasons, a gene knockout system may be considered more reliable. It would be beneficial for this study to generate ESCs with an ATM gene knockout to reliably determine the role of ATM in ESCs.

7.4 Conclusion

Overall, this study provides novel understanding for the role of B-Myb during S phase in mouse ESCs. The exact mechanism for how B-Myb interacts with the cellular machinery is still not fully elucidated, although, the multiple future experiments described would help to characterise this further. The findings presented contribute to understanding of how stem cells can maintain a highly stable genome whilst also presenting a highly proliferative cell cycle. In addition, this data not only has the potential to contribute to the understanding of cancer cell behaviour, but also represents a potential avenue for the discovery of novel anti-cancer therapeutic strategies.

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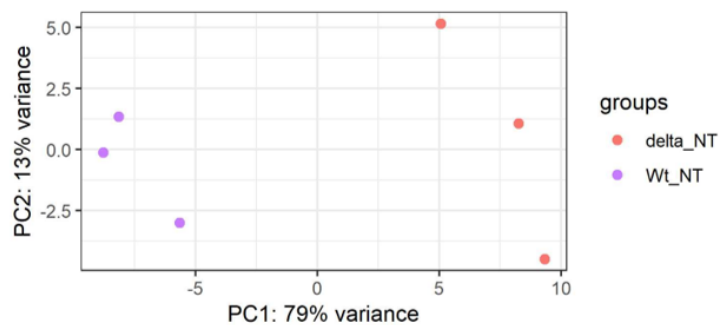
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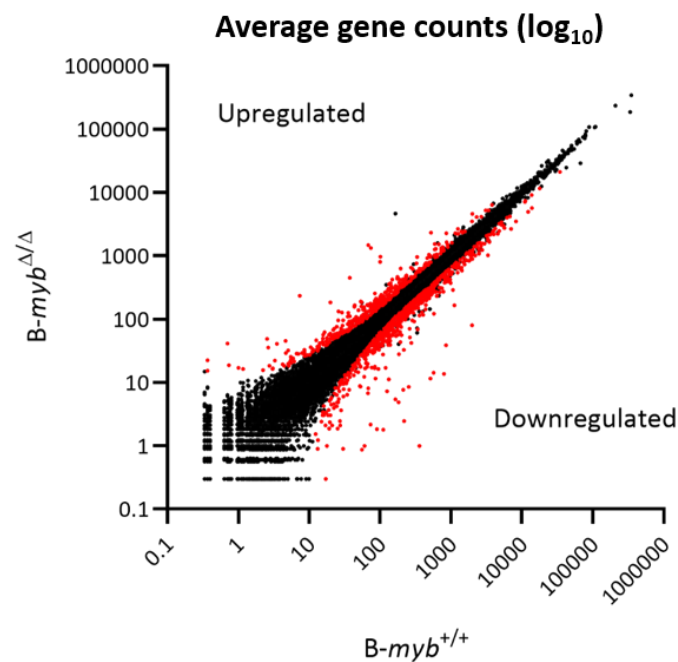
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Appendix

A

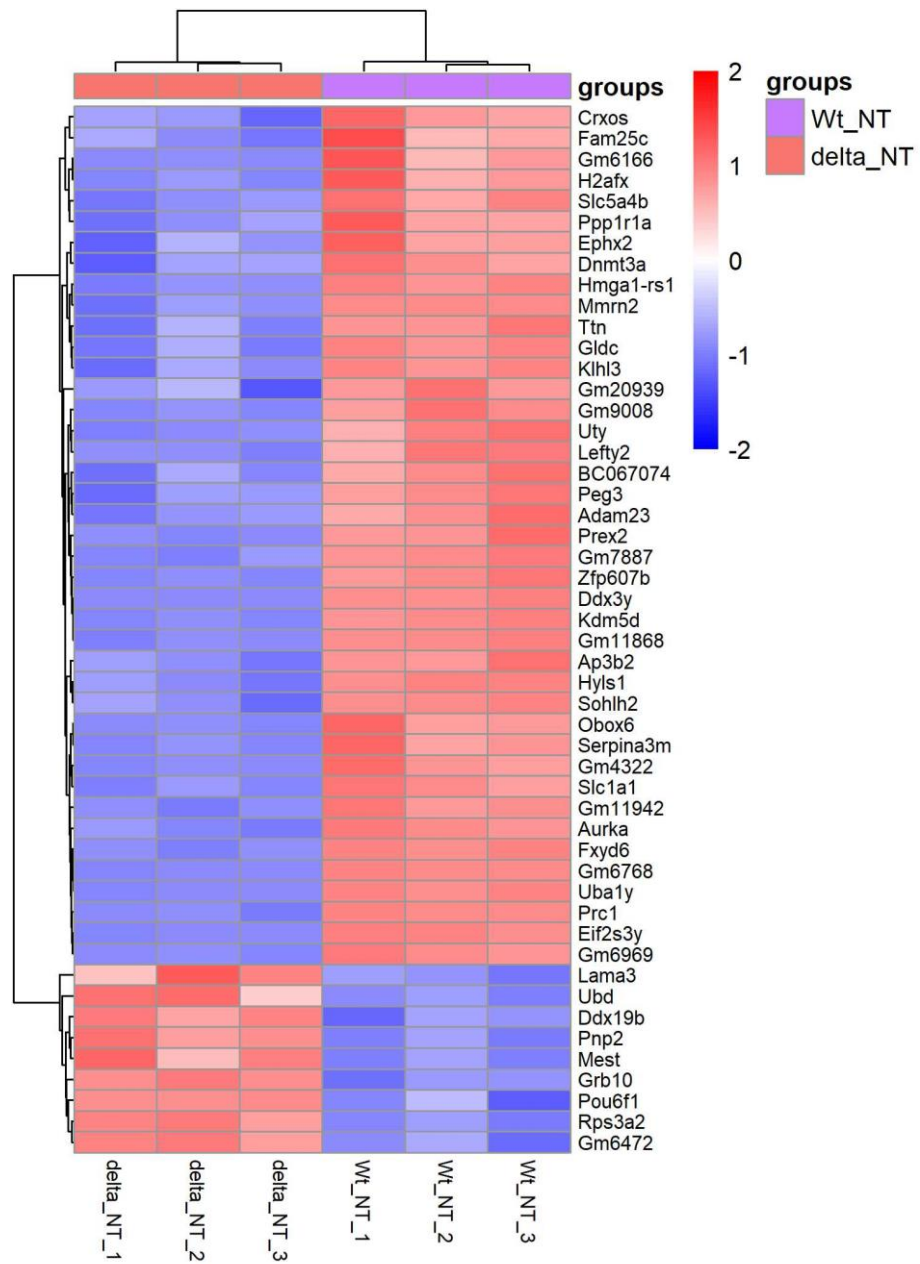


B



A) Principal component analysis (PCA) in the B-myb $^{+/+}$ versus the B-myb $^{\Delta/\Delta}$ groups PCA analysis was performed and plot generated by Dr Boris Noyvert from the CRUK Birmingham Centre. PCA plot demonstrating the clustering of individual samples from the B-myb $^{+/+}$ and the B-myb $^{\Delta/\Delta}$ groups analysed by RNA sequencing.

B) Differential expression analysis in B-myb $^{+/+}$ versus the B-myb $^{\Delta/\Delta}$ Differential expression analysis was performed by Dr Boris Noyvert. The resulting gene counts for each gene in the B-myb $^{+/+}$ versus the B-myb $^{\Delta/\Delta}$ were plotted using GraphPad prism. Each dot represents a single gene and red dots represent those genes with a p-value less than 0.05 and a fold-change of at least 1.5.



Top 50 genes with the most significantly altered expression in *B-myb*^{ΔΔ} ESCs Heatmap, generated by Dr Boris Noyvert through DeSeq2 analysis and in R. Genes downregulated are in blue and upregulated in red; scale represents z-score. In the top 50 most significantly changed genes, 41 are downregulated, and 9 genes are upregulated in the *B-myb*^{ΔΔ}.