The DNA replication stress checkpoint transcriptional response and its role in replication stress tolerance

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I, Anna Elizabeth Herlihy, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.
Abstract

DNA replication stress is defined as the slowing down or stalling of DNA replication forks. The DNA replication stress checkpoint prevents replication stress from developing into DNA damage and subsequent genome instability. This checkpoint response maintains E2F-dependent cell cycle transcription through inactivation of E2F6. However, the role and importance of transcription in response to replication stress remains largely unknown.

Work presented in this thesis shows that in mammalian cells, unlike yeast, active protein synthesis is required for an efficient checkpoint response. Many checkpoint effector proteins, which are E2F targets, are found to be unstable and so require sustained E2F-dependent transcription to maintain levels during replication stress. Maintaining correct protein levels is necessary for specific functions of the checkpoint response – replication fork stalling, stabilisation and protection, and resolving stalled forks after stress. E2F-dependent transcription is therefore required to prevent DNA damage following replication stress. Importantly, maintaining E2F-dependent transcription during replication stress in checkpoint-compromised cells is sufficient to prevent DNA damage.

These results suggest that sustained E2F-dependent transcription is a key mechanism in the replication stress response. Activated oncogenes increase E2F activity, driving uncontrolled proliferation, thought to be at the basis of oncogene-induced replication stress, an early event in tumourigenesis. Data presented shows E2F-dependent transcription is also required to prevent DNA damage during oncogene-induced replication stress; suggesting oncogenic cells rely on E2F-dependent transcription for tolerance to high levels of replication stress.

Given the important role of E2F-dependent transcription in the replication stress re-
sponse, the mechanism of E2F6-dependent repression is also investigated.

Overall my work establishes that E2F-dependent transcription is a key mechanism in the response to DNA replication stress and is necessary during oncogene-induced replication stress. The regulation and functions of this transcriptional response are elucidated, showing a far greater role than previously suspected in the response to DNA replication stress.
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List of Abbreviations

4OH-T - Hydroxytamoxifen
Aph - Aphidicolin
ATM - Ataxia Telangiectasia Mutated
ATR - Ataxia Telangiectasia and Rad3-related protein
ATRIP - ATR Interacting Protein
CDK - Cyclin-dependent kinase
CFS - Common Fragile Site
Chx - Cycloheximide
CMG - Cdc45/Mcm2-7/GINS
dNTP - Dinucleotide triphosphate
Doxy - Doxycycline
DSB - Double strand break
HU - Hydroxyurea
MEF - Mouse Embryonic Fibroblast
ORC - Origin Recognition Complex
PcG - Polycomb group
pre-RC - Pre-Replicative Complex
RNR - Ribonucleotide reductase
RPA - Replication protein A
ssDNA - Single stranded DNA
WT - Wild type
1. Introduction

1.1. The cell cycle

The cell cycle comprises a series of events, following a precise temporal order, that are required to coordinate cellular growth and proliferation to allow a cell to undergo cell division. The cell cycle is separated into distinct phases, starting with Gap phase 1 (G1), the first growth phase. Cells then pass the commitment point, and are committed to complete a full cell cycle, and enter Synthesis phase, S phase. Genome duplication occurs in S phase through the process of DNA replication. Cells then proceed through Gap phase 2 (G2) to M phase, where the cell divides (Morgan, 2007). It is essential that the DNA is faithfully replicated and equally divided between the two daughter cells. The daughter cells either continue to enter a new cell cycle, or exit the cell cycle and enter G0, or quiescence. Cell cycle progression is controlled by Cyclin-dependent kinase (CDK) activity, which is regulated by cell cycle transcription (Woo and Poon, 2003). In particular, waves of transcription regulate the accumulation of cell cycle phase specific cyclins that in turn regulate the key cell cycle transitions - G1/S, G2/M and M/G1. In addition, a number of checkpoints exist at these transition points which can arrest the cell cycle when problems occur (Malumbres and Barbacid, 2009).

To ensure the cell faithfully replicates all of its components and divides into two identical daughter cells, each transition, and its regulatory transcriptional network, must be tightly controlled. Entry into S phase, and therefore entry into the cell cycle, is driven by the activation of the G1/S wave of transcription. This wave of transcription comprises a large number of genes whose expression rises in late G1 phase and is then repressed in S phase (Bertoli et al., 2013b). This transcriptional network encodes for many of the components needed during S phase, such as those required for DNA replication and repair. Deregulation of G1/S transcription is found in every
type of cancer and promotes uncontrolled cell cycle entry and proliferation, one of the hallmarks of cancer (Hanahan and Weinberg, 2000). The upstream signalling pathway regulating G1/S transcription includes many oncogenes and tumour suppressors such as Myc, Ras, Cyclin/CDKs, CDK Inhibitors and pRb (Chen et al., 2009; Stevens and La Thangue, 2003). The mechanism controlling the regulation of G1/S transcription activation and repression is conserved from yeast to human, although the proteins involved are not (Bertoli et al., 2013b). In mammalian cells, G1/S transcription is controlled by the E2 promoter binding factor (E2F) family of transcription factors and is known as E2F-dependent transcription (Stevens and La Thangue, 2003; Tsantoulis and Gorgoulis, 2005).

1.2. E2F-dependent transcription

E2F-dependent transcription plays a key role in controlling cell cycle progression. In particular, it is responsible for controlling G1/S transcription, but has also seen to be involved in G2/M target regulation (Ishida et al., 2001). E2F-dependent transcription is regulated by a family of eight transcription factors, E2F1 to E2F8 (Tsantoulis and Gorgoulis, 2005), with their dimerisation partners DP1 or DP2 (Girling et al., 1993; Zhang and Chellappan, 1995). In addition, there are three co-regulatory proteins known as the pocket proteins, pRb, p107 and p130, which interact with E2F transcription factors and are required for regulation (Helin et al., 1993; Sardet et al., 1995). Many members of the E2F family show considerable sequence homology, with E2F1 to E2F6 and DP1 and DP2 sharing DNA binding and dimerisation domains. E2F1 to E2F5 also share transactivation and pocket protein binding domains (Stevens and La Thangue, 2003). The E2F transcription factors are divided into 3 main subgroups. E2F1, E2F2 and E2F3a function primarily as activators whose activity is constrained by pRb binding (Helin et al., 1993; Kovesdi et al., 1986; Lees et al., 1993). pRb binding inactivates the E2Fs and prevents the binding of the transcription initiation complex, and also recruits HDACs and nucleosome remodellers to alter chromatin structure and repress transcription (Tsantoulis and Gorgoulis, 2005). E2F3b, E2F4 and E2F5 act primarily
as repressors in conjunction with the pocket proteins p107 and p130 (Sardet et al., 1995). E2F4 and E2F5 are cytoplasmic proteins and require pocket protein binding for nuclear localisation and subsequent transcriptional repression (Gaubatz et al., 2001). The final group E2F6, E2F7 and E2F8 display pocket protein independent repression (Cartwright et al., 1998; De Bruin et al., 2003; Gaubatz et al., 1998; Logan et al., 2005; Morkel et al., 1997; Trimarchi et al., 1998). The DP proteins are required to optimise DNA binding, and may also influence binding specificity to some extent (Tao et al., 1997). The situation is even more complex, as depending on the specific context the activators can sometimes function as repressors and visa versa. In addition, compensation also occurs, for example E2F4 compensates for E2F6 loss in mouse embryonic fibroblasts (MEFs), although the reverse is not true (Giangrande et al., 2004). The different transcription factors also display specificity to different subsets of E2F targets, although this can also vary (Tao et al., 1997).

E2F-dependent transcription is responsible for regulating G1/S transcription in mammalian cells. In G0, E2F4 binds to promoters with the pocket protein p130 and represses E2F-dependent transcription (Takahashi et al., 2000). During early G1 phase, the binding of pRb to the activators E2F1, E2F2 and E2F3a prevents transcriptional activation (Helin et al., 1993). Mitogens cause increased Cyclin D1 levels, which results in a Cyclin D1/CDK4 complex which phosphorylates pocket proteins and sequesters the CDK inhibitor p27, this allows for Cyclin E/CDK activity that further phosphorylates pocket proteins and targets p27 for degradation (Sherr and McCormick, 2002). Cyclin E dependent inactivation of pRb relieves inhibition of the activator E2Fs, which robustly activates E2F-dependent transcription. This activation of E2F-dependent transcription must act as an irreversible switch. To ensure this a positive feedback loop is activated whereby E2F-dependent transcription encodes a further increase in levels of Cyclin E, which further phosphorylates and inactivates the pocket proteins (Lundberg and Weinberg, 1998) to ensure the switch like behaviour of this transcriptional wave. E2F-dependent transcription also encodes for the S phase Cyclin/CDKs, these
phosphorylate targets in the pre-Replicative Complex to initiate DNA replication (Woo and Poon, 2003; Zegerman and Diffley, 2009). This mechanism of activation ensures a wave of transcription controlling the transition from G1 to S phase, with the coordinated initiation of DNA replication once the cell has entered S phase.

The repression of E2F-dependent transcription in S phase involves multiple negative feedback loops to ensure robust inactivation. E2F targets include Cyclin A and Cyclin E, which both contribute to indirect negative feedback loops to turn off E2F-dependent transcription. These Cyclin/CDKs act to inhibit p27, a CDK inhibitor, thereby relieving inhibition and increasing Cyclin A/CDK activity (Montagnoli et al., 1999). The up-regulation of Cyclin A and removal of p27 inhibition allows Cyclin A to phosphorylate the activator E2Fs, this results in dissociation from promoters and a loss of activation of E2F-dependent transcription (Xu et al., 1994). The E2F transcriptional repressors, E2F6, E2F7 and E2F8, are E2F targets themselves, which constitutes an additional negative feedback loop. These repressors accumulate during S phase, bind promoters and inactivate E2F-dependent transcription (Giangrande et al., 2004; Westendorp et al., 2012). Altogether these regulatory mechanisms ensure a wave of transcription, triggered by the activation of cyclin/CDK activity, which peaks at the G1/S transition, and is repressed in S phase. The presence of both positive and negative feedback loops in this mechanism ensures that transcription displays a switch-like activation, for cell cycle entry commitment, and a robust inactivation, to prevent inappropriate transcription and possible genomic instability.

Robust inactivation of E2F-dependent transcription is required in S phase in order to separate the cell cycle phases (Giangrande et al., 2004), and is thought to prevent cellular damage due to inappropriate expression of E2F targets. It has been shown that overexpression of individual E2F targets can result in the misregulation of DNA replication and subsequent DNA damage and genome instability. Cyclin E overexpression prevents correct Mcm helicase loading (Ekholm-Reed et al., 2004) and increases origin firing (Jones et al., 2013), whereas overexpression of the licensing factors Cdc6 and
Cdt1 causes re-replication (Liontos et al., 2007), both result in replication stress, DNA damage and genome instability (Teixeira et al., 2015). In addition, collisions between the transcription and replication machinery has been seen to cause replication stress and genome instability (Helmrich et al., 2011; Jones et al., 2013; Kotsantis et al., 2016; Tuduri et al., 2009, 2010). Deregulated E2F-dependent transcription may increase the frequency of collisions and so induce genome instability.

1.3. DNA replication

Before division a cell must replicate its DNA such that each daughter cell receives a complete and faithfully replicated genome. Failure to fully replicate DNA results in daughter cells receiving an incomplete genome. This can result in loss of gene function, which may ultimately lead to cell death or drive cancer development (Hanahan and Weinberg, 2000). To ensure the genome is duplicated in a timely manner, DNA is replicated during S phase in a semi-conservative process, initiated at multiple DNA replication origins throughout the genome (Cayrou et al., 2011). This process is tightly controlled to ensure DNA is replicated once and only once during each cell cycle. Replication initiation consists of two distinct stages temporally separated during the cell cycle. Licensing of origins occurs in G1 phase under conditions of low CDK activity, whereas firing of origins and replication initiation requires high CDK activity and occurs in S phase (Yeeles et al., 2015). High CDK activity inhibits origin licensing in S phase and therefore prevents re-replication. There are many more origins licensed in G1 than are used in each S phase (Cayrou et al., 2011). Origins can be constitutive, flexible or dormant (Fragkos et al., 2015). Constitutive origins are used in all cells in every cell cycle, whereas usage of flexible origins varies between cell types and cell cycles in a largely stochastic manner. Dormant origins are not used under normal conditions, but they are important to ensure complete genome duplication in response to DNA replication stress (Ge et al., 2007).

The licensing of replication origins occurs in conditions of low CDK activity during G1
phase, by the loading of a pre-Replicative Complex (pre-RC) to origins, this pre-RC comprises the Origin Recognition Complex (ORC), Cdc6 and Cdt1. The ORC consists of six subunits, Orc1 - Orc6, and establishes the sites of replication origins (Fragkos et al., 2015). Cdc6 and Cdt1 are then recruited, these factors are involved in loading of the Mcm2-7 double hexamer helicase (Cook et al., 2002; Rialland et al., 2002). In G1 phase the firing of origins is prevented by the low levels of CDK activity.

During S phase the Dbf4-dependent kinase (DDK or Cdc7-Dbf4 complex) and Cyclin/CDKs phosphorylate pre-RC components. This drives the recruitment of the full Cdc45/Mcm2-7/GINS (CMG) complex, which comprises Cdc45, Mcm2-7, GINS complex, RECQL4 (Sld2 in yeast) and Mcm10. This allows for DNA replication to be initiated bidirectionally outwards from a limited number of licensed origins. Activation of the CMG complex in mammalian cells is dependent on CDK phosphorylation of Treslin (Sld3 in yeast) and its subsequent interaction with TOPBP1 (Dbp11 in yeast) and the CMG complex (Labib, 2010). This activation results in the unwinding of DNA and the double Mcm hexamer separating to form two replication forks proceeding bidirectional out from each origin. The replication fork contains all the proteins required for DNA replication including the DNA helicase, DNA polymerases, PCNA and checkpoint mediators that monitor replication fork integrity. During DNA replication, the helicase unwinds the two strands of DNA revealing short stretches of single stranded DNA (ssDNA), which is protected by the ssDNA binding protein Replication Protein A (RPA) (Zou et al., 2006). DNA is replicated in a 5’ to 3’ direction, which allows continuous replication of one strand, the leading strand, whereas the other strand, the lagging strand, is replicated in a discontinuous manner with Okazaki fragments.

Origin licensing must be inhibited during S phase to prevent re-licensing and re-replication, which can result in gene amplification and DNA damage (Liontos et al., 2007). Re-replication is prevented by a number of mechanisms. First, dormant origins are disassembled by passive replication (Santocanale et al., 1999). Second, the high Cyclin/CDK levels in S phase result in phosphorylation of pre-RC components and prevents assem-
bly of new pre-RCs onto DNA in S phase (Nguyen et al., 2001). Third, the S phase protein geminin binds to Cdt1 and prevents new pre-RC formation (Wohlschlegel et al., 2000). Both Cyclins and geminin are degraded by the APC/C, preventing this inhibition in G1 (Yekezare et al., 2013). Finally, Cdt1 and Orc1 are targeted for degradation by Cullin-based E3 ubiquitin ligase activity (Li et al., 2003; Méndez et al., 2002). All of these regulatory mechanisms ensure the genome is replicated once and only once during each cell cycle.

1.4. DNA replication stress

During DNA replication, problems can result in DNA replication stress. The definition of replication stress is still evolving (Muñoz and Méndez, 2016). However, this work will use the emerging consensus definition that DNA replication stress is the slowing down or stalling of the replication fork which usually results in extended lengths of ssDNA (Macheret and Halazonetis, 2015; Toledo et al., 2013; Zeman and Cimprich, 2013). DNA replication stress has a number of consequences for the cell. The time taken to complete genome duplication increases due to the slow progression of forks and the global inhibition of late origin firing (Ge and Blow, 2010). Therefore, the end of S phase must be delayed to ensure all DNA is replicated before the cell enters mitosis. In addition, a stalled replication fork may result in inappropriate intermediate structures, which have to be resolved before replication can be continued to prevent DNA damage (Branzei and Foiani, 2010). A more prolonged period of stress can cause fork collapse, whereby the replisome, the complex of proteins associated with the replication fork, may dissociate from DNA, this can result in double strand breaks (DSBs) (Hanada et al., 2007). A collapsed fork cannot restart and replication cannot be initiated from this position as licensing is inhibited during S phase. In order for replication to be completed, a dormant origin close by must to activated to rescue this stalled fork and complete DNA replication (Petermann et al., 2010).

DNA replication stress can be experienced by cells under normal physiological condi-
tions or due to exogenous factors. There are a number of factors that can cause DNA replication stress, which I will now discuss in turn.

**Causes - component regulation**

As the building blocks of DNA, dinucleotide triphosphates (dNTPs) are essential for DNA replication. All four dNTPs - Adenine, Cytosine, Guanine and Thymine (A, C, G and T) - are required at correct levels for the efficient and accurate replication of DNA. dNTPs are produced by a series of enzymes, most notably the ribonucleotide reductase enzyme (RNR) (Aye et al., 2014). Low levels of RNR activity results in low levels of dNTPs, which is unable to support full efficient DNA replication. The replication fork therefore slows and DNA replication stress occurs. Replication stress caused by a low dNTP pool, such as following aberrant activation of Cyclin E, can be rescued by exogenous addition of nucleosides (Bester et al., 2011). To model replication stress experimentally, the drug Hydroxyurea (HU) is commonly used, this inhibits the ribonucleotide reductase enzyme (RNR) to reduce dNTP levels and cause DNA replication stress.

There are also many other components required for DNA replication both in the replisome complex and others. The misregulation of these components can also cause DNA replication stress. For example, inhibition of DNA polymerase by Aphidicolin (Aph) is known to cause replication stress. The loss of essential proteins can prevent DNA replication from occurring and so replication forks will stall. As there are so many replication origins which fire simultaneously the levels of proteins must be sufficient to support replication at all these replication forks simultaneously. A small reduction in a protein may result in some replication forks proceeding correctly, but other forks not containing enough components to replicate properly. As mentioned previously, E2F-dependent transcription encodes for many proteins required in S phase and particularly for DNA replication, such as Mcm2, Mcm5, Cdc45 and Polε subunit B (Bertoli et al., 2013a). Therefore, misregulation of E2F-dependent transcription may alter the levels of important replication proteins and may result in replication stress.
Causes - DNA characteristics

There are certain DNA sequences which are inherently difficult to replicate and so can cause replication stress. Repeats, such as dinucleotide, trinucleotide, inverted and tandem repeats can result in alternative DNA structures, which can block replication fork progression (Mirkin and Mirkin, 2007). In addition, these repeats often experience slippage, resulting in expansion of these repeats (Viguera et al., 2001). These repeat sequences, and others, can result in secondary DNA structures that are hard for replication forks to travel through. These include cruciforms, G-quadruplexes, hairpins and Z-DNA (Muñoz and Méndez, 2016). In addition to these structural challenges to DNA replication, sections of the DNA containing low origin density can also be difficult to replicate, due to a lack of dormant origins to rescue stalled forks.

Common fragile sites (CFSs) are specific sites of the genome which display high rates of replication fork stalling, DSBs and chromosomal rearrangements (Debatisse et al., 2012; Durkin and Glover, 2007). These CFSs are sensitive to breakage following even mild levels of replication stress (Gorgoulis et al., 2005; Helmrich et al., 2011; Tsantoulis et al., 2008). The exact cause of CFS expression is under debate. However, it is likely due to some or all of the DNA characteristics described above - difficult to replicate repeats, strong secondary structure and low origin density (Debatisse et al., 2012; Durkin and Glover, 2007).

Causes - obstructions to replication

DNA is not an isolated structure, proteins bound to DNA, which are vital for cellular function, may obstruct replication fork progression (Ivessa et al., 2003). Most obviously, DNA is packaged into chromatin and is therefore tightly associated with histone proteins. It has been seen that heterochromatic regions show increased sites of DNA damage, suggesting chromatin state can affect DNA replication (Rozenzhak et al., 2010; Szilard et al., 2011). There are a wide range of other proteins, such as the pre-RC at dormant origins and kinetochores at centromeres, which must be tightly bound to
DNA and may impede replication fork progression. Replication Fork Barriers also exist, which deliberately recruit proteins to stall replication forks. These are often unidirectional and help to prevent replication and transcription collisions, a cause of replication stress discussed below (Lambert and Carr, 2013).

DNA damage can form bulky lesions, which can stall replication forks (Zeman and Cimprich, 2013). There are many types of DNA damage and many mechanisms of repair depending on the specific damage and the cell cycle phase (Branzei and Foiani, 2008). Different types of DNA damage will impede replication fork progression in different ways. The outcome of replication forks reaching DNA damage also differs depending on the type of damage. For example, DNA adducts will stall forks and require translesion synthesis or template switch for continued replication, whereas single stranded DNA breaks will be processed to DSBs by the replication fork (Branzei and Foiani, 2010).

Another factor that can cause replication stress is collisions between DNA replication forks and transcriptional bubbles (Helmrich et al., 2011; Jones et al., 2013; Kotsantis et al., 2016). Whilst the genome must be replicated, transcription occurs simultaneously to encode for proteins required for cellular function. Replication forks and transcriptional bubbles move along the same template and can collide in-line or head-on (Zeman and Cimprich, 2013). These collisions cause topological problems, even before they collide, due to positive supercoiling between them and the reduced mobility caused by the tethering of the transcription machinery to the nuclear pore (Bermejo et al., 2011). This topological stress must be relieved by the action of topoisomerases (Tuduri et al., 2009). These collisions can be reduced by spatial and temporal separation between the two processes, but cannot be completely prevented, particularly in long or actively transcribed genes (Helmrich et al., 2011). Collisions can also result in R-loops, these are RNA:DNA hybrids formed between the DNA template strand and the nascent RNA transcript, with the exclusion of the non-template strand as ss-DNA (Helmrich et al., 2011; Kotsantis et al., 2016). R-loops are potentially damaging through hindering replication fork progression, the vulnerability of the exposed ss-
DNA and the possibility of transcription-coupled nucleotide excision repair resulting in DSBs (Muñoz and Méndez, 2016). R-loops are suppressed by Topoisomerase I and require RNase H1 for processing (Helmrich et al., 2011; Tuduri et al., 2009). Mutations that cause a general increase in transcription, often seen in cancer, will increase the probability of these collision and therefore cause replication stress (Kotsantis et al., 2016).

**Causes - DNA replication dynamics**

DNA replication is tightly regulated, as discussed in section 1.3. However, in some cases this control can be lost, resulting in replication stress. Both increases and decreases in the frequency of DNA replication initiation can result in DNA replication stress.

A reduction in the number of origins licensed in G1 phase can result in reduced levels of replication initiation in the following S phase (Shreeram et al., 2002). This under-replication means that each replication fork will have to travel further to completely cover the genome. This longer distance may increase the probability that the replication fork may stall. In addition, it may increase the time taken to replicate the DNA, increasing the chance of the cell entering mitosis without a completely duplicated genome (Hills and Diffley, 2014). Even if reduced origin licensing allows normal levels of replication initiation in S phase, this can still be detrimental to the cell by reducing the number of dormant origins available to rescue stalled replication forks (Blow et al., 2011; Ge et al., 2007). Reduced replication initiation may also result from a decrease in the levels of key firing factors, which are limiting for this process.

During S phase origins are fired through the action of a number of proteins, these include RecQL4, TOPBP1, treslin, Mcm10 and the kinases CDK and DDK (Yeeles et al., 2015). These firing factors are limiting for initiation and so increases in protein levels, as is often seen in cancer, can cause higher rates of replication initiation (Tanaka and Araki, 2011). Increased levels of these factors can also disrupt the temporal regulation
of origin firing, such as causing late origins to fire early, and this can also increase rates of replication initiation (Mantiero et al., 2011). This increase in replication initiation and over-replication can cause replication stress through the exhaustion of components required to support DNA replication (Mantiero et al., 2011). This will result in some forks being unable to replicate efficiently and so they will slow down or stall (Hills and Diffley, 2014). Increased replication can also increase the probability of collisions between replication forks and transcriptional bubbles, which, as discussed above, can cause further replication stress.

Re-replication, whereby misregulation allows licensing during S phase of already replicated DNA, can also cause replication stress, and is known to cause genomic instability (Liontos et al., 2007; Neelsen et al., 2013). Licensing is normally inhibited during S phase through a number of different mechanisms, described in section 1.3. However, if these regulatory mechanisms fail and licensing does occur during S phase, the same section of DNA may be replicated more than once in the same cell cycle (Vaziri et al., 2003). This can cause replication stress by a number of mechanisms. First, it will increase the overall number of active replication forks and so may deplete replication factors. Second, it may increase the chances of collisions with other replication forks or transcriptional bubbles. Finally, if re-replication is infrequent it may increase the probability of fork stalling, as the fork has a large distance to travel before meeting another replication fork (Neelsen et al., 2013).

1.5. DNA replication stress checkpoint response

Many factors, both endogenous and exogenous, can result in DNA replication stress, this stress can be experienced even in unperturbed cell cycles. If replication stress is left unchecked it will eventually lead to DNA damage and genome instability (Jones and Petermann, 2012). DNA replication stress would be particularly damaging if it resulted in the cell entering mitosis without a fully replicated genome. The cell has therefore evolved a DNA replication stress checkpoint response, conserved from yeast to man,
which acts to prevent DNA damage and subsequent genome instability (Jossen and Bermejo, 2013). The replication stress checkpoint signalling pathway primarily consists of extended lengths of ssDNA bound by Replication Protein A (RPA) resulting in ATR and then Chk1 kinase activation (Branzei and Foiani, 2009). There are a large number of checkpoint effector proteins regulated by this signalling pathway. The activity of these proteins carries out the checkpoint functions required for cellular tolerance to replication stress. The checkpoint signalling and checkpoint functions, discussed in detail below, with the resulting prevention of DNA damage is collectively referred to in this work as the DNA replication stress checkpoint response. This replication stress checkpoint response is considered distinct from the classical DNA damage response, referring to DSB-dependent activation of ATM and Chk2, as suggested in Toledo et al. (2011). The DNA replication stress checkpoint response is vital, as an inefficient checkpoint response results in an increase in DNA damage and subsequent genome instability (Cimprich and Cortez, 2008).

**Checkpoint signalling**

During DNA replication stress replication fork progression slows or stalls. This is thought to result in an uncoupling of the helicase, which continues to unwind DNA, and the blocked polymerase (Byun et al., 2005). This exposes extended lengths of ssDNA, which is bound by the ssDNA binding protein Replication Protein A (RPA). RPA binding functions to protect vulnerable ssDNA, but also acts as the signal of replication stress (Toledo et al., 2013; Zou et al., 2006). RPA present at stalled replication forks recruits the checkpoint sensor kinase ATR (Ataxia Telangiectasia and Rad3-related protein) and ATRIP (ATR Interacting Protein) (Zou and Elledge, 2003). It also is required to recruit Rad17, which is responsible for loading of the 9-1-1 complex, which recruits TopBP1, which ensures full ATR activation (Kumagai et al., 2006; Sørensen and Syljuåsen, 2012; Zou et al., 2003). ATR is a serine/threonine kinase of the PI-3-like kinase family (Abraham, 2001). Once activated by replication stress, ATR phosphorylates, amongst other targets, the checkpoint effector kinase Chk1 (Liu et al., 2000). This ATR-Chk1 pathway
is primarily induced by replication stress and the parallel ATM-Chk2 pathway is primarily induced by DNA damage. However, crosstalk has been seen between these two pathways (Bartek and Lukas, 2003). Recent work has also shown that ATR and Chk1 can have distinct and independent roles in the DNA replication stress checkpoint response (Buisson et al., 2015; Koundrioukoff et al., 2013). The role of post-translational modifications, such as phosphorylation, is well-established in the replication stress checkpoint response (Huen and Chen, 2008). In particular, activated Chk1 phosphorylates a wide variety of targets to alter their level and activity, in order to carry out the functions of the checkpoint response, as discussed below.

**Checkpoint functions**

The cell cycle must be arrested to allow time for replication stress to be resolved and DNA replication to be completed, before entering mitosis. The cell cannot be allowed to enter mitosis without a fully replicated genome, as this would result in catastrophic DNA damage. The progression into mitosis is controlled by CDK activity, with high CDK activity allowing cell cycle progression (Malumbres and Barbacid, 2009). This CDK activity is constrained by Wee1-dependent phosphorylation (McGowan and Russell, 1993). Opposing this, the phosphatase Cdc25 removes this inhibitory phosphorylation and increases CDK activity and cell cycle progression. In response to DNA replication stress, Chk1 increases Wee1 activity and also phosphorylates Cdc25 (Sørensen and Syljuåsen, 2012). Phosphorylation targets Cdc25 for Ubiquitin-dependent degradation, resulting in increased CDK inhibitory phosphorylation and hence cell cycle arrest (Sørensen et al., 2003).

The checkpoint response must stall ongoing replication forks during replication stress. In *Saccharomyces cerevisiae* this is dependent on the replisome components Tof1 (human Tim) and Mrc1 (human Claspin) (Hodgson et al., 2007; Katou et al., 2003). In mammalian cells this slowing of active replication forks in conditions of replication stress is dependent on Tipin (Tim interacting protein), which forms a stable complex with Tim (Unsal-Kaçmaz et al., 2007). Allowing DNA replication to continue under con-
ditions of replication stress would increase the levels of stress further and may result in DNA damage. For example if stress is due to low levels of dNTPs, or other replication components, allowing replication to continue would not only result in likely inaccurate replication, but would enhance the stress by depleting pools of dNTPs or other required proteins even further. An additional checkpoint function is the checkpoint-dependent upregulation of RNR activity to increase dNTP pools to reduce this source of stress (Aye et al., 2014; Zegerman and Diffley, 2009). Continued DNA replication during replication stress may also expose such high amounts of ssDNA that RPA is unable to protect all vulnerable DNA, resulting in replication catastrophe (Toledo et al., 2013). Stalled replication forks must be stabilised to prevent the dissociation of replisome components and prevent the formation of aberrant and damaging DNA structures (Calzada et al., 2005; Lopes et al., 2001). This stabilisation is dependent on Chk1 (Petermann et al., 2010).

A key checkpoint function involves the regulation of replication origin firing. Late origin firing is inhibited by ATR and Chk1 preventing the formation of new replication factories. This directs replication components to sections of the genome already being replicated and experiencing stress. Along with global inhibition of origin firing, dormant origins local to stress are fired (Ge and Blow, 2010). This is thought to be dependent on the stochastic firing of dormant origins, which due to nearby fork stalling, have not been passively replicated and disassembled (Santocanale et al., 1999). This combined regulation prevents the possibility of further stalled forks in regions which have not yet started replication, but ensures replication is completed in regions experiencing replication stress (Ge et al., 2007).

Once the stress has been resolved DNA replication must be completed. The mechanism of replication restart depends on the condition of the replication fork. There are many different proposed mechanisms of replication restart and the details have not been fully elucidated. Following a transient stalling of the replication fork it has been seen in *Escherichia coli* and *Saccharomyces cerevisiae* that replication can continue fol-
ollowing a repriming event downstream of the lesion, on both the lagging and leading strands (Lopes et al., 2006; Yeeles and Marians, 2011). Fork remodelling by helicases, such as BLM, WRN or SMARCAL1, may mediate replication restart by re-annealing ss-DNA or by fork regression and degradation (Petermann and Helleday, 2010). After short periods of replication stress the fork is only stalled, meaning the replisome is still intact, and replication may restart directly from this fork via a Holliday junction intermediate in a Rad51 dependent manner (Petermann et al., 2010). In this model helicases, such as BLM and WRN, cause fork regression into a Holliday junction in a chicken foot structure. End processing by the exonuclease Mre11 forms a lagging strand gap or 3′-overhang, which is required for Rad51 recruitment and filament formation. Rad51 filament formation and stability depends on XRCC3 and BRCA2, which limits DNA resection (Schlacher et al., 2011). The Rad51 filaments catalyse homology search and strand exchange to form a D-loop. This allows replication restart with the homologous sequence acting as a template for DNA polymerases; the Holliday junction is then resolved. This process is distinct from DSB repair (Petermann et al., 2010). After long periods of replication stress, replication forks collapse and the replisome dissociates, preventing replication restart and resulting in fork-associated DSBs (Hanada et al., 2007). The resolution of these collapsed forks and DSBs requires repair mechanisms such as Homologous Recombination. Mre11 and Exo1 carry out DNA end resection on the DSB and repair proceeds in a similar manner as described above via D-loop and Holliday junction intermediates (Jones and Petermann, 2012). To complete DNA replication new origin firing is required from nearby dormant origins. Once the normal programme of DNA replication is restored, the cell cycle arrest must be reversed to allow the cell to continue into mitosis with a fully replicated genome.

The checkpoint response and E2F-dependent transcription

Recent work from the de Bruin lab has established E2F6, the repressor of E2F-dependent cell cycle transcription in S phase, as an important target of Chk1. Chk1-dependent phosphorylation inactivates E2F6 and prevents E2F6-dependent repression of tran-
scription. This sustains E2F-dependent transcription in S phase during replication stress (Bertoli et al., 2013a). This maintenance of E2F-dependent cell cycle transcription is seen to be required during replication stress to prevent cell death, although the mechanism and reason for this had not been established. Despite being required for cell viability following replication stress, the role of transcription in the response to DNA replication stress has not been well studied. In fact, previous work has shown that in the yeast model *Saccharomyces cerevisiae* active translation is not required for cell viability following DNA replication stress (Tercero et al., 2003). As active translation is not required this would therefore suggest that a transcriptional response to DNA replication stress is also not required. Interestingly, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* also maintain G1/S cell cycle transcription through the checkpoint dependent phosphorylation and inhibition of the transcriptional repressor Nrm1 (Bastos de Oliveira et al., 2012; Caetano et al., 2011; Chu et al., 2007; de Bruin et al., 2008; Dutta et al., 2008; Gomez-Escoda et al., 2011; Ivanova et al., 2013, 2011; Travesa et al., 2012). Together this work demonstrates that there is a checkpoint dependent transcriptional response to DNA replication stress; however, the role and importance of this response has not been clearly demonstrated.

### 1.6. Oncogene-induced replication stress

Oncogene-induced replication stress has recently been recognised to be a very early event in tumourigenesis (Bartkova et al., 2005; Gorgoulis et al., 2005) and has been proposed to be a hallmark of cancer (Macheret and Halazonetis, 2015). It is thought that replication stress caused by oncogene activation or tumour suppressor inactivation results in DNA damage and DSBs in pre-cancerous lesions (Tsantoulis et al., 2008). This activates the DNA damage response, which acts as an initial barrier to tumourigenesis (Bartkova et al., 2006; Di Micco et al., 2006; Halazonetis et al., 2008). The DNA damage checkpoint is a well-established response which detects and repairs DNA damage through ATM-dependent signalling (Ciccia and Elledge, 2010; Jackson and Bartek, 2009). If the level of damage is too great the DNA damage response medi-
ates oncogene-induced senescence or apoptosis (Bartkova et al., 2006; Di Micco et al., 2006). However, oncogene-induced replication stress is also a driver of genome instability and this situation creates an environment in which mutations that bypass the DNA damage checkpoint are selected for, which allows for continued tumour progression (Halazonetis et al., 2008). As such, oncogene-induced replication stress has a major role in the evolution of cancer (Lecona and Fernández-Capetillo, 2014).

Mechanisms of oncogene-induced replication stress

There are many different mechanisms behind oncogene-induced replication stress, some of which have been clearly established and others which have yet to be fully understood. These are all based on oncogene activation misregulating DNA replication, thereby causing replication stress by the mechanisms discussed earlier in section 1.4. Some mechanisms of oncogene-induced replication stress will be specific, linked to the signalling pathways and components misregulated by particular oncogenic activity. However, some may act as general mechanisms of oncogene-induced replication stress, such as increased transcription and replication collisions. Individual oncogenes can cause replication stress through a number of different mechanisms depending on the context, as demonstrated below for Cyclin E, and these causes may change as tumours evolve (Hills and Diffley, 2014). I will discuss below a few mechanisms of oncogene-induced replication stress, although there are probably many more yet to be elucidated.

Activation of many oncogenes, often through the deregulation of the CDK-pRb-E2F pathway (Chen et al., 2009), results in uncontrolled proliferation, a hallmark of cancer. For example, overexpression of Cyclin D1 and Cyclin E accelerates S phase entry and reduces the length of G1 phase (Resnitzky et al., 1994). This may result in a shorter window for licensing of origins and therefore fewer origins are licensed, as seen for Cyclin E (Ekholm-Reed et al., 2004). As discussed above, reducing the number of licensed origins can cause replication stress by forcing each replication fork to travel further, it is therefore more likely to stall. In addition, reduced origin licensing results
in fewer dormant origins, which are important to complete replication following repli-
cation fork stalling.

Activation of oncogenes can also deregulate both the frequency and temporal pattern
of replication origin firing. Cyclin E has also been seen to induce replication stress
through this increase in replication initiation (Jones et al., 2013). This over-replication
may cause stress by depleting essential replication factors, the depletion of nucleotide
pools was demonstrated as one mechanism of Cyclin E induced replication stress (Bester
et al., 2011). A similar mechanism may in part describe the stress caused by re-replication
following Cdt1 and Cdc6 overexpression (Liontos et al., 2007).

Increasing the rate of replication initiation following oncogene activation may also re-
result in stress through an increase in the frequency of collisions between replication
forks and transcription bubbles and R-loop formation. This was seen to be a cause be-
hind Cyclin E induced replication stress (Jones et al., 2013). The probability of collisions
could also be increased through higher levels of transcription, as is seen specifically
following HRAS\textsuperscript{V12} overexpression (Kotsantis et al., 2016). As many oncogenes alter
transcriptional regulation, this mechanism may be a general mechanism of oncogene-
induced replication stress.

Understanding the mechanisms of oncogene-induced replication stress may prove in-
strumental in understanding the evolution of cancer. Mechanisms specific to individ-
ual oncogenes may prove useful in designing targeted cancer therapies. However, a
more general approach would be to target a mechanism of tolerance to the higher
levels of replication stress experienced by oncogenic cells (Boyer et al., 2016). For ex-
ample, inhibitors of the key replication stress checkpoint kinases, ATR and Chk1, are
promising as cancer therapies (Lecona and Fernández-Capetillo, 2014).

**Oncogene-induced replication stress and E2F-dependent transcription**

The deregulation of E2F-dependent transcription may contribute to oncogene-induced
replication stress. Activation of many oncogenes, including Myc, Ras and CDK/Cyclins,
or inactivation of tumour suppressors, such as pRb and CDK Inhibitors, result in the deregulation of E2F-dependent transcription (Chen et al., 2009). As E2F-dependent transcription controls the G1 to S phase transition, an increase in E2F activity results in uncontrolled S phase entry and hence uncontrolled proliferation (Resnitzky et al., 1994). As described above, uncontrolled proliferation is thought to result in oncogene-induced replication stress through a number of mechanisms. In addition, E2F-dependent transcription encodes for many components required for correct DNA replication, such as Cyclin E, Cdt1 and Cdc6. Deregulation of E2F-dependent transcription by oncogenic activity may therefore result in the inappropriate activity of these E2F targets and hence cause oncogene-induced replication stress (Bester et al., 2011; Jones et al., 2013; Liontos et al., 2007).

1.7. E2F6-dependent repression

Transient activation of E2F-dependent transcription during the G1 to S phase transition drives S phase entry, but transcription is sustained during the checkpoint response to DNA replication stress (Bertoli et al., 2013a). Further understanding the regulation of E2F-dependent transcription will therefore aid our knowledge of both cell cycle regulation and the cellular response to replication stress. This is particularly important in the context of oncogene-induced replication stress, which is influenced by both aspects of E2F-dependent transcription. E2F6 has been identified as the transcriptional repressor responsible for repressing E2F-dependent transcription during S phase (Giangrande et al., 2004), and is inactivated by Chk1 phosphorylation during the checkpoint response to DNA replication stress to maintain E2F-dependent transcription (Bertoli et al., 2013a). Understanding the mechanism of E2F6-dependent repression will therefore help our understanding of E2F-dependent transcriptional regulation.

E2F6 was first identified as the mouse homologue EMA through a yeast two-hybrid screen with the dimerisation partner DP-1 (Morkel et al., 1997). Human E2F6 was then
identified independently by three labs in 1998 through sequence homology to other E2F family members (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). All of these studies identified E2F6 as containing a conserved DNA binding domain and DP dimerisation domain, but lacking the transactivation and pocket protein binding domains. As the sequence would predict E2F6 functions in a heterodimer with the DP proteins in a sequence specific DNA binding manner, however it is unable to activate transcription or bind pocket proteins. E2F6, and its mouse homologue EMA, is however identified as a transcriptional repressor in all studies. These studies also showed that the binding affinity to an alternative CCC containing motif was higher than to the consensus CGC E2F binding site (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). In addition, Trimarchi et al., 1998 show that E2F6 has a lower DNA binding affinity than E2F1 and E2F4 for each of five sequences tested, although it is possible that this may not always be the case when the E2F binding sites are considered in the context of the genome. Functional characterisation of E2F6 is seen in the E2F6 -/- mouse. This mouse is viable, healthy and fertile, despite a defect in spermatogenesis. In addition, embryonic fibroblasts from the E2F6 -/- mice show no proliferation defects. This may be due to the observed compensation of E2F6 loss by E2F4 (Giangrande et al., 2004). However, E2F6 -/- mice display homeotic transformations of the axial skeleton in a manner similar to Polycomb mutant mice (Storre et al., 2002).

These initial studies identifying and characterising E2F6 function all demonstrate that E2F6 is able to prevent activation of both endogenous or exogenous E2F1 and E2F4 activity in CAT or luciferase reporter assays (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). This block in activation is seen in a dose-dependent manner. These studies also all demonstrate that the mechanism of E2F6-dependent repression is not through the sequestration of the DP proteins, as addition of DP proteins is unable to prevent E2F6-dependent repression (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). Although Trimarchi et al., 1998 show that a low level of
repression can be mediated by DP sequestration when E2F6 is unable to bind DNA.

However, beyond these insights, these studies show very varying evidence as to the mechanism of E2F6-dependent repression. As repression is independent of DP protein sequestration but is dependent on sequence specific DNA binding, this suggests E2F6 acts as a dominant negative inhibitor, with repression being dependent on E2F6 competing with the activating E2Fs for binding to E2F-specific promoters. However, the evidence does not establish whether repression is mediated through the simple displacement of activating E2Fs, a loss of activation, or through a further recruitment of repressive proteins such as Polycomb group proteins, an active repression.

A number of experiments suggest that E2F6-dependent repression is mediated by displacement of activators alone. Cartwright et al., 1998 shows that a Gal4-E2F6 fusion protein does not display intrinsic repressive function, suggesting E2F6 is an inhibitor of E2F-dependent transcription through a displacement mechanism alone. In addition, they show that overexpression of a mutant E2F1 protein, lacking the the transactivation and pocket protein binding domains, is able to function in a similar, if slightly weaker, manner to E2F6 in blocking wild type E2F1 transactivation. Further ChIP based studies show that the promoter occupancy of E2F1 increases in response to silencing E2F6 (Oberley et al., 2003), and that E2F6 replaces E2F1 at promoters in S phase (Bertoli et al., 2013a). These studies do not determine whether this was an active process or not, as would be needed for an efficient mechanism of repression. All of this evidence suggests that E2F6 functions as an inhibitor of activating E2Fs and repression is achieved through a loss of activation, or displacement of activator E2Fs.

In contrast to this, a number of studies have shown E2F6 contains independent repressive function. This suggests that repression is mediated by E2F6 recruiting additional repressive proteins to promoters. First, in direct contrast to Cartwright et al., 1998, Gaubatz et al., 1998 demonstrate that a Gal4-E2F6 fusion protein shows repressive properties in a luciferase assay. In addition, they map this repressive function specifi-
cally to the C terminal 173-281 amino acids. This is the basis of defining the repression
domain of E2F6. It is unclear why such similar approaches yield such different results,
however they are carried out in different cell lines - C-33A and U2 OS - which may ac-
count for some differences. Taken together these two studies do not define the mech-
anism of E2F6-dependent repression, with both mechanisms possible. Another study
identifies the MBT domain-containing protein L3MBTL2 as a co-repressor of E2F6, with
loss of L3MBTL2 resulting in impaired E2F6 repression (Trojer et al., 2011). This evi-
dence would support the recruitment of additional factors to promoters as the mech-
anism of E2F6-dependent repression.

Polycomb group (PcG) protein complexes are responsible for the maintenance of long-
term transcriptional repression through the alteration of chromatin structure. These
proteins could be candidates to mediate E2F6-dependent repression. Following this,
E2F6 has been identified in at least 4 separate multi-subunit complexes containing
Polycomb group proteins, including RYBP, Ring1A, Bmi1, MEL-18, mph1 (Trimarchi
et al., 2001), Ring1, Ring2, MBLR, YAF2 (Ogawa et al., 2002; Trojer et al., 2011), EPC1,
EZH2 (Attwooll et al., 2005) and PHC3 (Deshpande et al., 2007). Two of these proteins,
RYBP and EPC1, have been identified as binding to E2F6 via amino acids contained in
the repression domain identified previously in Gaubatz et al., 1998. However, a fur-
ther study found no overlap between chromatin binding of E2F6 and the PcG protein
RYBP (Giangrande et al., 2004). In addition to PcG proteins, some of these complexes
contained other proteins responsible for gene expression including histone methyl-
transferases and the co-repressor L3MBTL2. However, it should be noted that the
functional significance of the interactions between E2F6 and Polycomb group proteins
was not tested in any of these studies.

E2F6 has been shown to have a role in repression of E2F-dependent transcription dur-
ing S phase (Bertoli et al., 2013b; Giangrande et al., 2004), however, evidence is also
seen for a potential role in G0/quiescence (Deshpande et al., 2007; Gaubatz et al.,
1998; Ogawa et al., 2002). Overexpression of E2F6 in NIH3T3 cells was unable to pre-
vent S phase entry in cycling cells, however, it did prevent cells entering S phase from G0 (Gaubatz et al., 1998), supporting an important role for E2F6 in G0. In direct contrast, overexpression of E2F6 in U2 OS cells was able to increase the number of cells in S phase, but in RAT-1 fibroblasts was unable to induce S phase entry from G0 (Cartwright et al., 1998), supporting no role for E2F6 in G0 but an important role in S phase entry in cycling cells. This may be due to the different cell lines used, but conclusions can therefore not be drawn as to the major cell cycle phase in which E2F6 is important.

Further studies demonstrate the presence of E2F6 and PcG complexes in G0 bound at E2F promoters (Ogawa et al., 2002) or in punctate polycomb bodies (Deshpande et al., 2007). It should be noted that the studies in Ogawa et al., 2002 also show E2F4 and p130 as binding to E2F promoters in G1 and not G0, which does not following knowledge of E2F-dependent transcriptional regulation. E2F6 is also seen at E2F promoters specifically in S and G2 phase and not G0 (Giangrande et al., 2004), and in complex with PcG proteins in proliferating cells (Attwooll et al., 2005; Trojer et al., 2011). These studies show that E2F6 may function as a repressor either in S phase in proliferating cells, or in G0 in quiescent cells.

When considering E2F6-dependent repression, both the mechanism of action and cell cycle dependent function show a number of alternative possibilities. Repression may be mediated through the displacement of activator E2Fs, or through the subsequent recruitment of repressive proteins such as the Polycomb group complex. It is possible that in a biological context only one of these mechanisms describes E2F6-dependent repression. However, due to the evidence for both mechanisms, it is very likely that both are involved in E2F6-dependent repression. It may be that both mechanisms act simultaneously, or in different contexts. The repression mediated by E2F6 may have important roles in either S or G0 phases of the cell cycle. Again, it is highly likely that both cases can be true with E2F6 playing an important role in the regulation of E2F-dependent transcription in both of these situations, with the mechanism of repression possibly depending on the specific cell line or context. It could also be imagined that
the mechanism of E2F6-dependent repression was dependent on the cell cycle phase, with recruitment of Polycomb group proteins being required for the more long-term, stable repression in G0, and the faster acting displacement mechanism primarily being responsible for repression during S phase. It is also possible that both mechanisms act in the same cell cycle phase but through difference dynamics. For example it may be that displacement of the activator E2Fs initially represses transcription, but that this is consolidated through recruitment of PcG proteins. However, these ideas are only theoretical and have not yet been tested in the biological context.

Understanding the mechanism of E2F6-dependent repression throughout the cell cycle not only adds to our knowledge of cell cycle transcriptional regulation control, but may help our understanding of cancer mutations. As well as changes in levels of E2F6, cancer associated point mutations have also been found in E2F6. These mutations are found throughout the protein, however they are concentrated in the DNA binding and repression domains. Mutations in the DNA binding domain would be expected to affect E2F6-dependent repression whether it was via displacement or PcG protein recruitment. However, mutations in the repression domain should not affect E2F6-dependent repression if the mechanism is solely through displacement. Therefore understanding the mechanism of E2F6-dependent repression may help to understand the impact of specific cancer mutations. If the two mechanisms are responsible for repression in different situations, this may mean mutations could act very differently, and this knowledge may be very interesting in the context of understanding cancer evolution in these mutations.

1.8. Research Aims

The aim of my thesis work is to investigate the DNA replication stress checkpoint transcriptional response and its role in replication stress tolerance. It is known that in response to DNA replication stress, the checkpoint response maintains E2F-dependent cell cycle transcription, which is important for cell viability in mammalian cells (Bertoli
et al., 2013a). However in yeast, despite this transcriptional response being conserved, active protein synthesis is not required for cell viability following replication stress (Tercero et al., 2003). In contrast to post-translational modifications, which have a well-established role in the replication stress checkpoint response, relatively little is known about the role of the transcriptional response to DNA replication stress. My PhD work therefore aimed to establish the role and importance of this transcriptional response in the tolerance to DNA replication stress.

E2F-dependent transcription is sustained in response to DNA replication stress. It is also known that many oncogenes and tumour suppressors deregulate E2F activity. As E2F-dependent transcription controls entry into S phase and therefore entry into the cell cycle, this deregulation of E2F-dependent transcription drives S phase entry and hence uncontrolled proliferation. This can ultimately lead to oncogene-induced replication stress, an early event in tumourigenesis (Bartkova et al., 2005; Gorgoulis et al., 2005). It is unknown whether E2F-dependent transcription is also sustained in response to oncogene-induced replication stress, and what role this may have in the tolerance to this stress. I therefore investigated the role of E2F-dependent transcription in oncogene-induced replication stress, to further understand the complex relationship linking E2F-dependent transcription and oncogene-induced replication stress.

Finally, E2F6 is the transcription factor responsible for repression of E2F-dependent transcription in S phase. The inactivation of E2F6 by Chk1 is the mechanism behind sustained E2F-dependent transcription during replication stress (Bertoli et al., 2013a). However, the mechanism of E2F6-dependent repression has not been fully established. Evidence has been seen supporting both the displacement of activator E2Fs (Cartwright et al., 1998; Oberley et al., 2003) and the recruitment of Polycomb group proteins (Attwooll et al., 2005; Deshpande et al., 2007; Ogawa et al., 2002; Trimarchi et al., 2001) as the mechanism behind E2F6-dependent repression. I therefore aim to investigate the mechanism of E2F6-dependent repression. This will give a more detailed understanding of the regulation of the transcriptional response to DNA replica-
tion stress. In addition, it may give insight into the function of different E2F6 mutations found in cancers.

Altogether this work will give a greater understanding of the DNA replication stress checkpoint transcriptional response and its role in replication stress tolerance. It will establish the importance of this transcriptional response, but also the mechanisms behind its role in the DNA replication stress checkpoint. This will be extended to an understanding of the role of the E2F-dependent transcription in oncogene-induced replication stress, which will have important implications for understanding the role of E2F activity in cancer evolution.
2. Materials and Methods

2.1. Cell Lines - Chapter 3

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media (Gibco)</th>
<th>Media Supplements</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPE1 hTERT</td>
<td>DMEM (41965)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140). In DMEM/F12 only - 3.5 % Sodium Carbonate (Gibco, 25080)</td>
<td>5 µg/ml Blasticidin</td>
</tr>
<tr>
<td></td>
<td>or DMEM/F12 (31331)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK293 T-Rex E2F6 cl1</td>
<td>DMEM (41965)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140)</td>
<td>5 µg/ml Blasticidin, 100 µg/ml Zeocin</td>
</tr>
<tr>
<td>RPE1 TetON E2F6 cl6</td>
<td>DMEM/F12 (31331)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140), 3.5 % Sodium Carbonate (Gibco, 25080)</td>
<td>5 µg/ml Blasticidin, 100 µg/ml Zeocin</td>
</tr>
<tr>
<td>T98G</td>
<td>DMEM (41965)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.1: Chapter 3 cell lines and maintenance conditions

HEK293 T-Rex E2F6 were previously described (Bertoli et al., 2013a).

RPE1 TetON E2F6 cells were constructed for this work by Cosetta Bertoli. Cells were transfected with Lipofectamine 2000 (Invitrogen, 11668-027) and OptiMEM1 (Gibco, 51985) following manufacturers instructions using the plasmid pcDNA4/TO E2F6. Transformed cells were selected in 5 µg/ml Blasticidin and 200 µg/ml Zeocin and colonies...
originating from single cells were tested for Doxycycline-dependent E2F6 overexpression.

### 2.2. Cell Lines - Chapter 4

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media (Gibco)</th>
<th>Media Supplements</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPE1 TetON E2F6 c-Myc-ER</td>
<td>DMEM/F12 (21041)</td>
<td>7 % Charcoal treated* FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140), 3.5 % Sodium Carbonate (Gibco, 25080)</td>
<td>5 µg/ml Blasticidin, 100 µg/ml Zeocin, 2 µg/ml Puromycin</td>
</tr>
</tbody>
</table>

* Charcoal treated FBS was prepared by adding 0.5 g Charcoal and 0.05 g Dextran to 500 ml of FBS and warmed at 37 °C with stirring for 2.5 hours and then filtered through a 0.2 µl filter.

RPE1 TetON E2F6 c-Myc-ER cells were constructed by Cosetta Bertoli for this work. Cells were obtained by retroviral infection of RPE1 TetON E2F6 cells using the pBABE c-Myc-ER plasmid (Addgene plasmid 19128) (Ricci et al., 2004). Infected cells were selected in puromycin 5 µg/ml and the surviving polyclonal cells used for the assays.

### 2.3. Cell Lines - Chapter 5

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media (Gibco)</th>
<th>Media Supplements</th>
<th>Antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293 T-Rex E2F6 cl1 (as Chapter 3)</td>
<td>DMEM (41965)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140)</td>
<td>5 µg/ml Blasticidin, 100 µg/ml Zeocin</td>
</tr>
<tr>
<td>HEK293 T-Rex E2F6 ∆C 1-178 cl6 &amp; cl3</td>
<td>DMEM (41965)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140)</td>
<td>5 µg/ml Blasticidin, 100 µg/ml Zeocin</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>-------------</td>
<td>---------------------------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>RPE1 TetON HA-E2F6 cl10 &amp; cl14</td>
<td>DMEM/F12 (31331)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140), 3.5 % Sodium Carbonate (Gibco, 25080)</td>
<td>5 µg/ml Blasticidin, 100 µg/ml Zeocin</td>
</tr>
<tr>
<td>RPE1 TetON HA-E2F6 ∆C 1-178 cl1 &amp; cl2</td>
<td>DMEM/F12 (31331)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140), 3.5 % Sodium Carbonate (Gibco, 25080)</td>
<td>5 µg/ml Blasticidin, 100 µg/ml Zeocin</td>
</tr>
<tr>
<td>RPE1 TetON HA-E2F6 ∆N 129-281 cl8 &amp; cl11</td>
<td>DMEM/F12 (31331)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140), 3.5 % Sodium Carbonate (Gibco, 25080)</td>
<td>5 µg/ml Blasticidin, 100 µg/ml Zeocin</td>
</tr>
<tr>
<td>RPE1 TetON Empty</td>
<td>DMEM/F12 (31331)</td>
<td>10 % FBS (Sigma, F7524), 1 % Penicillin-Streptomycin (Gibco, 15140), 3.5 % Sodium Carbonate (Gibco, 25080)</td>
<td>5 µg/ml Blasticidin</td>
</tr>
</tbody>
</table>

**Table 2.3:** Chapter 5 cell lines and maintenance conditions

HEK293 T-Rex E2F6 ∆C 1-178 cl6 and cl3 were constructed for this work. Cells were transfected with Lipofectamine 2000 (Invitrogen, 11668-027) and OptiMEM1 (Gibco, 51985) following manufacturers instructions using the plasmid pcDNA4/TO E2F6 ∆C 1-178. This plasmid was constructed by cloning the ∆C 1-178 fragment of E2F6 into the BamHI and EcoRI sites in the pcDNA4/TO plasmid. Transformed cells were selected in 5 µg/ml Blasticidin and 200 µg/ml Zeocin and colonies originating from single cells
tested for Doxycycline-dependent E2F6 overexpression.

RPE1 TetON HA-E2F6 wild type, ΔC 1-178 and ΔN 129-281 were constructed for this work. The three plasmids were cloned into the BamHI and EcoRI sites of the pcDNA4/TO vector starting with the sequence:

\[
\text{CACC ATG GCC TACCTACGACGTGCCGACTACGCC TCCCTCGGATCC} \quad \text{E2F6 sequence}
\]

where blue shows linker sequences, green shows start codon and yellow shows the HA tag sequence. Cells were transfected with 4µg DNA with Lipofectamine 2000 (Invitrogen, 11668-027) and OptiMEM1 (Gibco, 51985) following manufacturers instructions using the three plasmids described. Transformed cells were selected in 5 µg/ml Blasticidin and 200 µg/ml Zeocin and colonies originating from single cells tested for Doxycycline dependent E2F6 overexpression.

### 2.4. Treatments

<table>
<thead>
<tr>
<th>Drug</th>
<th>Abbreviation</th>
<th>Pre-treatment time before HU</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyurea</td>
<td>HU</td>
<td>-</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Chx</td>
<td>10 minutes</td>
<td>10 µg/ml</td>
</tr>
<tr>
<td>(Sigma, C4859)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Doxy</td>
<td>1 - 2 hours as specified</td>
<td>2 or 4 µg/ml as specified</td>
</tr>
<tr>
<td>Aphidicolin</td>
<td>Aph</td>
<td>-</td>
<td>2.4 mM</td>
</tr>
<tr>
<td>Hydroxytamoxifen</td>
<td>4OH-T</td>
<td>-</td>
<td>100 or 200 nM as specified</td>
</tr>
<tr>
<td>(Sigma)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.4: Treatment concentrations and conditions*
2.5. Statistics

Statistical significance was analysed using a number of different tests as appropriate for each experiment and indicated in each figure legend. The Wilcoxon sum rank two sided test was carried out using the R function Wilcox.test. This is a non-parametric test for unmatched samples and was therefore used for data that was not necessarily normally distributed. ANOVA was used for multiple pairwise comparisons followed by the appropriate post hoc test; ANOVA reduces the rate of false positives following multiple comparisons. One-way or two-way ANOVA was used depending on the number of independent variables being analysed. Two-way ANOVA, with multiple comparisons of the means performed by Tukey’s honest significant differences, was carried out with Prism. One-way ANOVA, with multiple comparisons of the means performed by Sidak’s, was carried out with Prism. The Student’s T test, unpaired two-tailed, was used for comparisons between two data sets and was carried out with Prism. Key throughout: n.s. $P \geq 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

When appropriate in chapter 4 only S phase cells were analysed to remove possible cell cycle effects. S phase cells were selected based on RPA2 staining which reveals two populations of cells - a non or low staining population of non-S phase cells and an RPA2 staining population of S phase cells. The divide between these populations varies due to staining and microscope settings and so was defined for each individual experiment, the same threshold was used for all conditions within each experiment.

Table 2.5 summarises the different statistical tests used for each figure. When only S phase cells were analysed the RPA2 threshold used for each repeat is stated in brackets.
<table>
<thead>
<tr>
<th>Figure</th>
<th>Test</th>
<th>Figure</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Wilcoxon</td>
<td>4.3</td>
<td>Wilcoxon (20)</td>
</tr>
<tr>
<td>3.2</td>
<td>Wilcoxon</td>
<td>4.4</td>
<td>Wilcoxon</td>
</tr>
<tr>
<td>3.3</td>
<td>1 way ANOVA</td>
<td>4.7</td>
<td>Wilcoxon (20/20/20)</td>
</tr>
<tr>
<td>3.4</td>
<td>Wilcoxon</td>
<td>4.8</td>
<td>Wilcoxon (20/5)</td>
</tr>
<tr>
<td>3.6</td>
<td>Wilcoxon</td>
<td>4.10</td>
<td>Wilcoxon (10)</td>
</tr>
<tr>
<td>3.7</td>
<td>Wilcoxon</td>
<td>5.3</td>
<td>1 way ANOVA</td>
</tr>
<tr>
<td>3.10</td>
<td>T test</td>
<td>5.4</td>
<td>1 way ANOVA</td>
</tr>
<tr>
<td>3.11</td>
<td>Wilcoxon</td>
<td>5.5</td>
<td>1 way ANOVA</td>
</tr>
<tr>
<td>3.17</td>
<td>T test</td>
<td>5.6</td>
<td>1 way ANOVA</td>
</tr>
<tr>
<td>3.18</td>
<td>T test</td>
<td>5.7</td>
<td>T test</td>
</tr>
<tr>
<td>3.19</td>
<td>2 way ANOVA</td>
<td>5.8</td>
<td>1 way ANOVA</td>
</tr>
<tr>
<td>3.21</td>
<td>Wilcoxon</td>
<td>5.9</td>
<td>T test</td>
</tr>
<tr>
<td>3.22</td>
<td>Wilcoxon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.23</td>
<td>Wilcoxon</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.24</td>
<td>Wilcoxon</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.5: Statistical tests and S phase selection

2.6. Immunofluorescence (extracted, chromatin-bound)

For all immunofluorescence experiments except figures 3.19 and 5.11 cells were extracted before fixing in order to stain only chromatin-bound proteins. Cells were pre-extracted for 1 minute in ice cold PBS with 0.2% Triton-X100 (Sigma, T8787) (0.15% in HEK293 cells) and then fixed for 20 minutes in 3.7% Formaldehyde (Sigma, 252549). Coverslips were processed similarly to Toledo et al., 2013 - coverslips were blocked for 1 hour in 5% BSA. They were then incubated overnight at 4 °C in primary antibodies RPA32 (RPA2) (1:500, Ms, MABE285, Millipore) and Phospho-Histone H2A.X (γH2AX) (Ser139) (1:400, Rb, 20E3, Cell Signaling Technology). Coverslips were washed in PBS and then incubated in secondary antibodies for 1 hour at room temperature with
Alexa Fluor 488 (1:1000, goat anti-mouse IgG (H+L), Life Technologies, A11029) and Alexa Fluor 647 (1:1000, goat anti-rabbit IgG (H+L), Life Technologies, A21244). They were washed again in PBS and incubated for 5 minutes in Hoechst 33342 trihydrochloride, trihydrate (1:10,000, Invitrogen, H3570), washed and mounted with Fluorosheild (Sigma, F6182). Once dry, coverslips were sealed with clear nail varnish. Images were obtained with a Leica TCS SP5 63x objective lens. Maximum projection images were processed in Fiji - the Hoechst channel was used to segment the nuclei with a Median filter with a radius of 2, Otsu thresholding, watershed, then particles selected with a minimum area of 15. Segmentation was manually checked and corrected or excluded when necessary. Due to the misregulation of nuclear structure following c-Myc-ER induction experiments in chapter 4 were analysed without a watershed step. The mean gray value of each channel was measured for each nucleus. This was completed using a semi-automated macro in Fiji, the code is displayed below. This macro was developed with significant help from Betheney Pennycook.

To complete a max projection of all open images:

```java
//to get a list of all open images to manipulate:
imgArray = newArray(nImages);
for (i=0; i<nImages; i++)
{selectImage(i+1);
 imgArray[i] = getImageID();}

//Take each image in the array in turn
for (i=0; i< imgArray.length; i++)
{selectImage(imgArray[i]);

//To perform a max projection
run("Z Project...", "start=1 stop=999 projection=[Max Intensity]");}

//To print the title of the final image, to use in the following macro
if (nImages>0) {
  title = getTitle();
  print("title: " + title);}

To complete the semi-automated image analysis of each image:

//n is the route of the file name, manually changed using the printed
//title from max projection macro. Series number also manually changed
//for each image
n = "File name.lif - Series000 - ";

//Select max projection of Hoechst channel. Run Median filter. Otsu
//Threshold. Watershed. Select nuclei with a minimum area of 15.
selectWindow( "MAX_" + n + "C=0");
run("Median...", "radius=2");
setAutoThreshold("Otsu dark");
run("Convert to Mask");
run("Watershed");
run("Analyze Particles...", "size=15.00-Infinity exclude clear add");

//Wait for manual correction of thresholding
waitForUser("", "Check Segmentation")

//Measure red (H2AX) channel
selectWindow( "MAX_" + n + "C=1");
run("Set Measurements...", "mean redirect=None decimal=2");
roiManager("Measure");

//Wait for manual copying of results
waitForUser("", "Copy H2AX results")

//Measure green (RPA2) channel
selectWindow( "MAX_" + n + "C=2");
run("Set Measurements...", "mean redirect=None decimal=2");
roiManager("Measure");

//Wait for manual copying of results
waitForUser("", "Copy RPA2 results")

//Save ROIs
roiManager("Save", ")
//Save threshold image
selectWindow("MAX_" + n + "C=0");
saveAs("png")
//Close all other windows
selectWindow("MAX_" + n + "C=0.png");close()
selectWindow("MAX_" + n + "C=1.png");close()
selectWindow("MAX_" + n + "C=2.png");close()
selectWindow(n + "C=0.png");close()
selectWindow(n + "C=1.png");close()
selectWindow(n + "C=2.png");close()
2.7. RNA Expression

RNA was extracted with the Qiagen RNeasy Plus Mini kit (Qiagen, 74134) following the manufacturers protocol. A Real Time Reverse Transcriptase qPCR SYBR assay was carried out with One Step Mesa Green qRT-PCR MasterMix for SYBR Assay no ROX (SYRT-032XNR, Eurogentec) following manufacturers instructions except replacing Mesa Green with Mesa Blue (Eurogentec). 80 ng of RNA was used per well in a reaction volume of 14 µl. Experimental triplicates were completed in each of three biological repeats. All experiments were normalised to GAPDH levels. A BioRad CTX Connect qPCR machine was used with the steps shown in table 2.6 and the primers detailed in table 2.7.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>30 min</td>
<td>x 1</td>
</tr>
<tr>
<td>95</td>
<td>5 min</td>
<td>x 1</td>
</tr>
<tr>
<td>95</td>
<td>3 sec</td>
<td>x 40</td>
</tr>
<tr>
<td>60</td>
<td>45 sec</td>
<td></td>
</tr>
<tr>
<td>Measure</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65-85</td>
<td>10 sec each</td>
<td>Melt Curve</td>
</tr>
</tbody>
</table>

Table 2.6: qRT-PCR cycles used

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence 5’ - 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Fw GAAATCCCATCACCATCTTCCAGG</td>
</tr>
<tr>
<td></td>
<td>Rv GAGCCCCAGCCTTCTCCATG</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Fw TGTCCTGGATGTAGCTGCCTTGA</td>
</tr>
<tr>
<td></td>
<td>Rv TGTCGCACCAGCTACCCCTGAAA</td>
</tr>
<tr>
<td>CtIP</td>
<td>Fw CTGGACTGACACGTGAAGG</td>
</tr>
<tr>
<td></td>
<td>Rv TGGAAATGACGGACGATGGCTTCT</td>
</tr>
</tbody>
</table>

Table 2.7: Primers used in qRT-PCR
2.8. Immunoblotting

Cell extracts were prepared in RIPA buffer (Tris-HCl pH 7.5 20 mM; NaCl 150 mM; EDTA 1 mM; EGTA 1 mM; NP40 1%; NaDoc 1%) containing phosphatase inhibitor cocktails 2 and 3 (1:1000, Sigma, P5726 and P0044) and protease inhibitor cocktail (1:1000, Sigma, P8340). Protein concentrations were measured using either the Pierce Coomassie (Bradford) Protein Assay kit (BioRad, 550-0006) or the Pierce BCA Protein Assay Kit (ThermoScientific, 23227) following manufacturers instructions. Equal concentrations of protein were then loaded onto NuPAGE Novex 4-12% Bis-Tris protein gels (Invitrogen, NP0322), and transferred onto nitrocellulose membranes by wet transfer.

Membranes were incubated with primary antibodies either overnight at 4°C or 1 hour at room temperature. The following antibodies were used, all in 5% milk in PBS/0.2% Tween at 1:1000 unless otherwise stated: Claspin (Rb, ab3720), CtIP (Rb, ab70163), Mre11 (Ms, ab214), Rad54 (Ms, ab11055), Histone H3 (Rb, ab1791) from Abcam. Chk1 (Ms, DCS-310, sc-56291), Cyclin E (HE12) (Ms, sc-247), Rad51 (Rb, H-92, sc-8349), Cdc7 (1:200, Ms, DCS-341, sc-56274), PCNA (1:2000, Ms, F-2, sc-25280), ATR (Gt, N-19, sc-1887), Myc (Ms, 9E10, sc-40), E2F6 (Gt, E-20, sc-8366) from Santa Cruz. GAPDH (1:3300, Ms, GT239) from GeneTex. Tubulin (1:2000, Ms, MAB3408), RPA34-20 (Ms, MABE285), E2F6 (Ms, MABE57) from Millipore. FANCD2 (1:27000, Rb, NB100-182) from Novus biological. RPA32 (RPA2) (S4/S8) (Rb, A300-245A-3) from Bethyl laboratories. Phospho-Histone H2A.X (γH2AX) (Ser139) (Rb, 20E3), Phospho-Chk1 (ser345) (Rb, 2341), p53 (Ser15) (Rb) from Cell Signaling Technology, all used in 5% BSA in PBS/0.2% Tween. Actin (Rb, A2066) from Sigma. HA high affinity (3F10, 11867423001) from Roche. RRM2 (Ms, M01, 1E1) from Abnova. E2F6 (Ms) a kind gift of Dr Jacky Lees.

Membranes were washed in PBS/0.2% Tween and incubated in secondary HRP antibodies for 1-2 hours at room temperature in 5% milk in PBS/0.2% Tween - Goat anti-mouse HRP (1:3333, Fisher Scientific, PA1-74421), Goat anti-rabbit HRP conju-
gated (1:3333, Fisher Scientific, PI-31460), Goat anti-Rat IgG1 HRP conjugated (1:200, Fisher Scientific, PA1-84708) or Rabbit anti-Goat IgG HRP conjugated (1:3333, Sigma, A5420) as appropriate. HRP was visualised using Luminata reagent (MerckMillipore, WBLUR0100) onto Amersham Hyperfilm ECL (GE Healthcare Life Sciences, 28906836). Tubulin, GAPDH or Actin were used as loading controls in whole cell extracts and Histone H3 was used as the loading control for chromatin preparations.

When appropriate western blots were quantified using Fiji following software instructions.

2.9. Flow Cytometry

Flow cytometry of DAPI/RPA2/\(\gamma\)H2AX was performed by Betheney Pennycook as previously described (Forment et al., 2012) using the antibodies Phospho-Histone H2A.X (\(\gamma\)H2AX) (Ser139) (1:200, Rb, 20E3, Cell Signaling Technology) and RPA2 (1:250, Ms, 9H8, ab2175, Abcam). Samples were measured on a BD LSRII flow cytometer using DIVA software (BD) and analysed using FlowJo software.

2.10. Fiber Analysis

Fiber analysis was performed by Cosetta Bertoli. Cells were labelled with 25 \(\mu\)M CldU and then 250 \(\mu\)M IdU following the timings described in the schematics. Fiber spreading and labelling was carried out as previously described (Petermann et al., 2010). Images were taken with a Leica TCS SPE2 and measured with ImageJ. 150-200 fibers were measured for each experiment.

2.11. Immunofluorescence (non-extracted)

Cells in figures 3.19 and 5.11 were not pre-extracted before fixation unlike all other immunofluorescence experiments to allow staining of all cellular proteins and visualisation of RPA2 foci. Cells were fixed for 20 minutes in 3.7% Formaldehyde (Sigma, 252549) and then permeabilised for 5 minutes in PBS with 0.2% Triton-X100 (Sigma,
Coverslips were processed similarly to (Toledo et al., 2013) - coverslips were blocked for 1 hour in 5% BSA. RPA32 (RPA2) (1:1000, Ms, MABE285, Millipore) primary antibody was incubated overnight at 4 °C. Coverslips were washed in PBS and then incubated for 1 hour at room temperature with Alexa Fluor 488 (1:1000, goat anti-mouse IgG (H+L), Life Technologies, A11029) secondary antibody. They were washed again in PBS and incubated for 5 minutes in Hoechst 33342 trihydrochloride, trihydrate (1:10,000, Invitrogen, H3570), washed and mounted with Fluorosheild (Sigma, F6182). Once dry, coverslips were sealed with clear nail varnish. Images were obtained with a Leica TCS SP5 63x objective lens. The percentage of cells containing RPA2 foci was manually counted on max projection images using the Cell Counter in Fiji.

2.12. Chromatin Preparation

Cell were scraped from the dish in 400 µl Buffer A (Hepes pH 7.9 10 mM; KCl 10 mM; MgCl 1.5 mM; Sucrose 0.34 M; Glycerol 10 %; DTT 1 mM) containing phosphatase inhibitor cocktails 2 and 3 (1:1000, Sigma, P5726 and P0044) and protease inhibitor cocktail (1:1000, Sigma, P8340). 0.1 % Triton was added for 8 minutes on ice. The nuclear fraction was pelleted at 1300 g, 5 min, 4°C and washed with 200 µl Buffer A and resuspended in 400 µl Buffer B (3 mM EDTA; 0.2 mM EGTA) with protease and phosphatase inhibitors as above, for 30 minutes on ice. The chromatin fraction was pelleted at 1700 g, 5 min, 4°C and resuspended in 150 µl 15 mM Tris/0.5% SDS. The sample was spun at 9600 g, 5 min, 4°C before use.

2.13. siRNA Silencing

siRNA transfections were performed with Lipofectamine 2000 (Invitrogen, 11668027) according to manufacturer instruction. Transfected cells were split 24 hours after transfection and then used 24 hours later for the experiments (-/+ HU 4 hr) or for control western blot, these originate from the same transfection.
Table 2.8: Sequences used for silencing

<table>
<thead>
<tr>
<th>Silencing</th>
<th>Figures</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>siCont (LacZ)</td>
<td>3.21, 3.22, 3.23, 3.24, 4.10</td>
<td>AACGUACCGGAAUACUUCGA</td>
</tr>
<tr>
<td>siE2F6</td>
<td>3.21, 3.23, 3.24, 4.10</td>
<td>AAGGAUUGUGCUAGCAGCUG</td>
</tr>
<tr>
<td>siE2F6-2</td>
<td>3.22, 3.24</td>
<td>AAACAGGUUGCAACGAAUU</td>
</tr>
<tr>
<td>Chk1</td>
<td>3.21, 3.22</td>
<td>SMARTpool: siGENOME CHEK1 siRNA (Dharmacon GE Life Sciences, M-003255-04-005)</td>
</tr>
<tr>
<td>siATR</td>
<td>3.23</td>
<td>UUGUAGAAUGGAUACUGA (validated Silencer Select sS36, Thermo Fisher Scientific)</td>
</tr>
</tbody>
</table>

2.14. Survival Assays

Survival assays in figure 4.9 were completed by Cosetta Bertoli. RPE1 TetON E2F6 c-Myc-ER cells were treated with 100 nM hydroxytamoxifen and/or 2 µg/ml Doxycycline for 48 or 72 hours as indicated. They were then diluted and grown in normal media in 10 cm dishes until colonies were visible. Cells were washed in PBS then fixed and stained with 8 ml 0.5 % methylene blue in 50 % ethanol for 20 minutes, then washed in distilled water 3 times.
3. The role of transcription in the response to DNA replication stress

The DNA replication stress checkpoint is a conserved cellular response, which prevents DNA damage in cells experiencing DNA replication stress (Jossen and Bermejo, 2013). Replication stress is the slowing or stalling of replication forks which usually exposes extensive single-stranded DNA (ssDNA) (Macheret and Halazonetis, 2015; Muñoz and Méndez, 2016; Toledo et al., 2013; Zeman and Cimprich, 2013), which is bound by the protein RPA (Zou et al., 2006). This is recognised by the checkpoint sensor kinase ATR (Zou and Elledge, 2003), which then activates the effector kinase Chk1 (Liu et al., 2000), thereby activating the DNA replication stress checkpoint response. The cellular response to replication stress includes arresting cell cycle progression (Sørensen et al., 2003), the stalling and stabilisation of active replication forks (Calzada et al., 2005; Lopes et al., 2001; Unsal-Kaçmaz et al., 2007), inhibiting late origins from firing (Ge et al., 2007), and once the stress has been resolved allowing DNA replication to resume (Petermann et al., 2010). Together this response prevents DNA damage and allows the faithful replication of the genome, preventing genomic instability.

The importance of post-translational modifications in this checkpoint response has been well established (Huen and Chen, 2008). In particular, the widespread phosphorylation of proteins by the checkpoint protein kinases ATR and Chk1 is a crucial element of checkpoint signalling (Abraham, 2001; Bartek and Lukas, 2003). Checkpoint protein kinase-dependent phosphorylation alters the activity of checkpoint effectors in many ways including altering interacting partners, activating or inhibiting specific protein functions or targeting proteins for degradation. However, the role of gene expression in the checkpoint response is less well understood. A transcriptional response to replication stress has been identified in both yeast and mammalian cells. This transcriptional response involves the maintenance of G1/S cell cycle transcription during
replication stress (Bastos de Oliveira et al., 2012; Bertoli et al., 2013a; Caetano et al., 2011; Chu et al., 2007; de Bruin et al., 2008; Dutta et al., 2008; Gomez-Escoda et al., 2011; Ivanova et al., 2013, 2011; Travesa et al., 2012). In mammalian cells, G1/S cell cycle transcription is controlled by E2F-dependent transcription. During DNA replication stress, Chk1 phosphorylates and inhibits the transcriptional repressor E2F6 to maintain E2F-dependent transcription (Bertoli et al., 2013a). The role and importance of this transcriptional response to replication stress has not been studied in detail. Previous work inhibiting translation during replication stress in yeast demonstrated that de novo protein synthesis, and hence also transcription, is not required to activate a DNA replication stress checkpoint response, or for the viability of cells following replication stress (Tercero et al., 2003). In contrast, work from our lab (Bertoli et al., 2013a) shows that the transcriptional response is important for cell viability following DNA replication stress in mammalian cells.

In this chapter I present work investigating the role of transcription in the response to DNA replication stress in mammalian cells. I find that, in contrast to yeast, active translation is required for an efficient DNA replication stress checkpoint response. Specifically, E2F-dependent transcription is required to prevent replication stress-induced DNA damage. I find that many checkpoint proteins are unstable and so sustained E2F-dependent transcription is required to maintain the levels of proteins involved in the checkpoint response. Importantly, my work shows that the maintenance of E2F-dependent transcription during replication stress is a key mechanism of the checkpoint response, and sufficient for many specific functions of the DNA replication stress checkpoint.

### 3.1. Active translation prevents DNA damage following DNA replication stress in mammalian cells

As previously discussed, active translation is not required for cell viability following DNA replication stress in yeast (Tercero et al., 2003), however a transcriptional re-
response has been seen to be required for cell viability following replication stress in mammalian cells (Bertoli et al., 2013a). I therefore assessed whether active translation was required for the response to replication stress in mammalian cells, by analysing the levels of DNA damage following replication stress with or without active translation. If active translation is required for the response to DNA replication stress, addition of the translational inhibitor Cycloheximide (Chx) would be expected to result in higher levels of DNA damage. DNA replication stress was induced by acute treatment with the drug Hydroxyurea (HU). HU treatment is widely used as a model for DNA replication stress. HU inhibits the ribonucleotide reductase enzyme (RNR) and so depletes the pools of dinucleotide triphosphates (dNTPs) and causes replication fork stalling.

Quantitative immunofluorescence was used to measure the intensity of chromatin-bound protein in single nuclei, similarly to Toledo et al., 2013. The intensity of Replication protein A (RPA2) was used as a read out for replication stress as it binds the extended lengths of ssDNA exposed during replication stress. There is also a cellular response to DNA damage, which is predominately coordinated by the activity of the ATM kinase. The hyper-phosphorylation of H2AX (γH2AX) is a marker of ATM activity and was therefore used as a read out for DNA damage.

Both ATR (Ward and Chen, 2001) and DNA-PKcs (Tu et al., 2013) can also phosphorylate H2AX. However, ATM is thought to be the major kinase responsible for γH2AX in response to DNA damage and is therefore widely used as a marker of DNA damage (Jones et al., 2013; Seiler et al., 2007; Toledo et al., 2013). In addition, previous work has shown that treatment with HU, with conditions comparable to this work, induces replication stress checkpoint signalling as seen by Chk1 phosphorylation, but not γH2AX (Bertoli et al., 2013a). In contrast, treatment with Camptothecin, which induces DNA damage, does result in γH2AX but not Chk1 phosphorylation. Taken together, although care must be taken in the interpretation of the results, γH2AX can be used as a marker of DNA damage. The combination of these two markers, RPA2 and γH2AX, at the level of individual nuclei allows the analysis of both replication stress
and DNA damage levels, and specifically the levels of replication stress-induced DNA damage, as established by Toledo et al., 2013.

This assay was used to assess the levels of DNA damage experience by RPE1 cells following a 2 hr treatment with HU. RPE1 (Retina Pigmented Epithelium) cells were chosen for these experiments for a number of reasons. This cell line is immortalised with hTERT and is near-diploid (www.ATCC.org). An immortalised cell line is preferable to a transformed cell line as checkpoint responses and E2F-dependent transcription are more likely to be intact. Importantly, we need the DNA replication stress checkpoint response to be intact in these cells, control experiments support this with HU treatment increasing levels of chromatin-bound RPA2 and γH2AX as expected. In addition, as these cells are epithelial in nature they are both adherent and relatively flat, these characteristics are advantageous for immunofluorescence imaging.

As expected, HU treatment in RPE1 cells causes a significant increase in the intensity of γH2AX signal as compared to no treatment, indicating the presence of some DNA damage following HU-induced replication stress (Figure 3.1). Inhibiting de novo protein synthesis with the translational inhibitor Chx causes a further increase in the levels of DNA damage in HU-treated cells. This increase in HU/Chx-treated cells compared to HU-treated cells is not significant at the short time-point of 2 hr, figure 3.1.

When this treatment is extended to 7 hr the levels of DNA damage following Chx treatment in HU-treated cells is significantly increased in one repeat, and shows a clear but non-significant increase in the other (Figure 3.2). This increase in DNA damage is seen in cells with high levels of RPA2 staining, suggesting this DNA damage results from replication stress. These data indicate that, unlike in yeast, mammalian cells do require active translation for an efficient response to DNA replication stress.

With this data set, and all the following experiments similar to this, I have decided to carry out statistical analysis on all the cells in the data set. It is possible to define non-S phase cells as those with low RPA2 levels, and carry out statistical analysis only
on S phase cells. This would make the analysis more specific to replication stress as it would remove cells which are not in S phase and therefore not experiencing DNA replication. In some cases there is a clear separate non-RPA2 staining population that can be defined as non-S phase. However, I have decided against this method for a number of reasons.

Firstly, this low RPA2 staining population is not always clearly separated. Cells with low levels of RPA2 are represented in black on all graphs for visual purposes only, the variation in how clear the two populations are separated can be see in many graphs,
Figure 3.2: Active translation is required to prevent DNA damage following replication stress. Top: Repeat 1. Middle: Repeat 2. Scatter graphs showing mean RPA2 intensity (x-axis) vs mean γH2AX intensity (y-axis) of chromatin-bound protein in individual nuclei in RPE1 cells after 7...
(continued) hr treatments as indicated. HU 0.5 mM, Chx 10 µg/ml with 10 min pre-treatment. 
P = increases in γH2AX intensity with the Wilcoxon test with HU compared to Control and HU 
Chx compared to HU. Black dots show low levels of RPA2 (RPA2 < 10 a.u.), orange and red dots 
show low and high levels of γH2AX respectively (arbitrary threshold γH2AX = 30 a.u.). Bottom: 
Representative images from repeat 1. Scale bar represents 20 µm.

such as in figure 3.1. Different experiments may therefore result in a different accuracy 
in the definition of S phase cells.

Secondly, this method requires a manual decision to be made as to the threshold of 
non-S and S phase cells. This is reasonable if only considering one experiment, however I am repeating these experiments many times and due to staining efficiency and 
microscope power the same threshold cannot be applied to all experiments. Defining 
a threshold manually in each experiment introduces an unnecessary biased variable 
when looking at many different experiments.

Finally, it is unclear what the treatments may do to RPA2 levels themselves. If a treat-
ment, such as translation inhibition, alters the levels of RPA2 this may result in the cell 
cycle status being incorrectly defined. An alternative to these experiments would be 
to carry out flow cytometry analysis where the DNA content could be measured to 
accurately define S phase cells.

However, in these experiments the short time-points used will limit the possibility of 
cell cycle effects. In addition, measuring both RPA2 and γH2AX intensity in the same 
nuclei allows an assessment of the levels of replication stress and DNA damage. There 
is a clear correlation in all experiments between high RPA2 levels and high γH2AX 
levels, strongly supporting that the changes in DNA damage seen are specifically due 
to high levels of replication stress.
3.2. Sustained E2F-dependent transcription prevents DNA damage following DNA replication stress

Previous work has shown that the maintenance of E2F-dependent transcription is required for cell viability following replication stress in mammalian cells (Bertoli et al., 2013a), however the mechanism of this tolerance has not been established. E2F-dependent transcription is induced during G1 phase; it is then repressed during S phase by the transcriptional repressor E2F6. As E2F6 is itself an E2F target this represents a negative feedback loop turning off transcription. During DNA replication stress Chk1 phosphorylates and inactivates E2F6, this removes the repression and results in E2F-dependent transcription being maintained during replication stress. To investigate the role of E2F-dependent transcription I used an inducible overexpression system. Doxycycline-induced overexpression of E2F6 prevents its inactivation by Chk1, and so E2F-dependent transcription is no longer maintained during replication stress.

Two E2F6-inducible cell lines were used in this work - RPE1 TetON E2F6 and HEK293 T-Rex E2F6, confirming the results are consistent across two different cell lines gives more validity to my findings. The advantages of the hTERT immortalised RPE1 cell line has been previously discussed on page 60; the Doxycycline-inducible cell line was constructed for this work as described in the Materials and Methods. The HEK293 T-Rex E2F6 cell line is also Doxycycline-inducible and was constructed in our lab previously (Bertoli et al., 2013a). Although a transformed cell line HEK293 (Human Embryonic Kidney) cells have been used extensively to characterise the interaction between the DNA replication stress checkpoint response and E2F-dependent transcription (Bertoli et al., 2013a). In addition, as with the RPE1 cell line, checkpoint signalling in control experiments show the expected responses to replication stress and DNA damage including increasing chromatin-bound RPA2 and γH2AX, and phosphorylation of Chk1 and p53. I therefore decided to also use this cell line in order to make use of this characterisation. Due to differences in these cell lines some experiments require
different timings, which were based on previous experiments. The RNA levels of E2F-dependent targets in HEK293 T-Rex E2F6 cells demonstrates the function of these inducible systems. The E2F-dependent targets Cyclin E and CtIP, which are also important proteins in the checkpoint response, are upregulated at the RNA level following 6 hr HU-induced replication stress. However, the overexpression of E2F6, induced with Doxycycline (Doxy) 2 hr prior to HU treatment, prevents this upregulation (Figure 3.3). The same response is also seen at the protein level in RPE1 TetON E2F6 cells in figure 3.15. The inducible E2F6 overexpression and subsequent prevention of sustained E2F-dependent transcription allows the study of the role of this transcriptional response during replication stress.

![Figure 3.3: Overexpression of E2F6 prevents the checkpoint-dependent maintenance of E2F-dependent transcription.](image)

To investigate whether E2F-dependent transcription was required to prevent DNA damage following HU-induced replication stress, RPE1 TetON E2F6 cells were treated with HU for 4 hr either alone, with Chx, or with Doxy treatment to overexpress E2F6 and prevent a sustained transcriptional response. The intensity of chromatin-bound RPA2 and γH2AX in individual nuclei was again analysed. Results at this time-point confirm the previous finding that HU treatment does cause some DNA damage, and that this DNA damage is significantly increased when translation is inhibited during replication.
stress. In addition, preventing a sustained E2F-dependent transcriptional response during replication stress significantly increases the levels of DNA damage compared to HU treatment alone, as shown by increased γH2AX intensity, figure 3.4. The effect

![Graphs showing mean RPA2 intensity (x-axis) vs mean γH2AX intensity (y-axis) of chromatin-bound protein in individual nuclei in RPE1 TetON E2F6 cells after 4 hr treatments as indicated. HU 0.5 mM, Chx 10 µg/ml with 10 min pre-treatment, E2F6 = Doxycycline 4 µg/ml with 2 hr pre-treatment. P = increases in γH2AX intensity with the Wilcoxon test with HU compared to Control and HU Doxy or HU Chx compared to HU. Black dots show low levels of RPA2 (RPA2 < 10 a.u.), orange and red dots show low and high levels of γH2AX respectively (arbitrary threshold γH2AX = 50 a.u.).]

Figure 3.4: Active translation and E2F-dependent transcription both prevent DNA damage following replication stress. Scatter graphs showing mean RPA2 intensity (x-axis) vs mean γH2AX intensity (y-axis) of chromatin-bound protein in individual nuclei in RPE1 TetON E2F6 cells after 4 hr treatments as indicated. HU 0.5 mM, Chx 10 µg/ml with 10 min pre-treatment, E2F6 = Doxycycline 4 µg/ml with 2 hr pre-treatment. P = increases in γH2AX intensity with the Wilcoxon test with HU compared to Control and HU Doxy or HU Chx compared to HU. Black dots show low levels of RPA2 (RPA2 < 10 a.u.), orange and red dots show low and high levels of γH2AX respectively (arbitrary threshold γH2AX = 50 a.u.).

seen with Doxy treatment is not as pronounced as with Chx treatment; this is expected as Doxy is a more specific treatment affecting gene expression of E2F-targets only, as opposed to blocking de novo synthesis of all proteins. The increased level of DNA damage seen during replication stress when translation or E2F-dependent transcription was prevented was confirmed by western blot. Compared to HU alone, HU Chx and HU Doxy treatment shows a small increase in levels of markers of DNA damage,
such as γH2AX and p53 phosphorylation (Figure 3.5).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
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<tbody>
<tr>
<td>Chx</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>E2F6</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Chk1 P Ser345</td>
<td>-</td>
<td>-</td>
</tr>
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<td>+</td>
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<tr>
<td>p53 P Ser15</td>
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<td>-</td>
</tr>
<tr>
<td>E2F6</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>GAPDH</td>
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<td>+</td>
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</table>

Figure 3.5: Preventing active translation or E2F-dependent transcription increases markers of replication stress and DNA damage. Western blot of whole cell extract in HEK293 T-Rex E2F6 cells following 2 hr treatments as indicated. HU 0.5 mM, Chx 10 μg/ml with 10 min pre-treatment, E2F6 = Doxycycline 2 μg/ml with 2 hr pre-treatment.

3.3. Active protein synthesis and sustained E2F-dependent transcription are required to prevent replication stress-induced DNA damage

The data presented so far suggests that active translation and specifically E2F-dependent transcription may be required to prevent replication stress-induced DNA damage, possibly due to a role in correct replication stress checkpoint function. However, to confirm that this effect is specific to replication stress, the effects of Chx and Doxy treatment alone need to be established. I therefore repeated the assay described above with no treatment, Chx or Doxy, all in untreated or HU-treated conditions. This was done in triplicate in HEK293 T-Rex E2F6 cells with 2 hr treatments, and in RPE1 TetON E2F6 cells with 7 hr treatments. As discussed before, the different timings used reflect cell lines differences and are based on previous experiments. The times used were as short as possible to prevent cell cycle effects. HEK293 T-Rex E2F6 cells treated with the protein synthesis inhibitor Chx or with Doxy, to prevent sustained E2F-dependent transcription, do not show a significant increase in the level of DNA damage, as measured by γH2AX levels, in the absence of HU treatment (Figure 3.6, Appendix A for
Figure 3.6: Active protein synthesis and E2F-dependent transcription are required to prevent DNA damage specifically following DNA replication stress in HEK293 T-Rex cells. Top: Scatter graph showing mean RPA2 intensity (x-axis) vs mean γH2AX intensity (y-axis) of chromatin-
bound protein in individual nuclei in HEK293 T-Rex E2F6 cells after 2 hr treatments as indicated. HU 0.5 mM, Chx 10 \( \mu g/ml \) with 10 min pre-treatment, E2F6 = Doxycycline 4 \( \mu g/ml \) with 1 hr pre-treatment. Arrows show change in mean on each axis, \( P = \) differences in mean intensity on each axis with the Wilcoxon test compared to control conditions (Control / HU as appropriate). Black dots show low levels of RPA2 (RPA2 < 10 a.u.), orange and red dots show low and high levels of \( \gamma H2AX \) respectively (arbitrary threshold \( \gamma H2AX = 30 \) a.u.). Bottom: Representative images, Scale bar represents 20 \( \mu m \).

(continued) Chx treatment alone shows a significant decrease in two repeats and a significant increase in \( \gamma H2AX \) levels in one repeat. Doxy treatment alone shows a decrease in \( \gamma H2AX \) levels in two repeats, one being significant, and one significant increase. Despite this variation it is clear that both inhibiting protein synthesis and preventing E2F-dependent transcription alone does not cause a consistent increase in levels of DNA damage. It is also seen that inhibiting translation causes a significant decrease in the level of RPA2 staining, indicating that active protein synthesis may be required to maintain RPA2 levels or required for DNA replication in unperturbed S phase. When cells are treated with Chx or Doxy in the presence of HU-induced replication stress an increase in the levels of DNA damage is seen when compared to HU treatment alone. Even at this short time-point of 2 hr Chx causes a significant increase in the levels of \( \gamma H2AX \) in two repeats, although a significant decrease is seen in one repeat. Preventing sustained E2F-dependent transcription, via E2F6 overexpression, causes an increase in the levels of DNA damage in all three repeats, and significantly in two repeats. These data indicate that in HEK293 T-Rex E2F6 cells active protein synthesis and E2F-dependent transcription are required to prevent DNA damage specifically following replication stress.

We see similar results when looking at immortalised RPE1 TetON E2F6 cells treated under the same conditions for 7 hr. Inhibiting translation alone in RPE1 cells does cause an increase in the levels of DNA damage, however the significant increase in \( \gamma H2AX \) levels seen under conditions of replication stress is much greater (Figure 3.7). Inhibiting protein synthesis causes a reduction in the levels of RPA2 staining, as with HEK293 cells, again suggesting that active protein synthesis is required for DNA repli-
Figure 3.7: Active protein synthesis and E2F-dependent transcription are required to prevent DNA damage specifically following DNA replication stress in RPE1 cells. Top: Scatter graph showing mean RPA2 intensity (x-axis) vs mean γH2AX intensity (y-axis) of chromatin-bound
protein in individual nuclei in RPE1 TetON E2F6 cells after 7 hr treatments as indicated. HU 0.5 mM, Chx 10 µg/ml with 10 min pre-treatment, E2F6 = Doxycycline 4 µg/ml with 1 hr pre-treatment. Arrows show change in mean on each axis, P = differences in mean intensity on each axis with the Wilcoxon test compared to control (Control / HU as appropriate). Black dots show low levels of RPA2 (RPA2 < 10 a.u.), orange and red dots show low and high levels of γH2AX respectively (arbitrary threshold γH2AX = 15 a.u.). Bottom: Representative images, Scale bar represents 20 µm.

Preventing a sustained transcriptional response via E2F6 overexpression does not cause a consistent increase in DNA damage in the absence of replication stress. The repeat shown (Figure 3.7) displays an increase in the levels of DNA damage with E2F6 overexpression alone, however, the other two repeats (Appendix A) both show a decrease. In contrast, in RPE1 TetON E2F6 cells under conditions of replication stress, an increase in the levels of DNA damage following E2F6 overexpression is seen in two of the three repeats, indicating that E2F-dependent transcription is likely to be required to prevent DNA damage in cells experiencing replication stress.

It should be noted that, as discussed previously on page 59, γH2AX is not solely the result of DNA damage and subsequent ATM activity, but can also be due to ATR or DNA-PKcs activity. However, results in Figure 3.5 demonstrate that HU treatment can induce ATR activation, as shown by a clear increase in Chk1 phosphorylation, without a concomitant increase in γH2AX. This suggests that the increases in γH2AX intensity are unlikely to be due to ATR-dependent phosphorylation in these conditions. In addition, if increased γH2AX was due to ATR-dependent phosphorylation, this would suggest that preventing E2F-dependent transcription enhances checkpoint signalling; this is in contrast to results discussed later in the chapter showing that the prevention of sustained E2F-dependent transcription has a negative impact on checkpoint functions. Altogether it is therefore likely that the increases in γH2AX intensity seen following inhibition of active protein synthesis and E2F-dependent transcription in conditions of replication stress reflect increases in DNA damage.

The results following protein synthesis inhibition show some variation. To summarise all the data, for each experiment I calculated the percentage change in mean γH2AX intensity.
intensity from HU treatment alone to HU Chx treatment. The statistics shown are calculated from the difference in mean $\gamma$H2AX intensity between the two conditions with the Wilcoxon test, as in the original figures. Although some variation is seen across these experiments, altogether these data show that inhibiting translation causes an increase in DNA damage following replication stress. Specifically, across the eight different experiments carried out in RPE1 cells at 2, 4 and 7 hr we see an increase in $\gamma$H2AX levels following protein synthesis inhibition in seven of these experiments, with four showing significant increases, and a non-significant decrease in one (Figure 3.8). The experiment was carried out in HEK293 cells three times, all at 2 hr, and we see a sig-

![Figure 3.8: Summary of all experiments showing active protein synthesis is required for an efficient DNA replication stress checkpoint response.](image)

The bar graph shows the percentage change in mean $\gamma$H2AX intensity from HU to HU Chx treatments. From left to right: experiments completed in RPE1 cells: 2 hr, 2 hr (Figure 3.1), 4 hr (Figure 3.4), 7 hr, 7 hr (Figure 3.2), 7 hr, 7 hr (Figure 3.7 and Appendix A), experiments completed in HEK293 cells: 2 hr, 2 hr, 2 hr (Figure 3.6 and Appendix A). Label in grey displays P value of difference in HU and HU Chx treatments with the Wilcoxon test, as seen on previous figures.
nificant increase in two experiments, but a significant decrease in one (Figure 3.8). Overall these data indicate that de novo protein synthesis is required for an efficient DNA replication stress checkpoint response to prevent DNA damage.

As with protein synthesis inhibition, preventing sustained E2F-dependent transcription with E2F6 overexpression also shows some variation across biological repeats. I therefore also summarised these experiments by showing the percentage change in mean γH2AX intensity from HU treatment alone to HU Doxy treatment (Figure 3.9), with statistics as described in the original figures. Taken together these data indicate that preventing sustained E2F-dependent transcription increases the levels of replication stress-induced DNA damage. A total of four experiments were carried out in RPE1 cells at 4 hr and 7 hr time-points. Increased DNA damage is seen with E2F6 overexpression in three of these experiments, with one showing a decrease. A further three experiments were carried out in HEK293 cells with all repeats showing an increase in γH2AX intensity, with two being significant. Altogether these show that sustained E2F-dependent transcription is required for an efficient checkpoint response to prevent replication stress-induced DNA damage.

The data presented so far shows that E2F-dependent transcription is required to prevent replication stress-induced DNA damage, despite some variability. This variability could be explained by the fact that replication stress only occurs in S phase, however, due to technical constraints of the cell lines used these experiments cannot be completed in synchronised cells. Therefore any effect seen on replication stress, which only occurs in S phase, will be diluted by other non-S phase cells in the population. Any change in the percentage of S phase cells in these experiments could explain the variability seen. It is possible to define S phase cells based on RPA2 levels, however, as discussed on page 60 this approach has some drawbacks and so I decided it was not appropriate for this data set. Another factor which could introduce variability across these experiments is the throughput of the assay. Images were taken manually and then analysed in a semi-automated manner in Fiji. Although high numbers of cells
Figure 3.9: Summary of all experiments showing sustained E2F-dependent transcription is required for an efficient DNA replication stress checkpoint response. Bar graphs showing the percentage change in mean γH2AX intensity from HU to HU Doxy treatments. From left to right: experiments completed in RPE1 TetON E2F6 cells: 4 hr (Figure 3.4), 7 hr, 7 hr, 7 hr (Figure 3.7 and Appendix A), experiments completed in HEK293 T-Rex E2F6 cells: 2 hr, 2 hr, 2 hr (Figure 3.6 and Appendix A). Label in grey displays P value of difference in HU and HU Doxy treatments with the Wilcoxon test, as seen on previous figures.

were analysed in each experiment (at least 100 cells per condition per experiment), the nature of the experimental design did limit this. To enhance the throughput of this assay Betheney Pennycook in the lab optimised a FACs protocol. In this assay 10,000 cells can be measured per condition and displayed in a density plot (Figure 3.10). This high throughput assay confirms the previous results showing E2F6 overexpression alone does not cause an increase in the levels of DNA damage. However, E2F6 overexpression during HU-induced replication stress causes an increase in the levels of DNA damage compared to HU treatment alone, confirming that the replication stress checkpoint requires sustained E2F-dependent transcription to function.
correctly to prevent DNA damage.

Figure 3.10: FACs analysis confirms that sustained E2F-dependent transcription is required for an efficient DNA replication stress checkpoint response. Density plot of FACs analysis showing RPA2 vs γH2AX staining in RPE1 TetON E2F6 cells after 7 hr treatments as indicated. HU 0.5 mM, E2F6 = Doxycycline 2 µg/ml with 2 hr pre-treatment. Quadrants define RPA2 or γH2AX −/+ cells (−/+ in red and blue respectively), percentage of double positive cells shown in grey. Around 10,000 cells were collected per condition. Logarithmic scale identical for all. Difference in γH2AX intensity compared to non E2F6 control in green with the Student’s T Test. ****P < 0.0001. Work completed by Betheney Pennycook.

3.4. The role of E2F-dependent transcription in Aphidicolin-induced replication stress is unclear

The work so far has used HU treatment to model the replication stress experienced by cells in physiological conditions. However, there are other treatments which also cause and model replication stress. Aphidicolin (Aph) inhibits DNA polymerases and induces replication stress, however it is unclear exactly how Aph treatment results in replication stress. I therefore investigated whether E2F-dependent transcription was required to prevent DNA damage resulting from Aph-induced replication stress. As before the experiments were carried out in triplicate in both HEK293 T-Rex E2F6 and RPE1 TetON E2F6 cells to ensure the effects were not cell line specific. It should be
noted that these experiments were done simultaneously with the experiments shown in Figures 3.6 and 3.7, therefore data for untreated and Doxy alone conditions are repeated in the full results shown in appendix A. As with HU, Aph treatment causes an increase in the levels of $\gamma$H2AX staining indicating that Aph is causing a low level of replication stress-induced DNA damage. The addition of Doxy to overexpress E2F6 and prevent E2F-dependent transcription in HEK293 T-Rex E2F6 cells results in an increase in the levels of DNA damage compared to Aph treatment alone, but only one of these repeats shows a significant increase (Figure 3.11). However, this does indicate that E2F-dependent transcription may also be important in the response to Aph-induced replication stress. Again in RPE1 TetON E2F6 cells Aph does cause an increase in the levels of $\gamma$H2AX indicating the presence of some replication stress-induced DNA damage. When E2F6 is overexpressed in addition to Aph treatment the level of $\gamma$H2AX decreases in two repeats and increases in one repeat, all of these are significant changes (Figure 3.11). These data may suggest that in contrast to HEK293 cells RPE1 cells do not necessarily require E2F-dependent transcription to prevent DNA damage following Aph treatment. From this set of data it is unclear whether E2F-dependent transcription plays a role in the response to replication stress induced by the drug Aphidicolin. The use of a higher throughput method such as the FACs analysis described above may help to clarify this issue.

There are a number of reasons why the effects seen with Aph may not match those seen with HU treatment. These two drugs cause replication stress in different ways which may affect checkpoint responses, influencing the results seen. In addition, the timings used were the same and were originally determined based on previous experiments with HU treatment, it may be that Aph affects DNA replication with different dynamics and so the timings used may not be optimal. The variation seen between the two cell types may suggest that the effects seen with Aph are not as robust and may not be as widely applicable as with HU. Due to this, and the large amount of previous data characterising the response to HU, I will continue to use HU to model replication
Figure 3.11: Aphidicolin-induced replication stress shows cell line variation in the requirement for sustained E2F-dependent transcription. Bar graphs showing the percentage change in mean $\gamma$H2AX intensity from Aph to Aph Doxy treatments. Aph 2.4 mM, E2F6 = Doxycycline 4 µg/ml with 1 hr pre-treatment. From left to right: 3 biological repeats at 7 hr in RPE1 TetON E2F6 cells, 3 biological repeats at 2 hr in HEK293 T-Rex E2F6 cells. Label in grey displays P value of difference in Aph and Aph Doxy treatments with the Wilcoxon test. Full data shown in Appendix A.

stress. However, this does highlight an important general point that although HU is being used to model replication stress this is a specific cause which may not model all endogenous forms of replication stress found under physiological conditions.

3.5. Active protein synthesis is required to maintain the levels of checkpoint proteins

The data presented so far suggests an important role for active protein synthesis in the cellular response to DNA replication stress, specifically through E2F-dependent
transcription. E2F-dependent transcription is responsible for the wave of transcription at the G1 to S phase transition, and many E2F targets are involved in the key processes occurring in S phase such as DNA replication, the replication stress checkpoint and DNA repair. I therefore investigated whether active protein synthesis was required to maintain or increase the levels of E2F target proteins involved in the checkpoint response. Some of these proteins, such as Claspin, also have an important role in unperturbed DNA replication (Petermann et al., 2008). Inhibiting protein synthesis, again with the drug Cycloheximide (Chx), reveals that many key checkpoint effectors are highly unstable, as seen by the drastic reduction in protein levels after 4 hr Chx treatment (Figure 3.12). This indicates that active protein synthesis may be required to maintain the levels of proteins needed during the checkpoint response.

![Figure 3.12: Checkpoint effector proteins are unstable.](image)

To investigate the protein stability of checkpoint effectors in more detail I carried out a time-course during HU treatment both with and without the translation inhibitor Chx in both HEK293 and RPE1 cells. In the absence of protein synthesis inhibition these checkpoint effectors, which are also E2F target genes, are all upregulated during HU-induced replication stress, as expected (Figure 3.13). Under conditions of replication stress when translation has been inhibited two types of protein regulation are seen. First, as with Cyclin E and Claspin, are proteins which are relatively stable and so do
not require active protein synthesis to maintain their levels at these short time-points. However, active protein synthesis is required to strongly upregulate protein levels, as is needed during replication stress. This translation-dependent upregulation of proteins is a relatively fast process, reflecting the need for a swift response to replication stress.

Figure 3.13: Active protein synthesis is required to both maintain and upregulate levels of checkpoint effectors during replication stress. Western blot of whole cell extract in HEK293 and RPE1 cells after different timepoints and treatments as indicated. HU 0.5 mM, Chx 10 µg/ml with 10 min pre-treatment. Asterisks mark unspecific bands. Quantification of western blots with all normalised to GAPDH levels and 0 hr timepoint. Blue shows HU treatment, red shows HU Chx treatment. Circles show HEK293 cells, triangles show RPE1 cells.

Second, as with CtIP and Chk1, are proteins which are inherently unstable and continued protein synthesis is required to maintain their levels during the replication stress response, and eventually upregulate protein levels. These proteins appear to be very unstable with translation inhibition resulting in drastic loss of protein in just 1 to 2 hr.
The instability of these proteins is perhaps not surprising when considering their function, Chk1’s inhibition of origin firing and cell cycle progression and CtIP’s role in DNA resection could be very deleterious to the cell if they persist at high levels once the stress has been resolved. If these proteins are inherently unstable this would protect the cell from DNA damage outside S phase. This describes just two proteins but would be expected to be true for many checkpoint effectors - their vital role in replication stress many become damaging if unchecked in unperturbed conditions, and so protein activity must be restricted to periods of replication stress. These data show that active protein synthesis is required for the correct dynamics of checkpoint effector protein levels in the response to replication stress. Active protein synthesis must both maintain levels of inherently unstable proteins and also strongly upregulate proteins to ensure protein levels are as required for an efficient checkpoint response to replication stress.

I also completed this time-course experiment in a third cell line - T98G. T98G are transformed, but maintain intact E2F-dependent regulation. I used these cells to confirm the role of active protein synthesis in checkpoint protein regulation in a third cell line. These data confirm the previous result that active protein translation is required for the correct dynamics of checkpoint effector protein levels (Figure 3.14). Again these data reveal proteins for which active translation is required to maintain their levels, and also proteins for which active translation is required for their upregulation. Chk1 and CtIP also show faster rates of degradation in HU-treated cells, suggesting that these inherently unstable proteins may also be targeted for degradation in a checkpoint-dependent manner, as has previously been seen for Chk1 (Park et al., 2015; Zhang et al., 2005). For Chk1 this was proposed to prevent inappropriate accumulation and the resulting delay in DNA replication and also limit the length of the checkpoint response (Zhang et al., 2005). The combination of checkpoint-dependent degradation and inherent instability may have important functions for other proteins as well. It would ensure the checkpoint response can be quickly turned off once check-
point signalling is removed, it would also prevent the presence of potentially damaging checkpoint proteins once the cell cycle has returned to unperturbed conditions. The potential harmful effects of these proteins in unperturbed conditions, as discussed on page 80, and the multiple levels of regulation indicates how important the correct and tight regulation of proteins levels may be, as will be discussed later in conditions of oncogene-induced replication stress.

![Figure 3.14](image)

**Figure 3.14: Active translation is required for the correct dynamics of checkpoint effectors in T98G cells.** Western blot of whole cell extract in T98G cells after different timepoints and treatments as indicated. HU 0.5 mM, Chx 10 µg/ml with 10 min pre-treatment.

### 3.6. E2F-dependent transcription is required for the correct protein dynamics of checkpoint effectors

The control of protein levels involves the coordinated regulation of transcription, translation and protein stability. Data presented so far shows that active translation is required to maintain and upregulate the levels of key checkpoint effectors during replication stress. Many of these proteins are E2F targets and so I tested whether E2F-dependent transcription is required for the correct regulation of protein levels during the response to replication stress. These experiments were again carried out in both
HEK293 T-Rex E2F6 and RPE1 TetON E2F6 cell lines to ensure the results were not cell line specific. Doxycycline-dependent overexpression of E2F6 reduces the levels of checkpoint proteins in both unperturbed and HU-treated conditions at a range of time-points and Doxy concentrations (Figure 3.15). In unperturbed conditions E2F6 overexpression represses E2F-dependent transcription and so proteins degraded due to inherent instability are not replaced. During HU-induced replication stress E2F6 overexpression prevents the checkpoint-dependent upregulation of these proteins and so protein levels are much lower than cells treated with HU alone.

Figure 3.15: E2F-dependent transcription is required for the correct expression of checkpoint effectors. Western blot of whole cell extract in HEK293 T-Rex E2F6 and RPE1 TetOn E2F6 cells after different timepoints and treatments as indicated. HU 0.5 mM, E2F6 overexpression (Doxycycline, 4 µg/ml for all except 2 µg/ml HEK293 16 hr. 1 hr pre-treatment for all except no pre-treatment for RPE1 24 hr and HEK293 16 hr.)

Overexpression of E2F6 and hence repression of E2F-dependent transcription may have some cell cycle effects, however these results are seen at a range of treatment times including at very short time-points of 2 hours which minimises any potential cell cycle effects. A limitation of this experimental design is that it is done in non-synchronised cells. This means that any effect, which would be presumed to be S phase dependent, may be diluted out by the non-S phase population. However, the
strength of the repression is strong enough to be seen despite the experiment being carried out in a non-synchronised population. It is assumed that the E2F6-dependent repression acts on cells in late G1 and S phase as this is when E2F-dependent transcription is activated. This experimental design does not confirm that the repression is solely in G1 and S phases of the cell cycle. It is possible that E2F6 could be repressing some proteins outside these phases if the genes are under the partial control of another transcription factor that E2F6 is able to override. These questions could be investigated using a cell line, such as T98G, which can be synchronised. It is also clear that the addition of Doxy during HU treatment does not reduce protein levels down to untreated conditions. This is in contrast to Chx treatment during HU which reduced protein levels to below that of untreated conditions. This is not entirely unexpected as Chx is a much stronger and robust treatment blocking all protein synthesis. E2F6 overexpression cannot completely prevent the HU-induced upregulation of these proteins, in particular of Cyclin E. This could be due to all S phase cells experiencing partial E2F6-dependent repression, or it could be that E2F6 overexpression is not effective in all S phase cells.

To investigate the requirement for E2F-dependent transcription in the regulation of checkpoint effector protein levels further in these non-synchronised cells I carried out a time-course experiment in HEK293 T-Rex E2F6 cells. Checkpoint effector E2F targets are again upregulated in response to HU-induced replication stress. The overexpression of E2F6 prevents this upregulation during the response to replication stress (Figure 3.16). Although as seen previously the effects of E2F6 overexpression are not as strong as Chx and the reduction in protein level is not complete. However, altogether these data show that during the checkpoint response to replication stress active protein synthesis, and specifically E2F-dependent transcription, is required for the correct and optimal regulation of checkpoint effector protein levels. This explains the dependence of the DNA replication stress checkpoint on a full transcriptional response to prevent DNA damage, as was seen in earlier experiments (Section 3.3).
3.7. E2F-dependent transcription is required for specific functions of the checkpoint response

The data so far suggests that E2F-dependent transcription is required for an efficient replication stress checkpoint response to prevent DNA damage and hence genome instability. The checkpoint response to replication stress acts on many processes to prevent further stress, such as arresting cell cycle progression, stalling and stabilising replication forks, inhibiting late origins from firing and resuming DNA replication and the cell cycle once the stress has been resolved. All these different functions combine for an efficient checkpoint response to prevent DNA damage. We therefore investigated which specific checkpoint functions require E2F-dependent transcription.

Replication fork stalling

During DNA replication stress ongoing replication forks must be stalled globally (Ge and Blow, 2010; Unsal-Kaçmaz et al., 2007), this restricts further stress by preventing DNA replication occurring under suboptimal conditions and prevents the exhaustion of replicative factors such as dNTPs. This stalling can be analysed by DNA fibre analysis. Nucleotide analogues such as CldU and IdU are added under specific conditions for known time periods. These analogues only become incorporated into actively replicating DNA. The DNA can be spread on cover slips and the analogues labelled and imaged by immunofluorescence. The length of DNA containing these analogues represents the distance of DNA replication progression in the conditions specified.
To assess the ability of HEK293 T-Rex E2F6 cells to stall replication under conditions of replication stress CldU (labelled purple) was added for 20 min, then IdU (labelled blue) was added with HU for 2 hr. Samples were prepared at the end of this 2 hr period of replication stress. The length of IdU tracks represents the length of DNA replication progress during HU-induced replication stress. Blue tracks were only measured if they directly followed a purple track to ensure that replication was not initiated part

Figure 3.17: E2F-dependent transcription is required for replication fork stalling during stress. DNA fiber analysis in HEK293 T-Rex E2F6 cells. Top: Left: Schematic and representative images. Right: Bar graphs showing IdU stained (blue) track length after 2 hr HU treatment −/+ E2F6 overexpression. HU 0.5 mM, E2F6 overexpression = Doxycycline, 2 μg/ml, 1.5 hr pre-treatment. Bottom: Left: Schematic. Right: Bar graphs showing IdU stained (blue) track length in untreated conditions −/+ E2F6 overexpression for 3.5 hr (equivalent to 1.5 hr pre-treatment and 2 hr HU treatment in Top panel) Doxycycline, 2 μg/ml or 4 μg/ml as indicated. P = differences with the Student’s T Test. Work completed by Dr. Cosetta Bertoli.
way through the treatment time. HU treatment results in a population of short IdU tracks as compared to track length before HU, figure 3.17, indicating an efficient replication arrest as expected. To investigate whether sustained E2F-dependent transcription was required in this process E2F6 was overexpressed and the length of IdU tracks measured. Following E2F6 overexpression the length of replication progression during stress was significantly increased (Figure 3.17). This effect is specific to replication stress as the same time of E2F6 overexpression alone, a total of 3.5 hr Doxy treatment equivalent to 1.5 hr pre-treatment and 2 hr during HU treatment, causes no significant change in replication progression (Figure 3.17 bottom panel). These data indicate that sustained E2F-dependent transcription is required for efficient fork stalling in the response to replication stress.

Replication fork stabilisation

Stalled replication forks must be stabilised during replication stress to prevent collapse and allow replication to resume once the stress has been resolved (Calzada et al., 2005; Lopes et al., 2001). The stability of replication forks can again be assessed using DNA fibre analysis with the addition of a 4 hr chase period of replication stress with no nucleotide analogues present. Previous work has shown that unstable stalled replication forks will give rise to shorter DNA tracks after this unlabelled chase period due to resection of the nascent analogue-containing DNA (Lossaint et al., 2013; Schlacher et al., 2011), however stable stalled replication forks will not undergo this shortening. The mean length of IdU tracks measured is significantly reduced when E2F6 is overexpressed compared to HU treatment alone (Figure 3.18). This indicates that preventing sustained E2F-dependent transcription during replication stress affects the stability of stalled replication forks. Comparing the DNA tracks seen in HU treatment alone without and with the 4 hr unlabelled chase period (Figures 3.17 and 3.18 respectively) the length of DNA tracks is slightly longer when the chase period is present. This may indicate that the nucleotide analogue is not completely washed out before the chase period and so some is incorporated in the chase period, giving slightly longer tracks.
However, comparing these two experiments, when E2F6 is overexpressed we see the opposite - the length of tracks are shorter following the chase period, despite the incomplete removal of nucleotide analogue demonstrated by the control experiments. This further supports the conclusion that E2F-dependent transcription is required for efficient replication fork stalling, and then efficient stabilisation of these stalled forks.

Figure 3.18: E2F-dependent transcription is required for stabilisation of stalled replication forks. DNA fiber analysis in HEK293 T-Rex E2F6 cells. Left: Schematic and representative images. Right: Bar graphs showing IdU stained (blue) track length after 4 hr chase with HU −/+ E2F6 overexpression. HU 0.5 mM, E2F6 overexpression (Doxycycline, 2 µg/ml, 1 hr pre-treatment). P = significantly shorter tracks with E2F6 overexpression, with Student’s T Test. Work completed by Dr. Cosetta Bertoli.

Resolution of stalled replication forks

Once replication stress is no longer present stalled replication forks must be resolved to allow DNA replication to continue and be completed. This resolution may consist of fork restart or the repair of a collapsed fork with replication completed through new origin firing (Petermann et al., 2010). I therefore investigated whether E2F-dependent transcription was required for the efficient resolution of stalled forks following stress by looking at the presence of RPA2 foci in cells, which indicate the presence of stalled replication forks. It should be noted that by the term ‘resolution’ I am describing both the restart of stalled forks and the repair of collapsed forks; this assay does not distinguish between the two mechanisms of resuming DNA replication.
HEK293 T-Rex E2F6 cells were treated with HU for 16 hr with or without E2F6 overexpression. Cells were then washed and released into normal medium for 7 and 9 hr. At each time-point cells were fixed, stained, imaged and the percentage of cells containing RPA2 foci counted. After 16 hr treatment (0 hr after release) both control cells (HU alone) and E2F6 overexpressing cells showed a similar percentage of cells containing RPA2 foci. This indicates a similar percentage of cells were in S phase and experiencing replication stress, this therefore excludes the possibility of cell cycle effects.

Following release from HU treatment control cells showed a significant reduction in the percentage of cells containing RPA2 foci at 7 hr, and a further significant reduction at 9 hr; this establishes that RPA2 foci are resolved, indicating that stalled replication forks are resolved. On the other hand, cells with E2F6 overexpressed during replication stress were unable to resolve RPA2 foci after release from HU with the percentage of cells containing RPA2 foci not decreasing, even after 9 hr. In fact an increase in the percentage of cells containing RPA2 foci was seen, although this was not significant. These data indicate that E2F-dependent transcription is required for DNA replication.

Figure 3.19: The resolution of stalled replication forks requires E2F-dependent transcription. Left: Bar graph showing percentage of HEK293 T-Rex E2F6 cells containing RPA2 foci at times shown after release from 16 hr HU −/+ E2F6 overexpression. HU 0.5 mM, Doxycycline 2 µg/ml. n = 3. >130 cells per condition per repeat. Error bars = SEM. *** P < 0.001, * P < 0.05 with ANOVA and Tukey’s. Right: Representative images. Scale bar represents 10 µm and is the same for all images.
to resume following DNA replication stress.

**Protection of replication forks**

The final checkpoint function investigated was the recruitment to chromatin of proteins required for the stabilisation and protection of stalled replication forks. Factors required for this process such as Rad51 and FANCD2 (Lossaint et al., 2013), Cdc7 (Yamada et al., 2014), PCNA (Ciccia and Elledge, 2010) and Claspin (McGowan and Russell, 2004) are recruited to chromatin after 7 hr HU treatment (Figure 3.20). It should be noted that some of these proteins, such as PCNA and Claspin, will be chromatin bound in unperturbed conditions as they are involved in DNA replication. The overexpression of E2F6 does not reduce levels of Claspin, Cdc7, FANCD2 and Rad51 in whole cell extract over this period, however it severely impairs the recruitment of these factors to chromatin. PCNA is reduced in whole cell extract by E2F6 overexpression, however its recruitment to chromatin is almost completely abolished. These data indicate that E2F-dependent transcription is required for the correct recruitment to chromatin of factors involved in replication fork protection.

![Figure 3.20: Formation of a protective fork complex at chromatin is dependent on E2F-dependent transcription.](image)

Altogether these data demonstrate that sustained E2F-dependent transcription is essential for specific key functions of the checkpoint response - replication fork stalling,
stabilisation and protection, and for resolving stalled replication forks after stress. These functions depend on the maintenance of checkpoint effector protein levels, which depends on sustained E2F-dependent transcription. Overall, E2F-dependent transcription is required for an efficient checkpoint response to DNA replication stress to prevent DNA damage, as seen in section 3.3.

3.8. Sustained E2F-dependent transcription is a key mechanism of the DNA replication stress checkpoint response

Data so far has shown that sustained E2F-dependent transcription is necessary for an efficient response to DNA replication stress. To investigate this further we tested whether the transcriptional response was a key mechanism of the DNA replication stress checkpoint. It is possible that one of the main roles of Chk1 in the checkpoint response is to phosphorylate and inhibit E2F6 and so sustain E2F-dependent transcription, which in turn, by inducing and maintaining protein levels, ensures the correct regulation of many checkpoint effector proteins. If this is the case upregulating E2F-dependent transcription alone in cells with a compromised checkpoint response should be sufficient to prevent DNA damage following DNA replication stress.

E2F activity is sufficient to prevent replication stress-induced DNA damage in checkpoint-compromised cells

In order to test the importance of the E2F-dependent transcriptional response Chk1 was silenced in RPE1 cells to compromise the checkpoint response. Silencing Chk1 in untreated conditions does not cause a significant increase in the levels of $\gamma$H2AX in our experimental conditions. This may be due to the relatively short time-points used or the fact that the silencing of Chk1 may not be complete. However, silencing Chk1 does significantly increase the levels of DNA damage seen following treatment with HU, as seen by increased levels of chromatin-bound $\gamma$H2AX (Figure 3.21). In these checkpoint-compromised conditions silencing the repressor E2F6, and so restoring the maintenance of E2F activity, significantly reduces the levels of DNA damage seen fol-
following HU treatment. This reduction in DNA damage is seen in both Western blot and immunofluorescence of γH2AX (Figure 3.21). A decrease in DNA damage is seen in HU-treated cells in immunofluorescence even when the checkpoint is not compromised, although this is not seen with a difference siRNA also targeting E2F6 (siE2F6-2, Figure 3.22). This indicates that not only is increased E2F activity protective in checkpoint-compromised conditions it may also be protective against replication stress.

**Figure 3.21: E2F activity is sufficient to prevent DNA damage in checkpoint-compromised cells.** RPE1 cells transfected with siRNA shown. Cells were split 24 hr after transfection and treatments carried out 24 hr later, all experiments originate from the same transfection. Untreated or HU-treated for 4 hr. HU 0.5 mM. Left: Column scatter graph showing mean chromatin-bound γH2AX intensity in individual nuclei. P = differences in mean γH2AX intensities with the Wilcoxon test. **** P < 0.0001, *** P < 0.001, * P < 0.05. Right: Top: Representative images. Scale bar represents 20 µm and is the same for all. Bottom: Western blot of whole cell extract. Work done in collaboration with Dr. Cosetta Bertoli; treatments and western blot by Dr. Cosetta Bertoli, imaging and analysis done by myself.
under normal conditions. It may be that E2F targets are limiting in the checkpoint response and so increasing E2F target expression increases the efficiency of the checkpoint response. Importantly, silencing E2F6 in untreated cells causes no significant change to the levels of γH2AX.

In order to confirm these results we repeated the experiment with a second siE2F6 sequence (siE2F6-2), and also analysed RPA2 intensities alongside γH2AX. These results confirm the previous finding that in HU-treated cells with a compromised checkpoint response, increasing E2F activity results in the reduction of DNA damage (Figure 3.22). Again this significant decrease in γH2AX intensity is seen specifically in cells with high levels of replication stress, as indicated by high levels of RPA2 staining. E2F activity is therefore specifically preventing replication stress-induced DNA damage in cells with a compromised checkpoint response, showing that sustaining E2F-dependent transcription is sufficient for key mechanisms of the cellular response to replication stress.

Recent work has shown that accumulation of the Ribonucleotide Reductase (RNR) enzyme subunit RRM2 plays a key role in preventing replication stress and genome instability, particularly when ATR is inhibited (Buisson et al., 2015; Lopez-Contreras et al., 2015). RRM2 is an E2F target and so could explain the protective effect of silencing E2F6. In all experiments we see RRM2 upregulated in response to HU treatment and reduced in response to Chk1 silencing, as might be expected. However, silencing E2F6 does not result in an increase in the levels of RRM2 in western blot (Figures 3.21, 3.23). RNR activity can also be regulated by allosteric modulation and subcellular localisation (Aye et al., 2014). RNR activity was not directly measured following E2F6 silencing and so I cannot exclude that this could have a role in the protective effects seen. However, if the protective effect were to be conferred by increased RRM2 activity, and hence increased levels of dNTPs, this would be expected to reduce the levels of replication stress, as would be seen by a reduction in RPA2 levels. In all four conditions where siE2F6-2 is used, the opposite is seen with three showing a non-significant increase and one showing a significant increase when compared to the non-siE2F6-2 control.
Figure 3.22: A second siE2F6 also prevents DNA damage in checkpoint-compromised cells.
RPE1 cells transfected with siRNA shown. Cells split 24 hr after transfection, treatments carried out 24 hr later, all originate from the same transfection. Control or HU-treated for 4 hr, HU 0.5 mM. Top: Scatter graphs showing mean RPA2 intensity (x-axis) vs mean γH2AX intensity (y-axis) of chromatin-bound protein in individual nuclei. Arrows show change in mean on both axis. $P =$ differences in mean intensity with the Wilcoxon test compared to non siE2F6 equivalent. Black dots show low levels of RPA2 (RPA2 < 20 a.u.), orange and red dots show low and high levels of γH2AX respectively (arbitrary threshold γH2AX = 50 a.u.). Bottom: Western blot of whole cell extracts. Experiments completed by Dr. Cosetta Bertoli, statistical analysis completed by myself.
Therefore the protective effect of increased E2F activity in checkpoint-compromised cells cannot be explained simply through the upregulation of RRM2.

Experiments so far have used Chk1 silencing to compromise the checkpoint response. The classical view is that there is a largely linear kinase cascade from ATR to Chk1 in the response to replication stress, therefore inactivation of Chk1 would compromise all global checkpoint responses. However, recent work has demonstrated that both ATR and Chk1 can have distinct and independent roles in the checkpoint response (Buisson et al., 2015; Koundrioukoff et al., 2013). We therefore repeated the experiment described above with ATR silencing to compromise the checkpoint response upstream and independently of Chk1. In HU-treated cells, silencing ATR does cause a significant increase in the levels of DNA damage. The effects of siATR appear less severe than those seen with siChk1, as has previously been reported (Buisson et al., 2015). Importantly, silencing E2F6, and so increasing E2F activity, is able to significantly reduce γH2AX levels, and this is seen specifically in cells undergoing replication stress (Figure 3.23). In addition, silencing E2F6 in cells with the ATR-dependent checkpoint compromised reduces the levels of RPA2 staining, indicating an overall reduction in replication stress. RPA2 is phosphorylated in response to replication stress, and silencing E2F6 in checkpoint-compromised cells is also able to reduce the level of this marker of stress as seen in western blot (Figure 3.23).

Overall these data show that cells with a compromised checkpoint response, through silencing of either Chk1 or ATR, are unable to tolerate DNA replication stress and so experience high levels of DNA damage. However, maintaining E2F activity alone is sufficient to reduce this replication stress-induced DNA damage. This indicates that sustaining E2F-dependent transcription is not just necessary for an efficient checkpoint response, but is a key mechanism in the DNA replication stress checkpoint response.
Figure 3.23: E2F activity is sufficient to prevent DNA damage in cells with siATR. RPE1 cells transfected with siRNA shown. Cells were split 24 hr after transfection and treatments carried
(continued) out 24 hr later, all experiments originate from the same transfection. Untreated or HU-treated for 4 hr. HU 0.5 mM. Top: Scatter graphs showing mean RPA2 intensity (x-axis) vs mean γH2AX intensity (y-axis) of chromatin-bound protein in individual nuclei. Arrows show change in mean on each axis. P = differences in mean intensities with the Wilcoxon text compared to non siE2F6 equivalent. Black dots show low levels of RPA2 (RPA2 < 50 a.u.), orange and red dots show low and high levels of γH2AX respectively (arbitrary threshold γH2AX = 50 a.u.). Bottom: Western blots of whole cell extracts. Experiments completed by Dr. Cosetta Bertoli, statistical analysis of immunofluorescence completed by myself.

**E2F activity is sufficient to rescue functions of the DNA replication stress checkpoint response important for replication restart**

Sustained E2F activity is sufficient to prevent replication stress-induced DNA damage in checkpoint-compromised cells. We next investigated whether sustaining E2F activity is sufficient to allow replication restart following replication stress in the absence of a Chk1-dependent checkpoint response. Once the stress has been removed cells must resume DNA replication. The ability to do so depends on many checkpoint functions, such as correctly stalling and stabilising replication forks.

DNA fibre analysis can be used to measure the restart of DNA replication following a period of replication stress. CldU (labelled purple) was added for 20 minutes and then washed out. HU was then added, with no nucleotide analogue, to induced replication stress for 2 hr. Cells were washed and then IdU (labelled blue) was added for 1 hr before samples were taken. This allows for the measurement of DNA replication progression before replication stress, in purple, and after a period of replication stress, in blue. With these timings we see that in the control cells, with an intact checkpoint response, the length of DNA tracks before and after replication stress is the same in our experimental setup. The measurements taken after were over a longer period of time, this probably indicates a slight delay in replication restart following replication stress.

In order to compromise the checkpoint response, Chk1 was inhibited during replication stress with the drug UCN01, this prevents the correct restart of DNA replication following stress, as seen by a significant reduction in track length following replication stress (Figure 3.24). This inability to restart DNA replication is due to a loss of the
checkpoint functions required to correctly stall and stabilise replication forks. However, silencing E2F6 and so sustaining E2F activity is able to rescue this defect in

Figure 3.24: E2F activity is sufficient to rescue replication restart in checkpoint-compromised cells. Top: Schematic showing fibre analysis in T98G cells and representative images. Scale bar represents 10 µm and is the same for all. Bottom: Bar graphs showing the length of DNA tracks before and after HU treatment with siRNA and treatments shown. HU 0.5 mM, UCN01 100 nM. P = differences in track length with the Wilcoxon test. ↑ indicates longer tracks after HU treatment, ↓ indicates shorter tracks after HU treatment. Work completed by Dr. Cosetta Bertoli.
checkpoint-compromised cells. Whereas control cells show a significant shortening of DNA tracks after replication stress in UCN01 treatment, in cells where E2F6 is silenced the length of DNA tracks after stress is significantly longer. These data show that E2F activity alone is sufficient for the restart of DNA replication even in cells with a compromised-checkpoint response, further supporting the model that maintaining E2F-dependent transcription is a key mechanism in the checkpoint response to DNA replication stress.

3.9. Summary

Data in this chapter indicates that the checkpoint maintenance of E2F-dependent transcription is a key mechanism in the response to DNA replication stress. Active protein synthesis, and specifically sustained E2F-dependent transcription, is required to maintain and increase E2F target protein levels. Many of these E2F targets are found to be unstable and so require sustained E2F-dependent transcription to maintain and upregulate their levels during the response to replication stress. My work reveals that sustained E2F-dependent transcription is required for many key functions of the checkpoint response; these include the stalling, stabilisation and protection of DNA replication forks and overall for resolving stalled forks once the stress has been removed (Figure 3.25). As E2F-dependent transcription is required for the response to replication stress, preventing this transcriptional response results in increased levels of DNA damage following HU-induced replication stress.

In addition, increasing E2F activity alone is sufficient to rescue checkpoint functions in cells with a compromised-checkpoint response, and limits the levels of DNA damage seen in these cells. Altogether these data indicate that E2F-dependent transcription is a key mechanism in the DNA replication stress checkpoint response in mammalian cells to prevent replication stress-induced DNA damage. The role and importance of post-translational modifications in the checkpoint response is well established, however, data presented here demonstrates that the transcriptional response
to DNA replication stress also plays a key role in the checkpoint response.

Figure 3.25: Sustained E2F-dependent transcription is a key mechanism of the checkpoint response to DNA replication stress. The DNA replication stress checkpoint response maintains E2F-dependent transcription and this is required for the correct regulation of checkpoint effector proteins. This in turn is necessary and sufficient for key checkpoint functions to prevent DNA damage and genome instability.
4. E2F-dependent transcription is required for tolerance to oncogene-induced replication stress

Data presented in chapter 3 shows that E2F-dependent transcription is required for the checkpoint response to replication stress. Importantly, my work suggests that sustained E2F activity is sufficient to prevent DNA damage in cells with a compromised checkpoint response. This shows that E2F-dependent transcription is protective for cells in the context of replication stress. However, this appears contradictory when considering oncogene-induced replication stress. Activation of certain oncogenes, such as c-Myc, Cyclin E and Ras, or inactivation of tumour suppressors, such as pRb, results in increased E2F activity (Chen et al., 2009). As E2F-dependent transcription drives the G1 to S phase transition this results in S phase entry and proliferation. This unscheduled S phase entry is thought to result in oncogene-induced replication stress through a variety of mechanisms (Hills and Diffley, 2014). In this setting an increase in E2F activity is thought to induce replication stress. However, my work in chapter 3 suggests that high levels of E2F activity are also important to tolerate replication stress as part of the checkpoint response.

In this chapter, I present work showing that during oncogene-induced replication stress, using c-Myc induction to model this, preventing sustained E2F-dependent transcription results in higher levels of DNA damage and cell death. Conversely, silencing E2F6 to increase E2F activity reduces DNA damage caused by c-Myc induction. Overall this data suggests that, although deregulation of E2F-dependent transcription may result in oncogene-induced replication stress, this upregulation is simultaneously required for tolerance to high levels of replication stress.
4.1. Inducible overexpression of c-Myc as a model for oncogene-induced replication stress

In order to test the role of E2F-dependent transcription in oncogene-induced replication stress a senior post doc in the group, Dr. Cosetta Bertoli, developed a stable inducible RPE1 TetON E2F6 c-Myc-ER cell line. This cell line allows Doxycycline-inducible overexpression of E2F6, as described in the previous chapter, which prevents the checkpoint-dependent maintenance of E2F-dependent transcription. In addition, treatment with hydroxytamoxifen (4OH-T) results in nuclear localisation of the c-Myc-ER fusion protein, thereby allowing c-Myc to function as a transcriptional activator (Ricci et al., 2004). I used CtIP as a representative E2F target, following the levels of this protein demonstrates the function of this cell line (Figure 4.1). Treatment with Doxycycline (Doxy) represses the levels of CtIP, as seen previously (Figure 3.15). Induction of c-Myc with 4OH-T increases E2F activity and an increase in CtIP levels is seen. Overexpression of E2F6 together with c-Myc activation is then able to repress the level of c-Myc induced CtIP (Figure 4.1).

Figure 4.1: RPE1 TetON E2F6 c-Myc-ER cells allow separate inducible induction of E2F6 and c-Myc. RPE1 TetON E2F6 c-Myc-ER cells. Top: Western blot of whole cell extract showing hydroxytamoxifen (4OH-T) dependent induction of c-Myc-ER in RPE1 TetON E2F6 c-Myc-ER cells compared to RPE1 cells, −/+ 4OH-T as indicated. Tubulin is loading control. Bottom: Western blot of whole cell extract after 72 hr treatments as indicated. E2F6 (Doxy 1 µg/ml), c-Myc (4OH-T 200 nM). CtIP is a representative E2F target which is increased with c-Myc induction, and repressed by E2F6 overexpression. Work completed by Dr. Cosetta Bertoli.

Activation of oncogenes that drive cell cycle entry can result in oncogene-induced replication stress, a very early event in tumourigenesis. In our system induction of
c-Myc activity models the c-Myc activation seen in oncogenic cells (Maya-Mendoza et al., 2014). The activation of c-Myc in these cells induces replication stress, as identified by the presence of RPA2 phosphorylation, a marker of replication stress, and high levels of γH2AX, a marker of DNA damage, (Figure 4.2). The levels of phosphorylated RPA2 induced by c-Myc are similar, although slightly higher, than the levels induced with HU treatment, equivalent to that used in chapter 3. However, HU treatment induces higher phosphorylation of Chk1 at these time points. The lack of Chk1 phosphorylation following c-Myc induction could be due to the very different timings needed for these treatments - 7 hr for HU but 24 and 48 hr for c-Myc induction. Altogether these data show that activation of c-Myc causes replication stress and subsequent DNA damage. The levels of replication stress following c-Myc induction and HU treatment are comparable, although some variation is seen. As with HU and Aphidicolin, discussed in section 3.4, c-Myc induction demonstrates a slightly different model of endogenous replication stress; the inherent differences between these models should be considered in the evaluation of these results. Overall, we conclude that our inducible c-Myc system is a good model of oncogene-induced replication stress.

### Figure 4.2: c-Myc-ER induction and HU-treatment induce comparable levels of replication stress.

Western blot of whole cell extract in RPE1 TetON E2F6 c-Myc-ER cells after 0.5 mM HU-treatment or 100 nM 4OH-T (c-Myc) treatment for the times indicated. Work completed by Dr. Cosetta Bertoli.

#### 4.2. E2F-dependent transcription may be required to prevent DNA damage following oncogene-induced replication stress

To test the role of E2F-dependent transcription I assayed the levels of DNA damage following induction of oncogene-induced replication stress with or without a normal E2F-
dependent transcriptional response. If E2F-dependent transcription is required for the
tolerance to oncogene-induced replication stress overexpressing E2F6 and preventing
a proper transcriptional response would result in increased levels of DNA damage, as
measured by the intensity of $\gamma$H2AX, a marker of ATM activity. As discussed on page
59, $\gamma$H2AX is not a completely unambiguous marker of DNA damage, which should be
considered in the interpretation of these results. However, it is a widely used marker
of DNA damage and is informative, especially when taken together with other assays,
such as cell viability.

RPE1 TetON E2F6 c-Myc-ER cells were treated with or without 4OH-T to induced c-Myc
expression and oncogene-induced replication stress, each condition with or without
Doxy treatment to overexpress E2F6 and prevent sustained E2F-dependent transcription. In the previous chapter I discussed on page 60 the decision to analyse all cells for
those experiments and not select S phase cells only. Although these concerns remain
true for these next experiments, the longer time points and presence of c-Myc activ-
ity means the potential for large cell cycle effects to alter the result is much greater. I
therefore only analysed S phase cells for experiments in this chapter.

RPA2 staining was used to define S phase cells. The distribution of RPA2 staining shows
two separated populations, a low staining non-S phase population and a RPA2 staining
S phase population. The threshold between these two populations varies slightly be-
tween each experiment due to variations in staining and microscope power, the defi-
nition of S phase was therefore defined individually for each experiment, although the
same threshold was used for all conditions within each experiment.

The percentage of S phase cells was analysed as a further control for the experiment.
As an oncogene, c-Myc activity should induce proliferation and result in a higher per-
centage of S phase cells. Whereas E2F6 overexpression represses E2F-dependent tran-
scription which would be expected to reduce entry into S phase and therefore reduce
the percentage of S phase cells. Analysing only S phase cells should remove the cell
cycle effects of these treatments. Therefore only cells able to experience replication stress, as they are undergoing DNA replication in S phase, were assessed for the requirement of E2F-dependent transcription in the response to oncogene-induced replication stress.

As expected a significant increase in the levels of DNA damage is seen following 72 hr c-Myc induction, which is likely to be the result of oncogene-induced replication stress. A significant increase in the levels of DNA damage is seen when E2F6 is overexpressed in c-Myc induced cells, suggesting that E2F-dependent transcription contributes to the tolerance of oncogene-induced replication stress, figure 4.3.

Figure 4.3: E2F6 overexpression may increase levels of $\gamma$H2AX in S phase cells following c-Myc induction. Top: Column scatter graph showing mean chromatin-bound $\gamma$H2AX intensity in individual S phase nuclei in RPE1 TetON E2F6 c-Myc-ER cells following 72 hr treatments as indicated. E2F6 = Doxycycline 2 $\mu$g/ml, c-Myc = hydroxytamoxifen (4OH-T) 100 nM, S phase cells defined as RPA2 > 20 a.u. P values in grey = differences in $\gamma$H2AX intensity with the Wilcoxon test. Mean and SD in red. Bottom: Bar graph showing percentage of S phase cells in each condition.

However, there are some caveats to this data set. Firstly, the distribution of S phase cells seen is surprising. In particular, c-Myc activation would be expected to induce proliferation and therefore increase the percentage of cells in S phase. With no treat-
ment 44% of cells are in S phase. However with all other treatments, including c-Myc induction, this is reduced to 10 - 18%. Although only S phase cells are analysed in this assay a difference such as this may indicate a problem with the experiment and so the results should be interpreted with care. The reduction in S phase cells following 72 hour c-Myc induction may indicate increased apoptosis, however I did not confirm whether or not this was the case.

Secondly, in these experiments E2F6 overexpression alone causes a significant increase in the levels of DNA damage seen when compared to untreated conditions. This may indicate that the result seen may not be specific to replication stress. However, this is inconsistent with work in the previous chapter where E2F6 overexpression alone does not cause a significant increase in DNA damage. Previous experiments were done over a period of 7 hours, whereas this analyses cells after 72 hours, which could explain the differences. This result is also not seen consistently in other experiments at these longer time-points, which I will discuss next. I therefore conclude that although this experiment indicates E2F-dependent transcription may be required in the response to oncogene-induced replication stress, the results should be interpreted with care and further experiments are required.

I decided to change the experimental set up and repeat this experiment at a shorter time point of 24 hours to try and reduce the problems seen after 72 hour treatments. This experiment was repeated in triplicate and the separate experiments are displayed in figure 4.4. E2F6 overexpression alone does not cause a consistent change in the levels of γH2AX, with a significant increase, a significant decrease and a non significant increase seen across the three repeats. The first repeat does not show an increase in the percentage of S phase cells or increased γH2AX levels following c-Myc induction, as would be expected with oncogene-induced replication stress. Repeats 2 and 3 show a more expected pattern with c-Myc induction increasing the percentage of S phase cells, however they show no significant change in the levels of DNA damage.
Figure 4.4: E2F6 overexpression has variable effects on levels of γH2AX in S phase cells following 24 hours c-Myc induction. Top: Column scatter graph showing mean chromatin-bound γH2AX intensity in individual S phase nuclei in RPE1 TetON E2F6 c-Myc-ER cells following 24 hr treatments as indicated. E2F6 = Doxycycline 2 µg/ml, c-Myc = hydroxytamoxifen (4OH-T) 100 nM, S phase cells defined as RPA2 > 20 a.u. for repeat 1, > 10 a.u. for repeat 2 and 3. P values in grey = differences in γH2AX intensity of S phase cells with the Wilcoxon test. Mean and SD in red. Bottom: Bar graph showing percentage of S phase cells in each condition.

A western blot of whole cell extract from repeat 1 shows a slight increase in CtIP levels following c-Myc induction but very low levels of γH2AX induction, figure 4.5, indicating successful c-Myc induction which has not yet caused high levels of replication stress. Together these data suggest that after 24 hours 4OH-T treatment c-Myc has not strongly induced replication stress in this experimental set up. However, it should be noted that repeat 3 does show an increase in γH2AX levels, although non significant, suggesting a low level of oncogene-induced replication stress may have been induced, figure 4.4. In this experiment E2F6 overexpression causes a further increase
in the levels of DNA damage, again indicating that E2F-dependent transcription may be required in the response to oncogene-induced replication stress.

![Figure 4.5: c-Myc induction only causes low levels of DNA damage after 24 hr treatment. Western blot of whole cell extract of RPE1 TetON E2F6 c-Myc-ER cells following 24 hr treatments as indicated. E2F6 = Doxycycline 2 µg/ml, c-Myc = hydroxytamoxifen (4OH-T) 100 nM. Top: Repeat 1. Bottom: Repeat 2. Both from same experiment as figure 4.4.](image)

**4.3. E2F-dependent transcription does prevent DNA damage following oncogene-induced replication stress**

The previous experimental setups have not been suitable to confidently address whether E2F-dependent transcription is required to prevent DNA damage following oncogene-induced replication stress. I decided on two improvements to the experimental design to address these problems. First looking at longer time points of 48 and 72 hours to ensure c-Myc induction causes oncogene-induced replication stress reproducibly. Second, I hypothesised that as c-Myc induction activates E2F activity and E2F6 overexpression represses E2F activity, it is possible that inducing them together would complicate the interpretation of the experiments. It could be imagined that if the treatments were closely balanced inducing them together could result in different outcomes in different cells depending on the exact balance of E2F activity at the time of induction. I therefore induced c-Myc activity first to establish oncogene-induced replication stress in these cells. E2F6 was then overexpressed in the final 24 hours to assess the require-
ment of E2F-dependent transcription in preventing further DNA damage from ongoing oncogene-induced replication stress. The intensity of chromatin-bound γH2AX and RPA2 proteins were analysed after 48 hours 4OH-T treatment to induce c-Myc activity, with Doxycycline-dependent E2F6 overexpression in the final 24 hours. Three biological repeats were completed of this experiment. In addition to immunofluorescence, western blot samples were prepared from the same dish in repeats 1 and 2. Results in figure 4.6 confirm that both c-Myc induction and E2F6 overexpression are successful in these experiments as seen by levels of the proteins themselves, and a typical E2F targets CtIP, as described previously in figure 4.1. In addition, the presence of γH2AX indicates that 48 hour c-Myc induction is likely to cause replication stress at these concentrations in this cell line, confirming this is an appropriate experimental design to test the role of E2F-dependent transcription in oncogene-induced replication stress.

The percentage of S phase cells in repeats 1 and 2 are as expected with E2F6 overexpression reducing and c-Myc induction increasing the percentage of S phase cells (Figure 4.7). Repeat 3 shows little change in the distribution of S phase cells. As ex-
pected c-Myc induction results in an increase in \( \gamma \)H2AX levels in all three repeats, although this increase is not significant in repeat 3. This suggests that these conditions are appropriately modelling oncogene-induced replication stress, although possibly to a lesser extent in repeat 3. In this experiment, as with the 24 hour experiment, E2F6 overexpression alone does not cause a consistent change in \( \gamma \)H2AX levels. Although the first repeat does show a significant increase in \( \gamma \)H2AX levels the further two repeats show no significant changes to \( \gamma \)H2AX levels with E2F6 overexpression alone. Importantly, the overexpression of E2F6 in cells experiencing oncogene-induced repli-

![Figure 4.7: 48 hour c-Myc induction consistently results in increase levels of DNA damage in S phase cells, which is enhanced by E2F6 overexpression. Top: Column scatter graph showing mean chromatin-bound \( \gamma \)H2AX intensity in individual S phase nuclei in RPE1 TetON E2F6 c-Myc-ER cells following 48 hr c-Myc induction as indicated with E2F6 overexpression in the final 24 hours as indicated. E2F6 = Doxycycline 2 \( \mu \)g/ml, c-Myc = hydroxytamoxifen (4OH-T) 100 nM, S phase cells defined as RPA2 > 20 a.u. for all. P values in grey = differences in \( \gamma \)H2AX intensity of S phase cells with the Wilcoxon test. Mean and SD in red. Bottom: Bar graph showing percentage of S phase cells in each condition.](image-url)
cation stress results in an increased level of DNA damage, as measured by $\gamma$H2AX intensity, across all three repeats. Although only the first repeat shows a significant increase, the presence of a consistent increase in every repeat again supports the hypothesis that E2F-dependent transcription may play a role in tolerance to oncogene-induced replication stress.

I then completed the experiment at the longer time-point of 72 hours c-Myc induction, which previously showed a significant increase in $\gamma$H2AX, figure 4.3. E2F6 was only

![Figure 4.8: E2F-dependent transcription is required to prevent DNA damage following oncogene-induced replication stress caused by c-Myc activity. Top: Column scatter graph showing mean chromatin-bound $\gamma$H2AX intensity in individual S phase nuclei in RPE1 TetON E2F6 c-Myc-ER cells following 72 hr c-Myc induction as indicated with E2F6 overexpression in the final 24 hours as indicated. E2F6 = Doxycycline 2 $\mu$g/ml, c-Myc = hydroxytamoxifen (4OH-T) 100 nM, S phase cells defined as RPA2 $> 20$ a.u. for repeat 1, $> 5$ a.u. for repeat 2. P values in grey = differences in $\gamma$H2AX intensity of S phase cells with the Wilcoxon test. Mean and SD in red. Bottom: Bar graph showing percentage of S phase cells in each condition.](image-url)
overexpressed in the final 24 hours to allow oncogene-induced replication stress to establish before E2F-dependent transcription was repressed. The experiment was repeated twice, I was unable to complete a third repeat due to technical issues and time constraints. In repeat 1 c-Myc does not cause an increase in the percentage of S phase cells, it is possible that at this longer time-point the c-Myc induction has started to induce cell death which could explain this. However, the second repeat shows the expected pattern of S phase cells. Importantly, both repeats show a significant increase in the intensity of $\gamma$H2AX staining following c-Myc induction, this indicates the presence of replication stress and subsequent DNA damage. There is also no significant change in the levels of $\gamma$H2AX following E2F6 overexpression alone. However, in both biological repeats preventing sustained E2F-dependent transcription in cells experiencing oncogene-induced replication stress, caused by 72 hours c-Myc induction, results in a significant increase in $\gamma$H2AX intensity; this demonstrates a requirement for E2F-dependent transcription in preventing DNA damage in response to oncogene-induced replication stress.

Across this set of data there is no consistent effect of E2F6 overexpression alone, indicating that any consistent effects seen are relevant specifically during oncogene-induced replication stress. Taken together these data show an interesting temporal pattern as the time of c-Myc induction proceeds. After 24 hours of c-Myc induction there is no clear induction of oncogene-induced replication stress, as demonstrated by inconsistent changes in $\gamma$H2AX levels. As such the effect of E2F-dependent transcription in the response to oncogene-induced replication stress is also not consistent. Although it should be noted that the only experiment showing an increase in $\gamma$H2AX intensity following c-Myc induction also shows a further increase with E2F6 overexpression. When the induction of c-Myc is analysed after 48 hours the levels of DNA damage are consistently increased, although in one repeat not significantly. Following this the effects of E2F6 overexpression in c-Myc induced cells are also not significant in all cases, but all repeats show a further increase in DNA damage when
E2F6 is overexpressed. Finally, after 72 hours c-Myc induction, a consistent and significant increase in DNA damage is observed, and this is significantly increased in all cases by E2F6 overexpression in c-Myc induced cells. I therefore conclude that in cells experiencing oncogene-induced replication stress sustained E2F-dependent transcription is required for an efficient checkpoint response to prevent DNA damage. This means that, although increased E2F activity can cause uncontrolled proliferation and subsequent oncogene-induced replication stress, the upregulation of E2F activity simultaneously acts to enhance the checkpoint response and provides a mechanism of tolerance to the high levels of replication stress experienced in oncogenic cells.

A number of other experiments could be carried out to further investigate the role of E2F-dependent transcription in preventing DNA damage following oncogene-induced replication stress. A limitation to these experiments is that although only S phase cells are analysed, the cell cycle phase in which the treatments is induced is not controlled. This could vary the results depending on whether c-Myc is originally induced during S phase or not. However, preliminary experiments revealed that although it is possible to arrest RPE1 cells in G1 phase through serum starvation and contact inhibition, the subsequent release into the cell cycle is not well synchronised. Although this would allow the induction of c-Myc to be standardised, the subsequent cell cycles would be affected by both c-Myc activity and the variation in release from arrest, this could make interpretation of the data difficult. Although other cells lines can be more easily synchronised, the untransformed nature of RPE1 cells still make them a valuable cell line for these experiments.

Although unable to resolved the non-synchronised induction of c-Myc, flow cytometry analysis could be used to increase the throughput and accuracy of this assay. This would allow the cell cycle phase to be more accurately determined with DNA levels, while also staining for $\gamma$H2AX levels in a higher number of cells than immunofluorescence allows. A further experiment, which could be informative, would be to follow the levels of replication stress or DNA damage markers in live cells. This would allow
the selection of cells induced with c-Myc in particular cell cycle phases. It could also be powerful as the effects of c-Myc induction and subsequent E2F6 overexpression could be followed temporally. This could be used to assess whether E2F-dependent transcription is required for the tolerance to acute or prolonged oncogene-induced replication stress, or whether it is protective at any stage.

4.4. **E2F-dependent transcription is required for cell survival following oncogene-induced replication stress**

Data presented in section 4.3 shows that E2F-dependent transcription is required in the response to oncogene-induced replication stress to prevent DNA damage. However, the replication stress checkpoint must not only prevent DNA damage, but also carry out a range of other functions to ensure the cell can continue to proliferate successfully. Therefore cell viability following replication stress is another important read out for the efficiency of the checkpoint response.

To assess whether E2F-dependent transcription was required for cell survival following c-Myc induction, in addition to preventing DNA damage, Dr. Cosetta Bertoli carried out a colony survival assay. Cells were left untreated or induced with c-Myc each with or without E2F6 overexpression. Treatments of 4OH-T and Doxy were added at the same time, unlike the experiments above, for 48 or 72 hr. Cells were then diluted and allowed to grow in normal, untreated medium for 10 days before being fixed and stained. The number of colonies indicates how many cells were able to survive the treatments and the size of the colonies indicates the growth potential of those cells.

After both 48 and 72 hr treatments there is no effect seen with E2F6 overexpression alone, figure 4.9. Induction of c-Myc results in a clear reduction in colony number, as expected as oncogene activation will induce cell death in many cells. Continued oncogene-induced replication stress is thought to create an environment allowing for mutations, the exact mutations will differ in each cell resulting in colonies with differing growth potentials. Consistent with this, the colony size is more heterogeneous
Figure 4.9: **E2F-dependent transcription is required for cell survival following oncogene-induced replication stress.** Colony formation assay showing the survival and proliferation of RPE1 TetON E2F6 c-Myc-ER cells following c-Myc induction and/or E2F6 overexpression for the times indicated. Treatments were induced simultaneously. E2F6 = Doxycycline 2 µg/ml, c-Myc = hydroxytamoxifen (4OH-T) 100 nM. After treatments cells were diluted in normal medium, grown for 10 day, then fixed and stained. Work completed by Dr. Cosetta Bertoli.

following c-Myc induction. Importantly, the overexpression of E2F6 in c-Myc treated cells reduces the number of colonies compared to c-Myc induction alone. This indicates that sustained E2F-dependent transcription is required for cell viability in response to oncogene-induced replication stress. This also supports a model where E2F-dependent transcription plays a key role in the tolerance to oncogene-induced replication stress.

### 4.5. Increasing E2F-dependent transcription is protective for cells following oncogene-induced replication stress

E2F6 overexpression increases levels of DNA damage following c-Myc induced replication stress. This suggests an important role for sustained E2F-dependent transcription in preventing DNA damage resulting from oncogene-induced replication stress. To test this model further we investigated whether the reverse was also true, if further increasing E2F activity could prevent DNA damage following oncogene-induced replication stress in this system. As previously, the levels of chromatin-bound protein in individual S phase nuclei were analysed. RPA2 was used as a marker of replication stress and γH2AX as a marker of DNA damage. c-Myc induction for 48 hours causes
high levels of DNA damage specifically in cells experiencing high levels of replication stress, as would be expected following oncogene-induced replication stress. Silencing E2F6 alone causes a small, 9.5 a.u., but significant decrease in the mean levels of γH2AX. However, silencing E2F6 in c-Myc induced cells results in a much larger, 37.1 a.u., significant decrease in the levels of c-Myc-induced DNA damage. Interestingly,

![Graph showing mean RPA2 intensity vs mean γH2AX intensity](image)

Figure 4.10: Increasing E2F activity is protective for cells following oncogene-induced replication stress. Scatter graph showing mean RPA2 intensity (x-axis) vs mean γH2AX intensity (y-axis) of chromatin-bound protein in individual nuclei in RPE1 TetON E2F6 c-Myc-ER cells transfected with the siRNA shown with or without 48 hours c-Myc induction. c-Myc = hydroxytamoxifen (4OH-T) 100 nM. Arrows show change in mean on each axis, P = differences in intensity of S phase cells on each axis with the Wilcoxon test compared to appropriate siCont. Black dots show non-S phase cells (RPA2 < 10 a.u.), orange and red dots show low and high levels of γH2AX respectively (arbitrary threshold γH2AX = 50 a.u.). Experiments completed by Dr. Cosetta Bertoli, statical analysis of immunofluorescence completed by myself.

silencing E2F6 is able to completely rescue the levels of γH2AX staining induced by c-Myc back to control levels, the mean levels of γH2AX between untreated siCont and c-Myc induced siE2F6 cells show no significant difference with a P value of 0.1598. These data strongly suggest that active E2F-dependent transcription is a key component of
the checkpoint response and is required to prevent DNA damage and subsequent cell
death following oncogene-induced, as well as HU-induced, replication stress.

4.6. Summary

Data in chapter 3 demonstrated that E2F-dependent transcription is a key mechanism in the checkpoint response to HU-induced replication stress, and is both required and sufficient for many checkpoint functions to prevent replication stress-induced DNA damage. Here I present evidence that this is also the case during oncogene-induced replication stress. The data presented reveals that E2F-dependent transcription is required to prevent DNA damage and subsequent cell death following c-Myc induced replication stress. Increasing E2F activity alone is protective and is sufficient to reduce the levels of c-Myc induced DNA damage. This has important implications when considering tumourigenesis.

Many oncogenes deregulate E2F activity and therefore disrupt the careful control of S phase entry and result in uncontrolled proliferation, which is thought to be at the basis of oncogene-induced replication stress. However, my work suggests that this deregulated E2F activity has an additional consequence. E2F activity is required for an efficient response to replication stress and so this deregulation simultaneously up-regulates the mechanism of tolerance to high levels of replication stress induced by oncogene activation or loss of tumour suppressors. So although oncogenic activity can result in high levels of replication stress (Bartkova et al., 2005; Gorgoulis et al., 2005), it can also increase the cellular response to tolerate replication stress. This allows oncogenic cells to tolerate high levels of replication stress and prevent DNA damage, figure 4.11.

This would predict that oncogenic cells may experience an increased reliance on E2F activity, and as such provides an interesting therapeutic possibility. Targeting this tolerance mechanism could be expected to be detrimental for cancer cells experiencing high levels of replication stress, but have limited effects on healthy cells which are ex-
Figure 4.11: Increased E2F activity both contributes to oncogene-induced replication stress and the tolerance to it. Activation of many oncogenes can deregulate E2F activity and subsequently result in uncontrolled proliferation and oncogene-induced replication stress. This increased E2F activity simultaneously acts as a mechanism of tolerance to the stress to prevent DNA damage and cell death.

experiencing much less replication stress. This approach, in particular in combination with treatments exploiting the loss of DNA repair pathways in cancerous cells, could prove therapeutically useful in killing cancer cells experiencing high levels of replication stress, without harming healthy cells.
5. Investigating the mechanism of E2F6-dependent repression

E2F-dependent transcription is controlled by a family of 8 transcription factors, as discussed in detail in section 1.2. E2F6 is the transcriptional repressor during S phase (Giangrande et al., 2004), and may play a role in repressing transcription in G0 (Deshpande et al., 2007; Gaubatz et al., 1998; Ogawa et al., 2002). During the checkpoint response to DNA replication stress, E2F6 is inactivated by Chk1 phosphorylation. This results in sustained E2F-dependent transcription, a key component of the checkpoint response, as shown in chapters 3 and 4. E2F6 is therefore an integral part in the correct regulation of E2F-dependent transcription.

Despite the important role of E2F6 in the cell cycle and the replication stress checkpoint, the molecular mechanism of E2F6-dependent repression has not been fully established. Work has shown that E2F6-dependent repression is independent of sequestration of the dimerisation partner DP proteins, but relies on its ability to compete with activator E2Fs for binding to E2F-specific promoters (Cartwright et al., 1998; Gaubatz et al., 1998; Trimarchi et al., 1998). I will use E2F1 as an example of a typical E2F activator when needed for simplicity. There is some evidence that the displacement of activator E2Fs is sufficient for E2F6-dependent repression (Cartwright et al., 1998; Oberley et al., 2003), left in figure 5.1. However, there is also evidence that E2F6 has repressive function independent of E2F promoter binding (Gaubatz et al., 1998) and mediates repression through the recruitment of co-repressors (Trojer et al., 2011) or Polycomb group proteins to promoters, right in figure 5.1. Polycomb group (PcG) proteins alter chromatin structure in order to regulate long-term gene repression. E2F6 has been identified in complexes with a range of Polycomb group proteins, supporting this mechanism (Attwooll et al., 2005; Deshpande et al., 2007; Ogawa et al., 2002; Trimarchi et al., 2001; Trojer et al., 2011). It may be that both of these mechanisms
are required for full E2F6-dependent repression either during different phases of the cell cycle or with different dynamics. For example, displacement could be responsible for repression during S phase, but the recruitment of Polycomb group proteins could be required for longer-term repression in G0. Alternatively, in both cell cycle phases displacement may initially repress transcription, with repression consolidated through the subsequent recruitment of Polycomb group proteins. I aimed to investigate which of these mechanisms is responsible for the repression of E2F-dependent transcription during S phase in our system.

![Figure 5.1: Proposed mechanisms of E2F6-dependent repression.](image)

**Figure 5.1: Proposed mechanisms of E2F6-dependent repression.** Top: During late G1 phase activator E2Fs, such as E2F1, are bound to promoters. Left: Displacement. E2F6 may displace the transcriptional activator E2F1 and hence result in a loss of activation. Right: Polycomb group protein recruitment. The binding of E2F6 to promoters may recruit Polycomb Group proteins to alter chromatin structure and result in active repression.

E2F6 is composed of a number of domains, see schematic in figure 5.2. The DNA binding domains allows specificity of binding to E2F promoters. The dimerisation domain is required for binding to the obligate binding partner DP-1 or DP-2, which is essential for efficient DNA binding. Both the displacement of activators, and the recruitment of repressive factors are dependent on E2F6 binding DNA in a sequence specific manner, therefore both of these domains will be essential for E2F6-dependent repression via either mechanism.
Figure 5.2: E2F6 protein domains. A schematic of the protein domains in transcriptional repressor E2F6. Below: Mutations referred to in this chapter.

The C terminal repression domain is so named as it is sufficient to repress transcription in a Gal4 fusion protein (Gaubatz et al., 1998), although it should be noted that an independent study did not reproduce this result (Cartwright et al., 1998). The repression domain is also required for binding to the Polycomb group proteins RYBP (Trimarchi et al., 2001) and EPC1 (Attwooll et al., 2005). Hence, this domain is expected to be required for repression via recruitment of Polycomb group proteins, but would not be expected to impact repression mediated by displacement of activators from promoters. An E2F6ΔC 1-178 mutant lacking the repression domain should be sufficient for displacement mediated repression, but unable to repress transcription through the recruitment of additional factors. This mutant, combined with my extensive characterisation of E2F6 wild type function in S phase and DNA replication stress, allows me to test which mechanism is responsible for E2F6-dependent repression in S phase.

In this chapter, I show that overexpression of the E2F6ΔC 1-178 mutant, containing no repression domain, partially represses E2F targets at the RNA and protein levels in a number of cell lines. In addition, functional assays demonstrate that overexpression of E2F6ΔC is sufficient to prevent an efficient checkpoint response, comparable to E2F6 wild type overexpression. Altogether these data suggest that the displacement of activator E2Fs, likely during S phase, is a mechanism of E2F6-dependent repression. However, my data does not exclude other mechanisms, such as recruitment of repressive factors, from also being required for full E2F6-dependent repression.
Further work is required to fully understand the relative contributions of different mechanisms of E2F6-dependent repression. In addition, it will be important to investigate the cell cycle phase dependence of these mechanisms. Elucidating the mechanism of E2F6-dependent repression not only adds to the understanding of cell cycle transcriptional regulation and the DNA replication stress checkpoint, but also gives the opportunity to gain deeper insight into cancer associated mutations found in E2F6. As mutations are seen clustered in both the DNA binding and the repression domains, knowledge of E2F6-dependent repression may explain the impact of these mutations in cancer development.

5.1. E2F6ΔC partially represses E2F-dependent transcription in HEK293 cells despite containing no repression domain

I previously demonstrated that inducible overexpression of wild type E2F6 was able to repress E2F targets at the RNA level, figure 3.3, in response to HU-induced replication stress. In order to analyse whether the repression domain was required for this E2F6-dependent repression I made cell lines in which E2F6ΔC, which does not contain the repression domain, could be inducibly overexpressed by addition of Doxycycline. HEK293 T-Rex cells were transfected with the pcDNA4/TO E2F6ΔC 1-178 plasmid.

![Image of Western blot](image)

**Figure 5.3: Selecting clones showing Doxycycline-inducible E2F6ΔC overexpression.** Western blot of whole cell extract in HEK293 T-Rex cells transfected with pcDNA4/TO E2F6ΔC 1-178 and selected in 5 µg/ml Blasticidin and 200 µg/ml Zeocin, with or without Doxycycline treatment, 2 µg/ml, for 24 hr. Clone 2 shows no E2F6ΔC overexpression, clones 7 and 9 show low levels of E2F6ΔC overexpression. Clones 3 and 6 show good E2F6ΔC overexpression and were selected for further work.
Cells were then diluted and selected in Blasticidin and Zeocin. Colonies originating from single cells were tested for Doxycycline-dependent E2F6 overexpression by western blot, figure 5.3. No E2F6ΔC overexpression is seen in clone 2 and clones 7 and 9 show low levels of E2F6 ΔC overexpression. Clones 3 and 6 show good Doxycycline-dependent E2F6ΔC overexpression, interestingly with concurrent repression of the E2F target Cyclin E. These clones were selected for further experiments.

I used these two clones, cl3 and cl6, to analyse whether E2F6ΔC overexpression was able to repress the E2F targets Cyclin E and CtIP at the RNA level. The experimental design was as in figure 3.3 on page 65, with 6 hours of treatment with and without HU and Doxycycline treatment. E2F6ΔC overexpression alone does not significantly change the levels of CtIP or Cyclin E RNA in either clone 3 or clone 6, figure 5.4. In the absence of HU treatment both clones show a slight but non-significant increase in CtIP levels, and a slight but non-significant decrease in Cyclin E levels, as is seen when E2F6 wild type is overexpressed, figure 3.3 in chapter 3. HU-induced replication stress causes an increase in both Cyclin E and CtIP levels in both clones, as expected. In HU-treated cells, the overexpression of E2F6ΔC in clone 6 causes a significant reduction in both Cyclin E and CtIP levels, as is seen following E2F6 wild type overexpression. This suggests that the overexpression of E2F6ΔC in this cell line is able to repress E2F-dependent transcription in the same way as E2F6 wild type overexpression, despite containing no repression domain. However, the overexpression of E2F6ΔC in clone 3 does not show the same result. Although overexpression of E2F6ΔC in HU-treated cells is able to reduce Cyclin E levels in this clone, this difference is not significant. In HU-treated cells, clone 3 E2F6ΔC overexpression results in no significant change in CtIP levels. Clone 3 shows more variation between the repeats of this experiment. In addition, in a functional assay used later, figure 5.11, this cell line does not show the expected control result without Doxycycline treatment. I conclude that this is a less reliable clone to use and therefore used E2F6ΔC clone 6 for further experiments.

When comparing E2F6 wild type and E2F6ΔC clone 6 overexpression exactly the same
effect is seen on RNA levels of the E2F targets CtIP and Cyclin E. This suggests that the mutant form of E2F6 containing no repression domain is able to repress as efficiently as the full length protein, supporting the displacement of E2F1 from promoters as the mechanism of E2F6-dependent repression, as the recruitment of Polycomb Group proteins is thought to be dependent on the repression domain.

**Figure 5.4: RNA expression reveals E2F6 ΔC represses E2F targets.** Bar graphs showing RNA expression levels in HEK293 T-Rex E2F6 ΔC cells, clone 3 (bottom) and clone 6 (top), following 6 hour treatment −/+ HU (0.5 mM) each −/+ E2F6 overexpression (Doxycycline, 2 µg/ml with 2 hour pre-treatment). Normalised to GAPDH and to no treatment. n=3, error bars show SEM. **** P < 0.0001, ** P < 0.01, * P < 0.05 with one-way ANOVA and Sidak’s.

Having identified E2F6 ΔC Clone 6 as the best clone to use for further experiments I compared the ability of the wild type and ΔC E2F6 proteins to repress RNA levels of E2F targets by repeating the previous experiment with the two cell lines simultaneously. I decided to investigate how consistent and stable the repression was by
analysing longer time points of 8 and 16 hours. Again each cell line was untreated or treated with HU to cause replication stress, each with and without Doxycycline treatment inducing E2F6 overexpression. After 8 hours treatment, overexpression of wild type E2F6 reduced Cyclin E RNA levels in both untreated and HU treated conditions, although not significantly. Overexpression of E2F6ΔC also shows a slight reduction in Cyclin E levels in both conditions, but to a lesser extent than the wild type (Figure 5.5). CtIP shows no significant change with both wild type or ΔC E2F6 overexpression.

![Figure 5.5: Overexpression of both WT and ΔC E2F6 does not consistently repress E2F targets at 8 hours. Bar graphs showing RNA expression levels in HEK293 T-Rex E2F6 Wild Type and ΔC (cl6) cells following 8 hour treatments −/+ HU (0.5 mM) each −/+ E2F6 overexpression (Doxycycline, 2 µg/ml with no pre-treatment). Normalised to GAPDH and to no treatment. n=3, error bars show SEM. **** P < 0.0001, ** P < 0.01 with one-way ANOVA and Sidak’s.](image)

When the treatment is taken to 16 hours, overexpression of wild type E2F6 is able to significantly reduce Cyclin E RNA levels during replication stress. However, overex-
pression of E2F6ΔC does not cause a significant change in HU-treated cells, despite showing a clear reduction, figure 5.6. As with 8 hour treatment, no significant changes are seen with CtIP RNA levels, although small reductions are seen with overexpression of both proteins. Taken alone the results here do not strongly suggest that E2F6ΔC is able to repress transcription, however the results seen with E2F6 wild type are not as strong as previously seen and so this should be taken with caution.

Figure 5.6: Overexpression of both WT and ΔC E2F6 does not consistently repress E2F targets at 16 hours. Bar graphs showing RNA expression levels in HEK293 T-Rex E2F6 Wild Type and ΔC (cl6) cells following 16 hour treatments −/+ HU (0.5 mM) each −/+ E2F6 overexpression (Doxycycline, 2 µg/ml with no pre-treatment). Normalised to GAPDH and to no treatment. n=3, error bars show SEM. **** P < 0.0001, ** P < 0.01 with one-way ANOVA and Sidak’s.

I do not know why the changes seen here at 8 and 16 hours are less clear than after 6 hours. The changes induced by HU treatment do appear to be smaller than seen previously in figure 5.4, so maybe all changes in these experiments are reduced. A
longer HU-induced S phase arrest might be expected to reduce variation between cells as more cells would accumulate in S phase. However, it is possible that instead, the longer arrest activates different signalling pathways, which may mask the effects of E2F6 overexpression. These data suggest that E2F6ΔC is able to repress expression of E2F mRNAs, however it appears to be less efficient than the wild type protein. It could be that the difference seen over longer time-points reveals an important insight in the function of the wild type and ΔC proteins. It could be hypothesised that initially E2F6-dependent repression depends on displacement of E2F1 alone, and so both the wild type and mutated proteins show equal function. Perhaps over longer time-points the recruitment of Polycomb group proteins is required to consolidate this repression, which E2F6ΔC cannot do. However, these data should be analysed with caution as the wild type protein does not consistently reduced E2F RNA levels significantly at these longer time-points.

Following the unclear results seen at 8 and 16 hours I repeated the experiment with E2F6 wild type and ΔC cell lines simultaneously, but at the original time point of 6 hours treatment. Overexpression of both E2F6 wild type and ΔC significantly reduces Cyclin E RNA levels in both untreated and HU treated conditions, figure 5.7. Although significant, the reduction seen following E2F6ΔC overexpression is less than seen with E2F6 wild type, although the increase induced by HU treatment is also reduced which could explain this difference. Surprisingly, when considering the previous data, there is no significant change in CtIP RNA levels with either E2F6 wild type or E2F6ΔC overexpression. The individual repeats show different results, which could be down to biological or technical variations, and this may explain the inconsistency with previous experiments. Overall, in this experimental set up, overexpression of E2F6 wild type and ΔC show the same effect on RNA levels of E2F targets, supporting displacement of E2F1 as the molecular mechanism of E2F6-dependent repression.

E2F6ΔC overexpression mimics E2F6 wild type overexpression when analysing RNA levels of E2F targets. I next looked at the effects of overexpression on protein levels.
Figure 5.7: RNA expression reveals E2F6ΔC represses E2F targets similarly to E2F6 Wild Type. Bar graphs showing RNA expression levels in HEK293 T-Rex E2F6 Wild Type and ΔC (c6) cells following 6 hour treatment −/+ HU (0.5 mM) each −/+ E2F6 overexpression (Doxycycline, 4 µg/ml with 2 hour pre-treatment). Normalised to GAPDH and to no treatment. n=3, error bars show SEM. **** P < 0.0001, *** P < 0.001, ** P < 0.01, * P < 0.05 with one-way ANOVA and Sidak’s.

RNA and proteins levels are not directly correlated due to differences in mRNA stability, translation rate, protein stability and turnover rates contributing to the overall protein level. Samples were prepared from the same experiment as figure 5.7 and run on western blots. Four repeats were quantified using the Gel Analyzer tool in Fiji. All results were normalised to GAPDH and to the non Doxy treated control. I normalised HU/Doxy to HU, rather than normalising to untreated, as the four repeats showed large variation in the extent of E2F target induction following replication stress. Normalising HU/Doxy to HU makes differences due to E2F6 overexpression clearer. Overexpression
of E2F6 wild type significantly represses both Cyclin E and CtIP in both untreated and replication stress conditions, figure 5.8, as seen previously at a number of time points, figure 3.15. Overexpression of E2F6ΔC represses levels of CtIP in both untreated and HU-treated conditions, although not significantly. The first three repeats all show a repression in both conditions, however the fourth repeat shows an anomalous increase. When this fourth repeat is excluded E2F6ΔC does significantly repress CtIP in HU-treated conditions. In HU-treated cells, E2F6ΔC is unable to repress a second E2F target Cyclin E, with no significant change seen. Together these data suggest that in HEK293 cells overexpression of E2F6ΔC, which lacks the repression domain, is able to repress E2F-dependent transcription; however, this repression is to a lesser extent
than with overexpression of the wild type protein.

5.2. **E2F6ΔC also partially represses E2F-dependent transcription in RPE1 cells**

Data from HEK293 cells demonstrates that E2F6ΔC overexpression is able to repress E2F-dependent transcription, although perhaps to a lesser extent than wild type overexpression. I wanted to assess the ability of E2F6ΔC to repress E2F-dependent transcription in a non-transformed cell line. I therefore made inducible E2F6 overexpression RPE1 TetON cell lines, as a tool for future experiments with E2F6 HA-tagged at the N terminal. In addition to the wild type protein and ΔC 1-178, which does not contain the repression domain, I also cloned E2F6ΔN 129-281, which does not contain the DNA binding domain, as a negative control. The ability to repress E2F-dependent transcription was again assessed following Doxycycline-induced E2F6 overexpression in untreated and HU-treated conditions. Two separately isolated clones each of E2F6 wild type, ΔC and ΔN were tested. RNA and protein levels of the E2F targets Cyclin E and CtIP were analysed after 8 hours treatment, this time-point was previously established in RPE1 cells to show differences with overexpression of E2F6 wild type, figure 3.15. I added a further negative control with an RPE1 TetON Empty cell line to ensure any effects seen following Doxycycline treatment are specifically due to protein overexpression and not unspecific effects on the cells.

Overexpression of HA-tagged E2F6 wild type reduces RNA levels of the E2F target Cyclin E in untreated conditions, and significantly reduces Cyclin E levels in HU-treated conditions, this is seen in both wild type clones. Overexpression of E2F6ΔC shows the same results as wild type, with clone 2 also able to significantly reduce Cyclin E levels even in untreated conditions, figure 5.9. E2F6ΔC does not contain the repression domain and so these data strongly support displacement of E2F1 as the mechanism of E2F-dependent transcription. The negative control, HA-E2F6ΔN, contains no DNA binding domain. The DNA binding domain should be required for both mechanisms
Figure 5.9: RNA expression reveals E2F6ΔC and E2F6ΔN represses E2F targets similarly to E2F6 Wild Type. Bar graphs showing RNA expression levels in RPE1 TetON HA-E2F6 Wild Type.
(continued) (clone 10 and 14) and ΔC (clone 1 and 2) and ΔN (clone 8 and 11) and Empty cells following 8 hour treatment −/+ HU (0.5 mM) each −/+ E2F6 overexpression (Doxycycline, 4 µg/ml with 1 hour pre-treatment). Normalised to GAPDH and to no treatment. n=3, error bars show SEM. **** P < 0.0001, ** P < 0.01, * P < 0.05 with one-way ANOVA and Sidak’s.

of repression, displacement and recruitment of PcG proteins to promoters, therefore overexpression of HA-E2F6ΔN should be unable to repress transcription. However, as seen in figure 5.9, E2F6ΔN is able to significantly repress transcription to the same extent as wild type. Results in the RPE1 TetON Empty cell line show no significant changes following Doxycycline, confirming the effects seen are specific to E2F6 overexpression.

The RNA levels of a second E2F target, CtIP, were also analysed. As seen in previous experiments the changes seen with CtIP are not as clear as those seen with Cyclin E. In HU-treated conditions, CtIP levels are slightly lower with E2F6 overexpression in both wild type clones and in E2F6ΔC clone 1, a slight increase is seen in ΔC clone 2. However, none of these changes are significant and so conclusions cannot be drawn. Surprisingly, overexpression of E2F6ΔN, containing no DNA binding domain, is able the significantly reduce CtIP levels in HU-treated conditions, thereby showing a stronger repressive ability than the wild type protein. This result will be discussed in detail in section 5.3. RPE1 TetON Empty again confirms that this result is specific to E2F6 overexpression. All clones show clear induction of E2F6, although a range of inductions are seen, from 9 to 58 fold. This could explain some differences between the cell lines. Clones were selected for similar overexpression levels, however, in some cases this was not possible, in particular with the E2F6ΔN clones.

As with HEK293 cells, western blots samples were prepared from the same experiment as figure 5.9. Western blots were quantified using Fiji software and normalised to the loading control GAPDH and to the appropriate non Doxy control, to take account of the large variation seen in protein level induction following HU treatment. Overexpression of E2F6 wild type has different effects on Cyclin E protein levels in
Figure 5.10: E2F6ΔC overexpression is unable to repress E2F target proteins. Right: Western
(continued) blot of whole cell extract in RPE1 TetON HA-E2F6 Wild Type (clone 10 and 14) and ∆C (clone 1 and 2) and ∆N (clone 8 and 11) and Empty cells following 8 hour treatment −/+ HU (0.5 mM) each −/+ E2F6 overexpression (Doxycycline, 4 µg/ml with 1 hour pre-treatment).

Left: Bar graphs showing the mean of quantification of all repeats. All normalised to GAPDH and the non E2F6 control. n=4 for wild type (clone 10 and 14) and ∆C (clone 1 and 2), n=3 for ∆N (clone 8 and 11) and empty, error bars show SEM. *** P < 0.001, ** P < 0.01 with two-tailed unpaired T-test.

untreated conditions depending on the clone. Clone 10 shows an increase, in contrast to previous results, but clone 14 shows a decrease consistent with previous results. However, in HU treated conditions both clones show significant reduction of Cyclin E protein levels following E2F6 overexpression, figure 5.10. Overexpression of E2F6∆C reduces Cyclin E levels in both untreated and HU-treated conditions in both clones, this decrease is significant in clone 1 in HU-treated conditions and in clone 2 in untreated conditions. This again shows the ability of the E2F6∆C mutant to repress E2F-dependent proteins. In agreement with the RNA data, overexpression of E2F6∆N shows repression in both conditions in both clones, despite this protein containing no DNA binding domain. The negative control RPE1 TetON empty shows no repression of Cyclin E following Doxycycline treatment.

Consistent with the RNA results, figure 5.9, which were taken from the same experiment, the change in the protein levels of CtIP are less clear than Cyclin E. Looking at overexpression of E2F6 wild type in both clones, the only significant repression is seen with clone 14 in HU-treated cells. Overexpression of clone 10 in untreated conditions shows an increase in CtIP, as was seen with Cyclin E. As this is inconsistent with all results seen previously, and the HU-treated conditions, I believe this to be an anomalous result. Altogether overexpression of E2F6 wild type results in slight repression of CtIP. Overexpression of the two E2F6∆C clones shows no significant changes to CtIP levels. An increase is seen following E2F6∆C overexpression in clone 1 untreated conditions and in both clones 1 and 2 in HU-treated conditions, however these increases are all very small (0.14, 0.016, 0.001 a.u.). E2F6∆C overexpression shows a slight decrease in untreated conditions in clone 2. These results show E2F6∆C is unable to repress CtIP
in RPE1 cells. Again, consistent with the RNA results, overexpression of E2F6△N surprisingly shows repression of CtIP in both untreated and HU-treated conditions, with clone 8 showing significant differences. RPE1 TetON Empty shows no clear change. All cell lines show a clear induction of E2F6 protein following Doxycycline treatment. The inductions vary in amplitude, although this is partly due to the method of quantification. The induced protein is normalised to the non Doxy control, which in many cases is background and is difficult to get an accurate measurement. Irrespective of this, it is clear that Doxycycline treatment induces E2F6 in all cell lines. RPE1 TetON Empty cells show a very slight increase in E2F6 levels following Doxycycline treatment, however this is not significant and nominal when compared to the other cell lines.

Taken together these data show that E2F6△C is able to repress the E2F target Cyclin E at both the RNA and protein level in RPE1 cells. This is not entirely consistent and not always significant, however the results seen with the HA-tagged E2F6 wild type clones are also not as clear as was previously seen in RPE1 TetON E2F6 cells in figure 3.15 on page 82. This could be due to experimental variation or may reflect a true difference in these different cell lines. In chapter 3, the RPE1 TetON E2F6 cell line used had no tag, however the construct used here contained an N terminal HA-tag. Although this tag is small in size and commonly used, I cannot rule out the possibility that it is affecting the function of the E2F6 protein. To remove this as a possibility I could make clones without the HA-tag and compare the repressive ability of these overexpression cell lines. Alternatively, C terminal HA-tagged overexpression cell lines could be used in order to keep the HA-tag, which will be a useful tool in further experiments, but help assess whether the tag is altering the function of the protein. In addition, these are stable cell lines and so the overexpression construct has been stably incorporated into the DNA, a different location of incorporation between the two cell lines could result in subtle differences. Overall, I conclude that E2F6△C mutant lacking the repression domain maintains some function as a transcriptional repressor, however it may not be as efficient as the wild type protein. This strongly suggests that the displacement
of transcriptional activators plays some role in the mechanism of E2F6-dependent repression, but other mechanisms may also contribute to the full function of the wild type E2F6 transcriptional repressor.

5.3. E2F6ΔN is able to repress E2F-dependent transcription despite containing no DNA binding domain

As seen in figures 5.9 and 5.10, the ΔN 129-281 mutant of E2F6 shows clear repressive function, and in some cases more efficient repression than the wild type protein. This protein does not contain the DNA binding domain and should therefore be unable to bind DNA or recruit proteins to DNA, and hence would be expected to be non-functional as a transcriptional repressor. This could suggest that E2F6 is able to repress transcription through an alternative mechanism other than through its canonical role as a E2F transcriptional repressor. Considering the large body of work analysing the role and function of E2F6 this would be very surprising. An alternative explanation would be due to the fact that these overexpression cell lines also contain endogenous wild type protein. It is possible that the endogenous protein, containing the DNA binding domain, is sufficient to recruit the strongly overexpressed mutant protein to promoters. If this is the case, once recruited to promoters the E2F6ΔN would be expected to functional to repress transcription through either mechanism, displacement or recruitment of Polycomb group proteins. It should be noted that the expression levels of E2F6ΔN following Doxycycline treatment are much higher than with any of the other cell lines (figure 5.9), despite this being the closest match in induced protein expression levels in all clones tested. It may be that the higher level of E2F6ΔN overexpression means this is an inaccurate comparison to the other cell lines.

A number of experiments need to be completed in order to confirm whether the ability of E2F6ΔN to repress transcription is a true biological result, or whether it is an artefact of the experimental design. First, analysing whether a reduced level of E2F6ΔN overexpression, through lower concentrations of Doxycycline, prevents the repression
of E2F targets. If this is the case, the ability of E2F6ΔN to repress in the previous results would be due to incomparable protein overexpression. Secondly, it should be confirmed whether or not E2F6ΔN is able to bind E2F promoters with ChIP experiments. I have attempted this experiment but technical difficulties in the ChIP protocol and time constraints have prevented me from analysing the ability of E2F6ΔN to bind E2F promoters. If E2F6ΔN is able to bind DNA promoters this would explain the repression seen, and means this is not a useful negative control for these experiments.

Finally, these experiments could be repeated in cell lines with no endogenous E2F6. Previous attempts in the lab to make an inducible knock down E2F6 cell line have experienced technical difficulties, partly due to the contribution of feedback loops in the regulation of E2F-dependent transcription. However, testing E2F6ΔN repression without endogenous E2F6 could be achieved through specific silencing of the endogenous E2F6 mRNA. The silencing of E2F6 would have to target the 3’UTR to prevent silencing of the overexpressed E2F6ΔN. A CRISPR knock out of endogenous E2F6 would be expected to have large cell cycle effects, which could confuse interpretation of the results. However, CRISPR interference (CRISPRi) could be used. In CRISPRi expression of a catalytically inactive Cas9 protein can be induced, which is recruited to a gene by a single guide RNA (sgRNA). The Cas9-sgRNA complex causes a steric block to transcription and represses gene expression (Larson et al., 2013). The sgRNA should be targeted to introns so that it would not also repress transcription of the E2F6ΔN construct. If silencing endogenous E2F6, by either of these methods, prevents E2F6ΔN dependent repression it would suggest that the endogenous protein is guiding the ΔN mutant to promoters and hence allowing repression. Although the experiments seen in figures 5.9 and 5.10 are surprising, on their own they do not necessarily show that E2F6 acts independently of its ability to bind target promoters, which could suggest it was not acting via its canonical role as a transcription factor. Further experiments are required to establish whether E2F6ΔN is able to bind DNA promoters or not; this is an important piece of information required before these data can be properly understood and interpreted.
5.4. E2F6ΔC overexpression prevents resolution of stalled replication forks

Work in this chapter has so far shown that overexpression of E2F6ΔC is able to partially repress E2F-dependent transcription, despite not containing the repression domain. To further analyse the requirement of the repression domain for correct E2F6 activity, I tested the functionality of the E2F6ΔC in an assay looking at the role of E2F6 in the replication stress checkpoint response. During replication stress replication forks are stalled, resulting in RPA2 foci. On release from HU-induced replication stress these foci are resolved over time. As shown in chapter 3, overexpression of E2F6 prevents sustained E2F-dependent transcription, a key component of the replication stress checkpoint response, and the resolution of RPA2 foci is no longer seen, figure 3.19 on page 88. To establish if E2F6ΔC, containing no repression domain, was sufficient to prevent sustained E2F-dependent transcription and therefore prevent the resolution of stalled RPA2 foci, I completed these experiments with the overexpression of E2F6ΔC.

Two HEK293 T-Rex E2F6ΔC clones were tested, clone 3 and clone 6. In the control experiment, with no Doxycycline treatment, the release from HU treatment alone should result in the resolution and therefore reduction of RPA2 foci. This is not seen in HEK293 T-Rex E2F6ΔC clone 3 cells, therefore this cell line cannot be used for this experiment. This clone also shows variability in other experiments (bottom, figure 5.4) and so I believe this clone is not reliable enough to use. On the other hand, the non-Doxycycline treated control clone 6 (top left, figure 5.11) does show the expected reduction in the percentage of cells containing RPA2 foci following release from HU-induced replication stress. The overexpression of E2F6ΔC during replication stress prevents the resolution of RPA2 foci on release from stress. I repeated this experiment with an additional time point to follow RPA2 foci levels 0, 7 and 9 hours after release from HU. Control cells, as expected, resolve RPA2 foci over time, but overexpression of E2F6ΔC prevents this resolution (top right, figure 5.4). The ability of E2F6ΔC to prevent this checkpoint func-
tion mimics the results seen with E2F6 wild type overexpression, figure 3.19. These results suggest E2F6ΔC overexpression is sufficient to prevent sustained E2F-dependent transcription and therefore prevent an efficient checkpoint response. This data supports the model of displacement as the mechanism of repression in this process.

![Figure 5.11: E2F6ΔC overexpression prevents resolution of stalled replication forks similarly to wild type.](image)

It is important to note that the percentage of cells containing RPA2 foci immediately after HU treatment is higher in these cells than in the E2F6 wild type cell line, figure 3.19. To establish whether this was due to experimental variation or reflects a true difference in the cell lines I repeated the experiment three times with HEK293 T-Rex E2F6 wild type and ΔC cells simultaneously. Unfortunately, the controls in all of these
repeats did not show resolution of RPA2 foci following release from HU-treatment alone. This is inconsistent with previous results (figures 3.19 and 5.11), and not expected based on knowledge of the DNA replication stress checkpoint, therefore these experiments cannot be used. It is important to complete this experiment with E2F6 wild type and ΔC side by side, and with a full three repeats of E2F6ΔC clone 6. This is required to confirm these initial experiments which indicate that E2F6ΔC is functionally equivalent to E2F6 wild type in its role in the DNA replication stress checkpoint response.

5.5. Further experiments are required to confirm the mechanism of E2F6-dependent repression

Work in this chapter has shown that overexpression of E2F6ΔC, which contains no repression domain, is able to repress E2F targets at the RNA and protein levels. However, in some cases this is not to the same extent as is seen with E2F6 wild type overexpression. In addition, E2F6ΔC is able to mimic E2F6 wild type in a functional assay looking at the replication stress checkpoint response. These data indicate that displacement of transcriptional activators plays a role in the mechanism of E2F6-dependent repression, but other mechanisms may also be involved. A number of key experiments are needed to extend this work and fully explain the mechanism of E2F6-dependent repression.

First, as discussed in detail in section 5.3, a number of experiments are required to explain the surprising result that E2F6ΔN is able to repress transcription whilst missing the DNA binding domain. Most importantly it should be confirmed whether or not E2F6ΔN is localised to DNA, perhaps through interaction with the endogenous protein. In addition, there is an important control concerning the E2F6ΔC mutant protein. E2F6ΔC contains no repression domain, the domain previously shown to be capable of recruiting Polycomb group proteins, but in my work shows repressive capacity. The binding of Polycomb group proteins was mapped to this region through CoIP of
transiently transfected E2F6 and RYBP (Trimarchi et al., 2001) or EPC1 (Attwooll et al., 2005) constructs. It is important to confirm that E2F6ΔC is unable to bind Polycomb group proteins in our system. This can be done through CoIP experiments utilising the HA-tag in these overexpression constructs and looking at a number of Polycomb group proteins. It would also be interesting to assess the ability of E2F6 wild type to bind Polycomb group proteins, particularly in different stages of the cell cycle. Work from the Nakatani and Hansen labs suggests that E2F6 may bind target promoters in a complex containing Polycomb group proteins and chromatin remodellers specifically in G0 (Deshpande et al., 2007; Ogawa et al., 2002). It could be that E2F6-dependent repression is mediated by Polycomb group protein binding only in G0, and that displacement functions as the mechanism of repression during S phase and replication stress, as my work suggests.

My work used Cyclin E and CtIP as typical E2F targets to analyse the repression mediated by E2F6 wild type and E2F6ΔC overexpression. It may be informative to extend this to a larger panel of targets to confirm the validity of the results. In addition, the results seen with these two targets do sometimes differ. It would therefore be interesting to investigate if the same mechanism and dynamics of repression are seen across all E2F6 targets.

The basis of the displacement mechanism is the ability of E2F6 to replace E2F1 at promoters, this can be tested directly through ChIP experiments looking at promoter occupancy of these transcription factors. It has previously been seen that E2F6 promoter occupancy increases as E2F1 promoter occupancy decreases during the G1 to S phase transition (Bertoli et al., 2013a) and that silencing E2F6 results in increased E2F1 occupancy at E2F promoters (Oberley et al., 2003). If displacement is responsible for repression, overexpression of E2F6 should reduce E2F1 promoter occupancy. It would be informative to test this with both wild type and ΔC overexpression. As E2F6ΔN has no DNA binding domain it should not be able to displace E2F1. However, overexpression of E2F6ΔN appears functional for repression, so it may be that E2F6ΔN can also
displace activator E2Fs and this will be important to establish.

In addition, as discussed in section 5.4, further experiments are required to confirm the ability of E2F6ΔC overexpression to prevent an efficient replication stress checkpoint response, as assessed with RPA2 foci resolution assays. These experiments were not repeated due to technical difficulties and time constraints. It would also be interesting to test the ability of E2F6ΔC to function in other biological assays. For example, whether overexpression of E2F6ΔC prevents the correct stalling and stabilisation of replication forks in a similar manner to E2F6 wild type, as assessed with DNA fibre assays.

Finally, some data indicates that E2F6ΔC may function more similarly to E2F6 wild type at earlier time-points. This leads to a hypothesis that repression is initially achieved through displacement of transcriptional activators, such as E2F1, but that repression is later consolidated through recruitment of Polycomb group proteins and subsequent chromatin modification. To analyse whether this is the case, RNA levels of E2F targets should be analysed following release from HU arrest where a repression of E2F-dependent transcription is seen. Overexpression of E2F6 wild type should increase the speed of this repression. Following the ability of E2F6ΔC to increase repression over time should reveal any differences in repression dynamics between the wild type and ΔC mutant proteins. This experiment would require endogenous E2F6 to be removed, as discussed previously through silencing targeting the 3’ UTR.

5.6. Summary

The regulation of E2F-dependent transcription plays an extremely important role in the checkpoint response to DNA replication stress. A crucial element of this regulation is relieving E2F6-dependent repression of E2F-dependent transcription in S phase, through Chk1 phosphorylation of E2F6, resulting in sustained E2F-dependent transcription. Despite this important role in repressing E2F-dependent transcription in S phase and in the replication stress checkpoint response, the mechanism of E2F6-
dependent repression has not been clearly established. Two mechanisms of repression have been proposed - the displacement of transcriptional activators or the recruitment of Polycomb group proteins. Data presented in this chapter shows that overexpression of a mutant form of E2F6, \(\Delta C\ 1-178\), which does not contain the Polycomb group protein-recruiting repression domain, represses E2F-dependent transcription. Although in some cases the repression seen is not as strong as that of the wild type protein. I also present data showing that overexpression of E2F6\(\Delta C\) prevents an efficient replication stress checkpoint response, as was previously seen with the wild type protein. Further work is required to fully establish the mechanism of E2F6-dependent repression. Overall, the data presented here shows that E2F6 is able to repress E2F-dependent transcription independent of its Polycomb group protein repression domain and therefore likely acts through the displacement of transcriptional activators at promoters. However, other mechanisms may also contribute to full transcriptional repression.
6. Discussion

The aim of my PhD was to investigate the DNA replication stress checkpoint transcriptional response and its role in replication stress tolerance. In response to replication stress, the checkpoint effector kinase, Chk1, phosphorylates and inactivates the repressor E2F6, thereby maintaining E2F-dependent transcription during S phase. I have established that sustained E2F-dependent transcription is required for an efficient checkpoint response to prevent replication stress-induced DNA damage. This is due to the need for sustained transcription for the correct regulation of checkpoint protein dynamics. This in turn is necessary, and sufficient, for key functions of the checkpoint response. In the context of oncogene-induced replication stress, where misregulation of E2F activity by oncogenes may result in high levels of replication stress, the increased E2F activity acts as a mechanism of tolerance to high levels of oncogene-induced replication stress (Bertoli et al., 2016). Elucidating this mechanism of tolerance could direct the identification of new cancer drug targets. Investigating this transcriptional regulation in more depth, I have shown that the displacement of activators is a mechanism of E2F6-dependent repression. However, other mechanisms are also likely to be involved. Further work is required to establish all the mechanisms of E2F6-dependent repression and their relevant roles during the cell cycle. Work presented in this thesis demonstrates that the transcriptional response to DNA replication stress has a key role in the cell’s ability to tolerate replication stress. A combination of regulatory mechanisms need to be considered in order to fully understand this response, a concept that is likely to be applicable to other signalling pathways and responses as well.

6.1. The role of transcription in the response to DNA replication stress

DNA replication stress results in a checkpoint response, which includes a transcriptional response. This involves the maintenance of G1/S cell cycle transcription, and is
conserved from yeast to man. G1/S cell cycle transcription is inactivated in S phase through a negative feedback loop, this feedback loop is inactivated by the replication stress checkpoint response in order to maintain transcription (Bastos de Oliveira et al., 2012; Bertoli et al., 2013a; Caetano et al., 2011; Chu et al., 2007; de Bruin et al., 2008; Dutta et al., 2008; Gomez-Escoda et al., 2011; Ivanova et al., 2013, 2011; Travesa et al., 2012). In mammalian cells, this is achieved through Chk1 dependent phosphorylation of the E2F transcriptional repressor E2F6. This inactivates E2F6 and relieves repression of E2F-dependent transcription. This transcriptional response was previously shown to be required for cell viability following DNA replication stress (Bertoli et al., 2013a). In contrast, work in yeast demonstrated that despite an analogous transcriptional response following DNA replication stress, this is not required for cell viability (Tercero et al., 2003).

Work presented in chapter 3 demonstrates that, in mammalian cells, active translation and specifically E2F-dependent transcription is required for an efficient checkpoint response to prevent replication stress-induced DNA damage. The importance of a sustained transcriptional response is explained by the finding that many E2F target proteins, important for the checkpoint response, have very short half-lives. Therefore, during an S phase arrest caused by DNA replication stress, sustained E2F-dependent transcription is required to maintain and then upregulate key checkpoint proteins. My work suggests that, as a result, sustained E2F-dependent transcription is required for many specific functions of the checkpoint response. These functions include the stalling, stabilisation and protection of replication forks and the resolution of stalled replication forks once the stress has been relieved. Furthermore, in cells with a compromised checkpoint response, the upregulation of E2F activity alone is sufficient to rescue checkpoint functions. Altogether, data presented in this chapter show that sustained E2F-dependent transcription is a key mechanism of the checkpoint response to prevent replication stress-induced DNA damage.

The important role of post-translational modifications in the regulation of the DNA
replication stress checkpoint response has been well established. In addition, a transcriptional response to DNA replication stress has been identified. The work presented in my thesis demonstrates that this transcriptional regulation is also key for the checkpoint response to replication stress. The role of transcription in this response is to ensure that the cell can maintain and correctly regulate the level of proteins important for replication stress tolerance. The sustained transcriptional response ensures maintenance of checkpoint effector proteins with short half-lives. This allows the checkpoint response to be quickly turned off once stress has been resolved, which may prove vital for cellular survival. It would be interesting to investigate whether the transcriptional response could be successfully mimicked by the prevention of protein degradation, or whether the synthesis of new proteins has a role in resetting the checkpoint response. These ideas will be discussed further in section 6.4.

6.2. **E2F-dependent transcription is required for tolerance to oncogene-induced replication stress**

Cells with activated oncogenes or inactivated tumour suppressors that drive proliferation induce replication stress. This oncogene-induced replication stress is an early event in tumourigenesis and is thought to be an important driver in cancer development (Bartkova et al., 2006, 2005; Di Micco et al., 2006; Gorgoulis et al., 2005; Halazonetis et al., 2008; Tsantoulis et al., 2008). Activation of certain oncogenes, such as c-Myc, Cyclin E and Ras, or inactivation of tumour suppressors, such as pRb, results in increased E2F activity. Deregulation of E2F activity is found in most, if not all, cancers (Chen et al., 2009). This inappropriate activation of E2F-dependent G1/S cell cycle transcription drives S phase entry and hence proliferation. This unscheduled S phase entry is thought to induce replication stress through a variety of mechanisms including: the deregulation of replication licensing and firing; exhaustion of replication factors; and increasing collisions between transcription bubbles and replication forks (Bester et al., 2011; Ekholm-Reed et al., 2004; Hills and Diffley, 2014; Jones et al., 2013; Liontos
et al., 2007; Tuduri et al., 2010). This body of work suggests that oncogene-induced deregulation of E2F-dependent transcription drives unscheduled S phase entry and ultimately results in oncogene-induced replication stress. This appears contradictory to my findings which show that E2F-dependent transcription is protective for cells in the context of replication stress.

Results shown in chapter 4 demonstrate that E2F-dependent transcription is required to tolerate oncogene-induced replication stress and prevent DNA damage and cell death. In addition, upregulating E2F activity is sufficient to reduce the levels of DNA damage following oncogene-induced replication stress. The induction of c-Myc, an oncogene acting in many cancers, is used to model oncogene-induced replication stress. This demonstrates the physiological relevance of E2F-dependent transcription in the replication stress checkpoint response, as it is required following stress modelled by both HU treatment and c-Myc induction. Importantly, my work suggests that although oncogenes may induce E2F activity and ultimately cause replication stress, E2F activity simultaneously acts as a mechanism of tolerance to oncogene-induced replication stress. Some cancer types experience and cope with much higher levels of replication stress than normal cells. Understanding this mechanism of tolerance may prove highly useful in further work investigating the role of replication stress in cancer development and for potential cancer treatments.

Importantly, my findings also uncover some general principles. Networks involved in cell cycle control, such as E2F-dependent transcription, are neither solely positive or negative but require the right balance. When oncogenic activity increases E2F activity this causes replication stress and has a damaging impact on the cells. On the other hand, my work reveals that E2F-dependent transcription is also required for an efficient replication stress checkpoint response, and hence has a positive impact on cells. This balance of network activities is in the context of replication stress during S phase. The temporal regulation E2F activity is also very important. The misregulation of specific E2F targets, for example through expression outside of the G1/S transition,
can induce replication stress and genome instability. This has been seen for Cyclin E (Jones et al., 2013; Teixeira et al., 2015) and the licensing factors Cdc6 and Cdt1 (Liontos et al., 2007). In addition, my work suggests that some checkpoint proteins display checkpoint-dependent degradation, as has been shown for Chk1 (Park et al., 2015; Zhang et al., 2005). This supports a model in which these proteins might also be detrimental to the cell outside the checkpoint response. To investigate the importance of a regulated balance of E2F-dependent transcription, it could be informative to analyse whether a whole network upregulation outside of G1/S would result in genome instability.

The deregulation of individual G1/S cell cycle transcription targets results in genome instability. However, this genome instability could be largely prevented by the deregulation of the entire regulon, as seen in fission yeast. In this context, non-essential G1/S target genes become essential following the deregulation of G1/S transcription (Caetano et al., 2014). In the context of cancer research, identifying proteins or processes which become essential for survival during cancer evolution is key for finding new therapeutic targets. Our work demonstrates that a promising approach would be to investigate which proteins or processes become essential following deregulated E2F-dependent transcription and replication stress. This could be done by inducing oncogene-induced replication stress and then screening for E2F targets which, when silenced, result in increased levels of replication stress-induced DNA damage or cell death. Understanding which proteins or processes become essential not only gives insight into the development of oncogene-induced replication stress, but also gives potential therapeutic targets specific to cancer cells.

I am using the term ‘proteins or processes’ as it is unknown whether the requirement for E2F-dependent transcription in the replication stress checkpoint response is dependent on a few key target proteins, a range of proteins responsible for key processes, or a more general upregulation of the whole transcriptional network. These proteins or processes will be required for tolerance to replication stress, and there-
fore will be sufficient to prevent DNA damage in cells with a compromised checkpoint response or misregulated E2F activity, and will become essential during cancer evolution. It should be noted that HU and c-Myc may induce replication stress in different ways, which could mean the proteins or processes which are required in these two situations may differ.

There are some key E2F targets, such as Chk1 and RRM2, which have an important role in the checkpoint response and have been proposed as ‘replication stress buffers’ (Lecona and Fernández-Capetillo, 2014). The upregulation of these proteins is shown to be protective against replication stress in both checkpoint-compromised and oncogenic conditions in mouse models (Lopez-Contreras et al., 2012, 2015). However, we see that loss of Chk1 can be rescued through increased E2F-dependent transcription, suggesting that the tolerance to replication stress via increased E2F activity is Chk1 independent. It has not been established whether any target alone is sufficient to prevent replication stress-induced DNA damage.

E2F-dependent transcription and the replication stress checkpoint response consist of complex signalling networks. It could be hypothesised that the upregulation of only a few key proteins, although conferring protective functions, may unbalance the coordinated response and so a full network upregulation is required. On the other hand, as the transcriptional response can show both positive and negative influences, it may be that full network upregulation has a neutral effect and the replication stress checkpoint instead relies on the specific upregulation of a subset of E2F targets. Understanding which of these situations is seen biologically may unveil properties of the checkpoint response. If only a few key proteins are sufficient, the functions of these proteins would reveal the most important elements of the checkpoint response. If, however, the full network response is required this provides an insight into the general importance of transcriptional regulation.

My work shows that E2F-dependent transcription is required for tolerance to oncogene-
induced replication stress, as modelled by c-Myc activation. Activation of many other oncogenes also results in oncogene-induced replication stress. As many oncogenes also misregulate E2F-dependent transcription, it would be expected that the same principles would apply as were seen for c-Myc activity. However, work has shown that c-Myc and Ras may induce distinct patterns of replication stress (Maya-Mendoza et al., 2014). It would therefore be important to establish whether E2F-dependent transcription is required for tolerance to oncogene-induced replication stress induced by the activation of other oncogenes. If this is a general mechanism of tolerance to replication stress induced by a range of oncogenes this would represent a very important insight for cancer research.

6.3. Investigating the mechanism of E2F6-dependent repression

E2F6 plays an important role in the checkpoint response to DNA replication stress, as it is the transcriptional repressor responsible for S phase repression of E2F-dependent transcription, and the target of the checkpoint response to allow sustained E2F-dependent transcription (Bertoli et al., 2013a; Giangrande et al., 2004). Despite this the mechanism of E2F6-dependent repression has not been fully established. However, two mechanisms of repression have been proposed based on previous experiments, mainly carried out in in vitro assays. The first is that E2F6 binding to promoters displaces transcriptional activators and this is sufficient for a loss of activation (Cartwright et al., 1998; Oberley et al., 2003). The second is based on the C terminal repression domain exhibiting independent repressive function (Gaubatz et al., 1998), and the identification of complexes containing both E2F6 and Polycomb group proteins. In this model E2F6 recruits co-repressors and Polycomb group proteins to promoters, resulting in active repression of E2F targets (Attwooll et al., 2005; Deshpande et al., 2007; Ogawa et al., 2002; Trimarchi et al., 2001; Trojer et al., 2011).

Work in this thesis reveals that E2F6-dependent repression relies, in part, on displacement of the activator E2Fs. Overexpression of a mutant protein not containing the
repression domain, which is thought to be required for Polycomb group protein recruitment, is able to repress E2F targets in a number of assays. It also shows functional activity equivalent to the wild type protein in biological assays looking at the response to replication stress. However, displacement alone does not account for the full mechanism of E2F6-dependent repression. This work reveals that a combination of mechanisms appear to be involved in full E2F6-dependent repression of E2F-dependent transcription.

As discussed in detail in section 5.5, there are a number of experiments required to confirm and extend this initial work. Importantly, it needs to be confirmed that the E2F6ΔC mutation cannot bind Polycomb group proteins, as would be expected based on previous binding site analysis (Attwooll et al., 2005; Trimarchi et al., 2001). It is also necessary to analyse and explain why E2F6ΔN, which does not contain a DNA binding domain, appears functional. The functional replication stress assays should be extended to investigate whether the wild type like functionality of E2F6ΔC is true in a range of different assays. These experiments would extend the current findings and confirm that displacement is a mechanism of E2F6-dependent repression.

Understanding the mechanism of E2F6-dependent repression could give further insight into the regulation of the DNA replication stress checkpoint response. Establishing the mechanism of repression may help understand how Chk1 phosphorylation inactivates E2F6. It is known that Chk1 inactivation is dependent on phosphorylation of E2F6 at Serines 12 and 52, a site close to the DNA binding domain (Bertoli et al., 2013a). It may be that Chk1 phosphorylation disrupts DNA binding of E2F6, or it could disrupt E2F6 recruitment of co-repressors and Polycomb group proteins. Understanding the mechanism of E2F6 inactivation by Chk1 will add another layer of understanding to E2F-dependent transcriptional regulation and the replication stress checkpoint.

In addition, understanding the mechanism of E2F6-dependent repression may give insight to the role of E2F6 mutations found in cancer. Mutations have so far been found
to cluster in both the DNA binding and repression domains. Mutations in the DNA binding domain would affect E2F6 repression mediated by both displacement of activators and Polycomb group protein recruitment. However, mutations in the repression domain should only affect repression due to Polycomb group protein recruitment. Fully understanding the mechanisms of E2F6-dependent repression and their contributions to repression may help to understand the role of these different mutations. Mutations affecting different mechanisms may contribute to cancer development in different ways and therefore require a different type of treatment.

Data presented in this thesis suggests that although displacement is a mechanism of E2F6-dependent repression, alone it cannot fully describe E2F6-dependent repression. Therefore, at least two mechanisms are likely to be responsible for full E2F6-dependent repression. This allows the possibility of a separation of function between the two mechanisms. This could be temporally, throughout the cell cycle, or through different dynamics of function. The first possibility is that the two mechanisms are active in different stages of the cell cycle. Experiments presented in this thesis show that preventing Polycomb group protein recruitment results in incomplete repression of E2F targets when compared to the wild type, however it does not appear to affect protein function in biological assays following replication stress in S phase. Taken together these data lead to the hypothesis that displacement may be the mechanism of repression in S phase, when swift changes in repression state are required, but that Polycomb group protein recruitment is the primary mechanism of repression during the more stable repression of G0. It could also be possible that both mechanisms are active in both stages of the cell cycle, but regulated with different dynamics. Displacement of activating E2Fs could act initially to repress E2F-dependent transcription, with the recruitment of Polycomb group proteins then required to consolidate this repression through altering chromatin structure. The incomplete repression seen in non-synchronised cells could also support this theory if E2F6ΔC was unable to fully repress transcription following initial successful displacement. In addition, if the dy-
namics of these processes mean S phase cells rely predominantly on displacement, this would also explain the ability of E2F6ΔC to mimic wild type in replication stress assays. Furthermore, experiments have indicated this could be the case, as at shorter time-points E2F6ΔC, which is able to repress via displacement, acts more similarly to E2F6 wild type than is seen at later time-points. Both these hypotheses should be tested and it would be very interesting to establish which, if either, is correct.

6.4. The coordinated regulation of the DNA replication stress checkpoint response

The role of post-translational modifications in regulating and coordinating the response to DNA replication stress has been widely studied (García-Rodríguez et al., 2016; Huen and Chen, 2008). Phosphorylation plays a key role, in particular through the activity of the sensor kinase ATR and the effector kinase Chk1. In addition to phosphorylation, other post-translational modifications such as ubiquitination and sumolation are also important. The combination and coordination of all of these post-translational modifications, such as through PCNA, is essential for an efficient DNA replication stress checkpoint response (Moldovan et al., 2007).

Work presented in this thesis demonstrates that post-translational modifications are not the only component of regulation in the replication stress checkpoint response. I show that the transcriptional response to replication stress, which is itself dependent on the phosphorylation of E2F6 by Chk1, is essential for an efficient checkpoint response. Active protein synthesis is also required to prevent DNA damage following replication stress. The levels of proteins involved in the checkpoint response are controlled by this regulation of transcription and translation. In addition, the degradation rate of a protein will also determine its final protein level. This degradation rate is controlled by two main factors. First is the inherent half-life of the protein, which will determine the degradation rates in normal situations. My work shows that many checkpoint effectors have very short half-lives, which may be an important aspect of
their function and control. In addition to inherent degradation rates, proteins can also be specifically targeted for degradation. Data in this thesis suggests that some proteins may be targeted for degradation in a checkpoint-dependent manner. This has also been seen previously for Chk1 (Park et al., 2015; Zhang et al., 2005).

The DNA replication stress checkpoint was previously known to rely on post-translational modifications. Work in this thesis demonstrates that the transcriptional response also has an important role. However, it is important to analyse transcription, translation, post-translational modifications and degradation in order to fully understand the DNA replication stress checkpoint response. It is highly likely that other regulatory mechanisms also depend on the combined and coordinated action of all of these elements.

6.5. The importance of a transcriptional response

The presence of a sustained transcriptional response to DNA replication stress means that proteins required for checkpoint functions, that have a short half-life, are maintained at the correct levels during the checkpoint response. This short half-life has a number of potentially positive implications for the checkpoint response, which I will now discuss.

Once the stress has been relieved a fast inactivation of the checkpoint response is important. This requires E2F-dependent transcription to be turned off quickly. In unperturbed conditions, E2F-dependent transcription encodes for the transcriptional repressor E2F6, which is then responsible for repressing transcription in S phase. This negative feedback loop is inactivated by checkpoint-dependent phosphorylation of E2F6 by Chk1. This network wiring means that E2F6, as an E2F target and therefore undergoing sustained transcription during replication stress, is poised to repress transcription as soon as Chk1 is inactivated.

In order to quickly and robustly turn off the checkpoint response, in addition to inactivating E2F-dependent transcription, proteins involved in checkpoint functions must
be removed. The short half-lives of checkpoint proteins, which I show in my work, would ensure this is an automatic mechanism. The removal of proteins would be enhanced by checkpoint-dependent degradation of proteins, as for Chk1 (Park et al., 2015; Zhang et al., 2005). This combination of inactivating transcription, short half-lives and checkpoint-dependent degradation would mean rapid changes in the proteome.

Following the removal of stress and checkpoint inactivation, DNA replication and the cell cycle must be restarted. The mechanism signalling DNA replication restart has not been established. It is possible that this network wiring, relying on sustained transcription of unstable proteins, could account for the mechanism of DNA replication restart. Active transcription of checkpoint proteins with short half-lives will result in high turnover rates. This will ensure that post-translationally modified proteins will be replaced by new, unmodified proteins as soon as the checkpoint is satisfied, which may signal for replication restart. Removing modifications such as phosphorylation can also be achieved through the action of phosphatases. However, the mechanism of new, unmodified proteins signalling for restart could have a number of advantages. First, it is widespread and so many different proteins could be replaced without the need for individual phosphatases. Second, it could be imagined to be a faster and more robust way of removing checkpoint-dependent modifications. Thirdly, ongoing checkpoint activation can be detrimental to the cell. Therefore, relying on a more automatic regulation, such as inherent degradation, could prevent indefinite checkpoint activation. Finally, this mechanism would directly link checkpoint inactivation and DNA replication restart. It would be interesting to test this proposed mechanism.

Although a transcriptional response to replication stress has many advantages, it must be tightly regulated to prevent damaging effects. This means E2F targets must be efficiently downregulated on checkpoint inactivation to prevent damage to the cell. The inappropriate expression of specific E2F targets, such as Cyclin E, Cdc6 and Cdt1 causes replication stress and genome instability (Jones et al., 2013; Liontos et al., 2007; Teix-
eira et al., 2015). It is highly likely that the misregulation of other E2F targets is also damaging to the cell, for example CtIP’s DNA resection could be detrimental if not correctly regulated. As discussed previously, misregulation of E2F activity by oncogenes is also a likely cause of oncogene-induced replication stress.

In addition to the loss of control of specific targets, the loss of regulation of E2F-dependent transcription may also have more general negative effects on genome stability. E2F-dependent transcription regulates the activity of a large number of target genes (Bertoli et al., 2013a). It has been seen that genome instability can be induced following an increase in collisions between transcriptional bubbles and replication forks (Helmrich et al., 2011; Jones et al., 2013; Tuduri et al., 2010). The loss of repression of E2F-dependent transcription outside the G1/S transition or in an unperturbed S phase would result in an increase in transcription-replication collisions and may cause genome instability.

6.6. Identifying tolerance mechanisms is key in cancer research

A key challenge in cancer research is how to harm cancer cells without affecting healthy cells. This can be achieved through targeting tolerance mechanisms required by oncogenic cells and not healthy cells. Work in this thesis identifies and characterises sustained E2F-dependent transcription as a tolerance mechanism to oncogene-induced replication stress. Sustained E2F-dependent transcription is required for the replication stress checkpoint response to prevent replication stress-induced DNA damage. Although E2F-dependent transcription is also required in non-oncogenic cells, the level of E2F target gene expression required is likely to be higher in many oncogenic cells due to increased levels of replication stress. This difference between normal and oncogenic cells could be exploited for cancer treatments in the future. This obviously raises the question of what level of checkpoint function is required to kill cancer cells but allow normal cells to cope with any replication challenges, and therefore not cause side effects.
Although a promising mechanism to target in cancer treatment, further research is required to fully understand the response. This is true of all cancer treatments as targeting a response not fully elucidated could have unexpected and damaging consequences. It was previously known that many oncogenes misregulated E2F-dependent transcription, and that this misregulation leads to uncontrolled proliferation and potential oncogene-induced replication stress. Targeting this pathway would therefore require a good understanding of the role of E2F activity, to ensure the checkpoint response is inhibited to such a level that it results in catastrophic DNA damage and cell death, rather than just a low increase in repairable DNA damage which could increase mutation rates and drive cancer development.

As discussed previously on page 147, it is unknown if the checkpoint dependence on E2F-dependent transcription is due to a few key targets or the whole transcriptional network. When considering possible cancer targets, it would be easier and more effective to target this mechanism of tolerance if only a few key components were required. Achieving specificity of action would be much more challenging to regulate the whole transcriptional response.

### 6.7. Future research opportunities

A range of possible new interesting areas of research are opened up based on the findings presented in this work. This thesis shows that in order to fully understand the cellular response to replication stress, including oncogene-induced replication stress, the transcriptional response must be considered, in addition to post-translational modifications and degradation rates. Understanding the complex interactions between transcription, translation, post-translational modifications and degradation rates, which together control the activity of proteins, would enhance our knowledge of the DNA replication stress checkpoint response. This integrated network wiring allows for rapid and robust responses, and so may be relevant to other signalling pathways, in particular stress responses.
Data in chapter 5 establishes that E2F6-dependent repression relies, in part, on the displacement of transcriptional activators. However, this is not responsible for all E2F6-dependent repression. It would be interesting to investigate and understand the relative contributions of different mechanisms of repression. There are indications that the two mechanisms could be responsible for different functions of repression. This may have further implications if found to be true, in particular in analysing E2F6 mutations found in cancer.

Most importantly, this work identifies sustained E2F-dependent transcription as a mechanism of tolerance to oncogene-induced replication stress. This reveals the exciting opportunity to research this further to both fully understand the mechanism and potentially exploit it in cancer treatments. An important question to answer is whether this tolerance is dependent on the upregulation of the whole network, or just a few key components. The outcome of this research will determine the best way to utilise this in potential cancer therapies. In oncogenic cells experiencing high levels of replication stress, much higher levels of E2F activity and checkpoint function will be required compared to normal cells. This opens up a potentially large therapeutic window to specifically target oncogenic cells. This approach could be particularly effective if used in combination with therapies targeting DNA repair mechanisms. Targeting the mechanism of replication stress tolerance would result in increased levels of DNA damage. Simultaneously targeting DNA repair mechanisms would prevent oncogenic cells from repairing this damage and the cells would be unable to continue proliferating.
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Finally, thank you to my friends and family for their support throughout my PhD and beyond. A special thank you to my fiancé Gary for celebrating and commiserating results he didn’t understand and for being there for me at every stage of my PhD.
A. Data in support of Chapter 3

The full data sets that are summarised in figures in Chapter 3 are presented in this appendix on the following pages. All are scatter graphs showing mean RPA2 intensity (x-axis) vs mean $\gamma$H2AX intensity (y-axis) of chromatin-bound protein in individual nuclei. Arrows show change in mean on each axis. $P =$ differences in mean intensity on each axis with the Wilcoxon test compared to control conditions (Control / HU or Aph as appropriate). Black dots show low levels of RPA2 (arbitrary threshold displayed under each figure), orange and red dots show low and high levels of $\gamma$H2AX respectively (arbitrary threshold displayed under each figure).
A.1. Data in support of Figures 3.8 and 3.9 in RPE1 TetON E2F6 cells

Data in figures 3.8 and 3.9 summarise the results of three biological repeats in RPE1 TetON E2F6 cells. All experiments used HU 0.5 mM, Chx 10 µg/ml with 10 min pre-treatment, E2F6 = Doxycycline 4 µg/ml with 1 hr pre-treatment. Repeat 1 is displayed in figure 3.7. Repeats 2 and 3 are displayed here.

![Graphs showing data from Figure A.1](image_url)

**Figure A.1:** Full data set of RPE1 TetON E2F6 cells summarised in figures 3.8 and 3.9 - Repeat 2. 7 hr treatment. Arbitrary threshold - RPA2 < 10 a.u., γH2AX = 30 a.u.
Figure A.2: Full data set of RPE1 TetON E2F6 cells summarised in figures 3.8 and 3.9 - Repeat 3. 7 hr treatment. Arbitrary threshold - RPA2 < 10 a.u., γH2AX = 30 a.u.
A.2. Data in support of Figures 3.8 and 3.9 in HEK293 T-Rex E2F6 cells

Data in figures 3.8 and 3.9 summarise the results of three biological repeats in HEK293 T-Rex E2F6 cells. All experiments used HU 0.5 mM, Chx 10 $\mu$g/ml with 10 min pre-treatment, E2F6 = Doxycycline 4 $\mu$g/ml with 1 hr pre-treatment. Repeat 1 is displayed in figure 3.6. Repeats 2 and 3 are displayed here.

Figure A.3: Full data set of HEK293 T-Rex E2F6 cells summarised in figures 3.8 and 3.9 - Repeat 2. 2 hr treatment. Arbitrary threshold - RPA2 < 10 a.u., $\gamma$H2AX = 30 a.u.
Figure A.4: Full data set of HEK293 T-Rex E2F6 cells summarised in figures 3.8 and 3.9 - Repeat 3. 2 hr treatment. Arbitrary threshold - RPA2 < 20 a.u., γH2AX = 30 a.u.
A.3. Data in support of Figure 3.11 in RPE1 TetON E2F6 and HEK293 T-Rex E2F6 cells

Data in figure 3.11 summarise the results of three biological repeats in both RPE1 TetON E2F6 and HEK293 T-Rex E2F6 cells. All experiments used Aph 2.4 mM, E2F6 = Doxycycline 4 μg/ml with 1 hr pre-treatment. These experiments were done simultaneously with previous experiments, therefore Control and E2F6 data are the same as displayed in the relevant repeats in figures 3.7, A.1, A.2, 3.6, A.4. Repeat 2 of HEK293 T-Rex E2F6 cells was completed separately and so no repeated data is shown.

![Figure A.5: Full data set of RPE1 TetON E2F6 cells summarised in figure 3.11 - Repeat 1. 7 hr treatment. Abitrary threshold - RPA2 < 10 a.u., γH2AX = 15 a.u.](image-url)
Figure A.6: Full data set of RPE1 TetON E2F6 cells summarised in figure 3.11 - Repeat 2. 7 hr treatment. Arbitrary threshold - RPA2 < 10 a.u., γH2AX = 30 a.u.

Figure A.7: Full data set of RPE1 TetON E2F6 cells summarised in figure 3.11 - Repeat 3. 7 hr treatment. Arbitrary threshold - RPA2 < 10 a.u., γH2AX = 30 a.u.
Figure A.8: Full data set of HEK293 T-Rex E2F6 cells summarised in figure 3.11 - Repeat 1. 2 hr treatment. Arbitrary threshold - RPA2 < 10 a.u., γH2AX = 15 a.u.

Figure A.9: Full data set of HEK293 T-Rex E2F6 cells summarised in figure 3.11 - Repeat 2. 2 hr treatment. Arbitrary threshold - RPA2 < 10 a.u., γH2AX = 15 a.u.
Figure A.10: Full data set of HEK293 T-Rex E2F6 cells summarised in figure 3.11 - Repeat 3. 2 hr treatment. Arbitrary threshold - RPA2 < 20 a.u., γH2AX = 30 a.u.
B. Published Work


Sustained E2F-Dependent Transcription Is a Key Mechanism to Prevent Replication-Stress-Induced DNA Damage

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This paper is included for reference on the following pages.
Sustained E2F-Dependent Transcription Is a Key Mechanism to Prevent Replication-Stress-Induced DNA Damage

Highlights

- Gene expression plays an essential role in the response to replication stress
- Key stress response functions depend on sustained E2F-dependent transcription
- E2F activity is a key mechanism to cope with and recover from replication stress
- E2F activity limits DNA damage resulting from oncogene-induced replication stress

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In Brief

Bertoli et al. establish a far greater role for transcriptional control in determining the outcome of replication-stress-induced events than previously suspected. Their data predict a model in which cells that experience oncogene-induced replication stress become addicted to E2F-dependent transcription to cope with high levels of replication stress.
Sustained E2F-Dependent Transcription Is a Key Mechanism to Prevent Replication-Stress-Induced DNA Damage

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**SUMMARY**

Recent work established DNA replication stress as a crucial driver of genomic instability and a key event at the onset of cancer. Post-translational modifications play an important role in the cellular response to replication stress by regulating the activity of key components to prevent replication-stress-induced DNA damage. Here, we establish a far greater role for transcriptional control in determining the outcome of replication-stress-induced events than previously suspected. Sustained E2F-dependent transcription is both required and sufficient for many crucial checkpoint functions, including fork stalling, stabilization, and resolution. Importantly, we also find that, in the context of oncogene-induced replication stress, where increased E2F activity is thought to cause replication stress, E2F activity is required to limit levels of DNA damage. These data suggest a model in which cells experiencing oncogene-induced replication stress through deregulation of E2F-dependent transcription become addicted to E2F activity to cope with high levels of replication stress.

DNA replication stress (RS) is defined as inefficient DNA replication that causes DNA replication forks to progress slowly or stall, making them susceptible to DNA damage (Abraham, 2001; Jackson and Bartek, 2009; McGowan and Russell, 2004). RS can be caused by many factors like deregulation of components required for DNA synthesis, a decrease or increase in the frequency of replication initiation, and factors that block replication forks. The ability of cells to cope with RS is largely dependent on the action of the RS checkpoint, a conserved signaling pathway that constantly monitors for the loss of integrity of the DNA replication fork (Branzei and Foiani, 2010). RS leads to the accumulation of single-stranded DNA (ssDNA), which is coated by the ssDNA-binding protein complex replication protein A (RPA) and activates the sensor kinase ATR and its downstream effector kinase Chk1 (Cimprich and Cortez, 2008). The activation of this checkpoint aims to prevent DNA damage, a potential source of genomic instability. The RS checkpoint arrests cell-cycle progression, arrests and stabilizes on-going forks to prevent their collapse, blocks initiation of replication from late origins, and finally, when the stress is resolved, allows replication to resume. A large body of evidence supports a critical role for post-translational modifications, such as phosphorylation, sumoylation, and ubiquitination, in the RS checkpoint response (Huen and Chen, 2008; Jackson and Bartek, 2009). Whereas these regulatory events have been shown to be major determinants of checkpoint functions, little is known about the role of transcription in the cellular response to RS. Previous work from our lab has shown that E2F-dependent cell-cycle transcription is part of the checkpoint transcriptional response (Bertoli et al., 2013a), but the importance of this for specific checkpoint functions remains largely untested.

Transcriptional control during the G1 and S phases of the cell cycle depends on the E2F family of transcription factors in mammalian cells (Bertoli et al., 2013b). Activation of E2F-dependent transcription (from now on referred to as E2F transcription) is tightly regulated, as it controls the entry of cells into S phase and into the cell cycle. Under physiological conditions, it is driven by cyclin-dependent kinases that are activated downstream of growth factor signaling (Bertoli et al., 2013b). Oncogenes, such as Ras, c-Myc, and cyclin E, deregulate E2F-dependent G1/S transcription to drive passage into S phase and cell proliferation. By accelerating S phase entry, these oncogenes can generate RS (Hills and Diffl ey, 2014). Upon S phase entry, E2F transcription is inactivated via a negative feedback loop involving the transcriptional repressor E2F6, an E2F target itself (Bertoli et al., 2013a; Giangrande et al., 2004). Our previous work showed that, in response to RS, the checkpoint actively maintains E2F transcription via Chk1-dependent phosphorylation and inactivation of E2F6 (Bertoli et al., 2013a). Here, we provide evidence that sustained E2F transcription functions to maintain the expression of many proteins with key roles in the RS checkpoint response. The expression of E2F-dependent targets is not just required but sufficient for accomplishing crucial checkpoint functions such as stabilizing on-going replication forks and allowing replication to resume after the arrest. Importantly, we find that, in the
context of oncogene-induced RS, where increased E2F activity drives proliferation, which is thought to cause RS, paradoxically E2F transcription is required to limit DNA damage levels. Thus, E2F transcription is a key mechanism in the tolerance to RS.

RESULTS

E2F Transcription and Active Protein Synthesis Are Required to Prevent RS-Induced DNA Damage

Our previous work shows that, in human cells, maintaining E2F transcription is important to prevent apoptosis in response to RS (Bertoli et al., 2013a). However, how it contributes to RS tolerance remains unknown. In yeast, protein synthesis is not required for cell viability during the cellular response to RS (Pellicci et al., 1999; Tercero et al., 2003). To test whether continuous expression of E2F target genes is important for RS response in human cells, we first tested whether de novo protein synthesis is necessary to prevent DNA damage following the cellular response to RS. RS was induced via acute treatment with hydroxyurea (HU), which depletes the pools of dNTPs by inhibiting ribonucleotide reductase (RNR) activity and results in replication fork stalling. To assay the levels of DNA damage, we measured the intensity of H2AX hyperphosphorylation (γH2AX), which is a hallmark of ATM activity and therefore a readout for DNA damage. Intensity was measured by quantitative immunofluorescence of chromatin-bound γH2AX in single nuclei, similarly to Toledo et al. (2013). Both 2 and 7 hr HU treatment results in a significant increase in γH2AX signal when compared to untreated control cells, indicating the presence of some DNA damage in cells experiencing RS (Figure 1A). When the protein synthesis inhibitor cycloheximide (Chx), which blocks translation and prevents de novo protein synthesis, was added in addition to HU treatment the extent of DNA damage (γH2AX intensity) was significantly increased compared to HU treatment alone (Figure 1A). Thus inhibiting protein synthesis increases the extent of DNA damage induced by RS, suggesting a requirement for de novo protein translation during the response to RS in mammalian cells. To quantify levels of DNA damage resulting more specifically from RS, we analyzed the chromatin recruitment of ssDNA-binding protein replication protein A2 (RPA) alongside γH2AX. RPA coats the extended amounts of ssDNA that occur during RS (Zou et al., 2006) and is used as an indicator of RS. We analyzed by quantitative immunofluorescence the intensity of both chromatin-bound RPA (marker of RS) and γH2AX (marker of DNA damage) in individual S phase nuclei; this allows us to analyze the extent of RS-induced DNA damage. These data show that the increase in DNA damage (γH2AX) seen following 7 hr Chx and HU treatment is highest in cells labeled with RPA, indicating that the DNA damage is resulting from RS (Figures 1B and S1A). Importantly, Chx alone does not generally cause an increase in γH2AX signal (Figures S1B and S2A). These findings indicate that sustained protein synthesis is required to prevent the occurrence of RS-induced DNA damage.

Next, we tested the contribution of sustained E2F transcription in preventing RS-induced DNA damage. Doxycycline-induced overexpression of the repressor E2F6 interferes with the checkpoint-dependent maintenance of E2F transcription (Figure S1C); preventing this response allows the study of its role following RS. If sustained E2F transcription is involved in the tolerance to RS then overexpression of the repressor E2F6, and subsequent loss of E2F transcription, would be expected to result in increased levels of DNA damage following HU-induced RS. As before, the intensity of chromatin-bound γH2AX and RPA in individual S phase nuclei was measured after 7 hr HU treatment. E2F6 overexpression was induced with a short 2 hr pre-treatment of Doxycycline in HU-treated or untreated cells. Results reported in Figures 1B, S2A, and S2B show an increase in γH2AX labeling upon E2F6 overexpression in HU treatment compared to HU treatment alone in both HEK293 TetE2F6 and RPE1 TetON E2F6 cells. This increase is seen in nuclei with high levels of RPA, indicating this is RS-induced DNA damage. E2F6 overexpression in untreated cells does not cause an increase in γH2AX levels. As expected, because only the E2F-dependent RS transcriptional response is compromised, the increase in γH2AX signal is less pronounced than that seen in Chx-treated cells. To confirm these results, we increased the throughput of the assay by adopting a protocol for fluorescence-activated cell sorting (FACS) analysis, based on Forment et al. (2012). This method provides a more quantitative way of measuring differences in both γH2AX and RPA staining in higher numbers of individual cells. This analysis confirms our results showing a significant increase in γH2AX with E2F6 overexpression in cells treated with HU but no significant change in untreated cells (Figure 1C). Increased DNA damage in E2F6-overexpressing cells in HU was also confirmed by western blot of whole-cell extract (WCE) (Figure S2C). These findings suggest that sustained E2F transcription is required to prevent RS-induced DNA damage in human cells.

Protein Synthesis and E2F Transcription Are Required to Maintain the Levels of Checkpoint Proteins

Our results suggest a role for active protein synthesis and specifically E2F transcription in the cellular response to RS. Because E2F cell-cycle target genes include most of the major DNA replication, repair, and checkpoint effectors, we hypothesized that...
active protein synthesis prevents RS-induced DNA damage by maintaining the levels of these proteins. To assess this, we analyzed the stability of a number of key checkpoint proteins during RS in HEK293, RPE1, and T98G cells (Figures 2A–2C, S3A, and S3B). As expected, these checkpoint proteins, which are all E2F targets, are upregulated during HU-induced RS. The addition of the translational inhibitor Chx reveals two types of proteins. (1) The first are proteins for which ongoing protein synthesis is required to significantly increase their abundance in response to RS. These proteins are relatively stable, and Chx addition only prevents the HU-induced accumulation but does not result in a loss of protein abundance, cyclin E, and claspin. (2) The second are proteins that are inherently unstable and so Chx treatment results in a dramatic loss of their abundance, CtIP, and Chk1. For these proteins, active protein synthesis during RS is mainly required to maintain their levels rather than to significantly increase abundance. Interestingly, this group includes checkpoint proteins that show increased degradation rates during the checkpoint response (Figure S3A), suggesting they are targeted for degradation in a checkpoint-dependent manner, as previously reported for Chk1 (Chang et al., 2003).

Figure 2. E2F Transcription and Active Protein Synthesis Are Required to Maintain and/or Upregulate the Levels of Checkpoint Proteins

(A) Western blot of WCE (whole-cell extract), RPE1 cells, treatment, and times shown.
(B) Western blot of WCE, HEK293 cells, treatment, and times shown.
(C) Quantification of (A) (RPE1) and (B) (HEK293), normalized to GAPDH and 0 hr.
(D) Western blot of WCE, RPE1 TetON E2F6 cells, treatment, and times shown. E2F6 overexpression, Doxy.
(E) Western blot of WCE, HEK293 T-Rex E2F6 cells, treatment, and times shown.
(F) Western blot of WCE, HEK293 T-Rex E2F6 cells, treatment, and times shown. See also Figure S3.

Overall, these data support the hypothesis that, to correctly regulate the level and activity of crucial checkpoint effector proteins, cells require active protein synthesis during RS.

Protein abundance is, among others, a function of both transcript levels (a function of transcription and mRNA stability) and protein stability. Our data suggest an important role for transcription, specifically E2F transcription, in preventing RS-induced DNA damage. To establish whether active transcription is required for maintaining protein levels during RS, we treated cells with the transcriptional inhibitor actinomycin D. In response to HU, protein levels were affected similarly by transcriptional and protein synthesis inhibition (Figure S3C), indicating that active transcription is required for maintaining optimal levels of proteins during RS. The same effect on protein levels was seen when just E2F transcription was inhibited, through doxycycline-induced overexpression of the repressor E2F6. As seen when inhibiting transcription or translation, preventing E2F transcription by E2F6 overexpression during RS results in a lower abundance of key checkpoint effector proteins in both HEK293 TRex E2F6 and RPE1 TetON E2F6 cells (Figures 2D–2F). These data suggest that sustained E2F transcription is required for maintaining optimal levels of key checkpoint proteins during the cellular response to RS.

Sustained E2F Transcription Is Necessary for the Arrest and Stabilization of Replication Forks

Our results indicate that sustained E2F transcription is required for the cellular response to RS. We therefore investigated which specific biological processes essential to the RS checkpoint response require sustained E2F transcription. An important process to prevent RS-induced DNA damage involves the arrest and stabilization of ongoing replication forks (Seiler et al., 2016).
2007). DNA fiber analysis was used to evaluate the arrest of ongoing forks during HU treatment (Lossaint et al., 2013), with and without active E2F transcription. DNA replication tracks were labeled with the nucleotide analog CldU (red) and then HU was added with the second analog IdU (green; Figure 3A). These nucleotide analogs are incorporated during ongoing replication and can then be visualized. The length of the green tracks displayed in the bar graph represents replication progression during RS. As expected, HU treatment alone induces a replication arrest, resulting in a population of short tracks. Strikingly, cells unable to sustain E2F transcription (+E2F6) as part of their checkpoint response continue to replicate their DNA further during HU treatment than control cells (Figure 3A). Importantly, overexpression of E2F6 in untreated cells does not affect the length of DNA tracks at these time points (Figure S3D). These data suggest that active E2F transcription is required to efficiently arrest replication forks in response to RS.

We then assessed the role of E2F transcription in stabilizing stalled replication forks during RS. Previous work has shown that an inability to stabilize replication forks during RS, over time, results in shorter DNA tracks in fiber analysis likely due to resection of the newly synthesized DNA (Lossaint et al., 2013; Schlacher et al., 2011). DNA fiber analysis was carried out in HU-treated cells with wild-type or overexpressed levels of E2F6. After the IdU pulse (green), the cells were chased in HU in the absence of nucleotide analogs for 4 hr (Figure 3B). If stalled replication forks are stable, the green tracks do not shorten; however, if the forks are not properly stabilized, this can result in shorter tracks, likely due to unchecked nuclease activity. The length of the green (IdU) tracks during the chase period is significantly reduced in E2F6 overexpressing cells (HU + E2F6) compared to control (HU; Figure 3B), suggesting that the maintenance of fork stability is compromised in the absence of a proper transcriptional response. Based on these results, we conclude that sustained E2F transcription is important for replication fork arrest and stabilization in response to RS.

**Protection and Resolution of Stalled Replication Forks Requires E2F Transcription**

Another important function of the RS checkpoint response is to resolve arrested replication forks to allow replication to resume.
once the stress has been relieved (Petermann and Helleday, 2010). This can be assessed by monitoring the number of cells containing RPA2 foci, which indicate stalled replication forks, at various times after release from HU-induced RS. To test whether there might be a role for E2F transcription in this process, cells were treated with HU with or without E2F6 overexpression and then washed and released into normal medium. After HU treatment (0 hr after release), control and +E2F6 show a similar percentage of cells containing RPA2 foci, indicating a similar number of cells in S phase and experiencing RS, excluding any cell-cycle effects of E2F6 overexpression (Figures 3C and 3D). After release from HU, control cells resolved RPA2 foci, as seen by a lower percentage of cells containing foci 7 and 9 hr after release. However, cells released from HU–E2F6, which were unable to sustain E2F transcription during RS, were unable to resolve arrested forks as indicated by the maintenance of high levels of RPA2 foci. To test this idea further, we monitored RPA2 foci resolution after HU release by in vivo time-lapse imaging, exploiting a HEK293 cell line stably expressing GFP–RPA2 (Movies S1 and S2). The inhibition of E2F transcription during HU treatment strongly delayed the resolution of RPA2 foci, confirming the previous results.

A critical step in the checkpoint response is the formation of a protective complex at stalled replication forks that enables fork stalling, stabilization, and restart (Branzei and Foiani, 2010). We therefore investigated whether sustained E2F transcription is required for the recruitment of factors involved in this process to chromatin. Strikingly, protective factors such as Rad51, FANCID2 (Lossaint et al., 2013), PCNA (Ciccia and Elledge, 2010), and Cdc7 (Yamada et al., 2013, 2014), which become bound to chromatin upon 7 hr HU treatment, show detective recruitment to chromatin when transcription is blocked by E2F6 overexpression (Figure 3E). Whereas overexpression of E2F6 during HU treatment reduces the levels of some of these proteins compared to HU treatment alone, they can still be detected in total lysate (Figure 3F). This shows that E2F transcription is required for the formation of a protective complex at forks during RS. Overall, these data show that sustained E2F transcription is required for specific checkpoint functions—replication fork stalling, stability, and protection during RS and resolving stalled forks after the arrest.

**E2F Activity Is a Key Mechanism of the Checkpoint Response to Prevent DNA Damage**

To determine whether maintaining expression of E2F targets is a key mechanism of the checkpoint response, we tested whether sustaining E2F transcription alone (by silencing the repressor E2F6) could rescue checkpoint functions in cells with a compromised checkpoint response (by silencing the checkpoint effector kinase Chk1). We first assessed whether activating E2F transcription (siE2F6) could prevent DNA damage from accumulating in checkpoint-compromised cells (siChk1), in response to 4 hr HU treatment. In cells treated with siChk1 and HU-induced RS, restoring E2F transcription (siE2F6) can indeed significantly reduce DNA damage levels (∇H2AX) as detected by western blot (Figures 4A and S4A) and quantitative immunofluorescence analysis in individual cells (Figures 4B and 4C). Importantly, the significant decrease in ∇H2AX signal is detected in RPA-labeled cells, and with a second siRNA targeting E2F6 (siE2F6-2) (Figures S4B and S4C). These data indicate that E2F transcription can reduce RS-induced DNA damage levels in cells with a compromised checkpoint response.

We next assessed whether activating E2F transcription (siE2F6) could also prevent DNA damage in response to HU treatment in cell depleted for ATR. ATR phosphorylates Chk1 in response to RS, but Chk1 can be activated when ATR is compromised (Buisson et al., 2015), and there are also ATR-dependent and Chk1-independent aspects of the RS response (Koundrioukoff et al., 2013). Following 4 hr HU, siATR-treated cells accumulate less ∇H2AX signal in RPA-labeled cells compared to that observed in siChk1-treated cells. However, we still observe a small but significant reduction in both ∇H2AX and RPA signal when E2F6 is co-depleted during HU treatment (Figures S4D and S4F), confirming the results obtained with Chk1. Furthermore, the co-depletion of E2F6 and ATR reduces the extent of RPA2 phosphorylation, another marker of ATM activity (Figure S4E). Overall, these results indicate that restoring E2F transcription can reduce RS-induced DNA damage in checkpoint-compromised cells.

Given the reported role of RNR enzyme in preventing RS, we evaluated the protein levels of the RNR subunit RRM2, a well-known E2F target. An increase in RRM2 has been recently shown to counteract RS and DNA damage induced by the inactivation of the checkpoint proteins ATR and Chk1 (Buisson et al., 2015). The levels of RRM2 increase in response to 4 hr HU (Figure S4E), according to our previous findings (Bertoli et al., 2013a), and the depletion of Chk1 decreases the levels of RRM2 (Figure 4A), supporting a role of Chk1 in maintaining RRM2 levels. Most importantly, the levels of RRM2 do not change following E2F6 depletion (Figures 4A and S4E), suggesting that the protective effect of
E2F6 depletion does not derive from increased levels of deoxyribonucleotides.

Next, we assessed whether activating E2F transcription (siE2F6) allows the replication fork protective complex to form in response to RS in checkpoint-compromised cells (siChk1). Control silenced cells do not yet show strong signs of RS after 4 hr HU treatment (Figures 4A, 4B, and 4C) and therefore display limited Rad51 and FANCD2 recruitment to chromatin (Figure 4D). In contrast, cells depleted of Chk1 (siChk1) experience high levels of RS-induced DNA damage in response to 4 hr HU treatment but limited protective complex formation. Strikingly, the inactivation of E2F6 in checkpoint-compromised cells (siE2F6 siChk1) can drive chromatin recruitment of FANCD2 and Rad51 (Figure 4D), correlating with the reduction in RS-induced DNA damage levels seen (Figures 4B and 4C). These data indicate that maintaining E2F transcription is required and sufficient to prevent the accumulation of RS-induced DNA damage, likely though driving the formation or maintenance of a protective complex at replication forks.

Next, we investigated whether maintaining E2F transcription, in cells with a compromised checkpoint response, is sufficient for resuming replication once the stress has been removed. DNA fiber analysis incorporating CldU was used to measure the length of DNA tracks, indicating the progression of DNA replication. Cells were then treated with 2 hr HU to cause RS, which is known to arrest replication. Then cells were washed and released into normal medium containing the iodU nucleotide analog and the length of DNA tracks measured. This shows the cells ability to resume replication following release from a period of RS. Control cells show similar track lengths before and after HU treatment (Figures 4E and 4F), showing they are able to resume replication following HU-induced RS. However, cells treated with a Chk1 inhibitor (UCN01), and therefore checkpoint-compromised, have significantly shorter tracks after HU treatment, indicating they are impaired in resuming replication. Strikingly, in this same checkpoint-compromised set-up (HU-UCN01) maintaining E2F transcription, via E2F6 depletion (siE2F6 or siE2F6-2) restores the cells’ ability to restart DNA replication following RS (Figures 4E, 4F, and 5A). These data suggest that sustained E2F transcription is sufficient, even without a proper checkpoint response, for the formation of a protective complex at stalled replication forks to allow replication to resume after HU treatment and to prevent DNA damage. Overall, these data suggest that sustained E2F transcription is an essential part of the checkpoint response mechanism to RS (Figure 4G).

**Tolerance to Oncogene-Induced RS Requires E2F Transcription**

Oncogenes, such as Ras, c-Myc, and cyclin E, induce E2F transcription to drive entry into S phase and cell proliferation. The generally accepted view is that this unscheduled S phase entry is at the likely basis of oncogene-induced RS, suggesting a direct link between E2F induction and oncogene-induced RS (Hills and Diffley, 2014). However, our data show that sustained E2F transcription is essential for key checkpoint mechanisms to prevent RS-induced DNA damage. This suggests that the upregulation of E2F transcription might inadvertently contribute to tolerance to oncogene-induced RS by preventing DNA damage. To investigate this possibility, we tested whether maintaining E2F transcription is required to prevent DNA damage caused by oncogene-induced RS. We created a stable, E2F6-inducible RPE1 cell line in which we can also induce oncogenic Myc activity (RPE1 TetON E2F6 c-Myc-ER). Importantly, the levels of RS induced by c-Myc-ER induction are comparable to the levels induced by HU treatment in our experimental conditions (Figures 5A and 5C), indicating these are within a physiologically relevant range. This cell line allows the separate induction of c-Myc with hydroxytamoxifen (4OH-T) to cause oncogene-induced RS (Figure 5B) and inducible overexpression of E2F6 with doxycycline to repres E2F-transcription. Taking ChTP as a representative E2F target gene, we observe a decrease in the repressor protein E2F6 alone does not result in a significant increase in γH2AX labeling in S phase cells (Figure 5D). However, overexpression of E2F6 in the presence of c-Myc induction (c-Myc/E2F6) significantly increases DNA damage during S phase compared to c-Myc induction alone (c-Myc), as indicated by increased γH2AX labeling (Figure 5D). In accordance with this, overexpression of E2F6 decreases the survival of cells following c-Myc induction, indicating that E2F transcription is required for cell survival following oncogene-induced RS (Figure 5D). These data suggest that tolerance to oncogene-induced RS depends on E2F activity.

Next, we tested whether, in response to oncogene-induced RS, as in response to HU, chromatin recruitment of fork-stabilizing proteins was impaired in the presence of E2F6 overexpression. c-Myc-induced RS results in the recruitment of ChTP and FANCD2 to chromatin. This is impaired by concurrent overexpression of E2F6 (Figure 5C), indicating that E2F transcription is also required in the response to oncogene-induced RS for the recruitment of fork-stabilizing proteins. Finally, we tested if a decrease in the repressor protein E2F6, and hence an increase in E2F2 transcription would reduce the levels of RS-induced DNA damage caused by oncogenic activity (c-Myc). c-Myc induction causes an increase in the levels of γH2AX. An increase in E2F2 transcription (siE2F6) reduced the levels of RS-induced DNA damage caused by oncogenic activity (Figures 5E and S5E). Together, these data support an important role for E2F transcription in the tolerance to oncogene-induced RS (Figure 5F).

**DISCUSSION**

The work presented here establishes that sustained E2F transcription is a key mechanism in the RS checkpoint response (Figure 4G). Our work shows that sustained E2F transcription is both required and sufficient for many essential functions of the checkpoint response, including fork stalling, stabilization, and resolution. Our data suggest that critical checkpoint proteins require sustained E2F transcription, which is actively maintained in a checkpoint-dependent manner, to correctly regulate their levels and likely activity during RS. Indeed, many of the proteins with critical roles in the stalling and stabilization of forks and restart of replication, including Brca1, Brca2, Rad51, ChTP, and BLM.
are E2F targets. Sustained E2F transcription during RS is required for the increase of certain checkpoint protein, whereas, for others, it is needed to maintain their levels due to their short half-lives. Importantly, we also find that, in the context of oncogene-induced RS, where increased E2F activity is thought to cause RS, E2F transcription is required to limit DNA damage levels (Figure 5F).

Currently, the mechanism that signals for the cell to resume replication is largely unknown. We propose that this particular network wiring, with coordinated control of transcription/translation and protein degradation, could enable the cell to quickly re-adjust the proteome once the stress has been resolved. Potentially, newly synthesized un-phosphorylated components replacing phosphorylated proteins at the replication fork could signal replication restart when the checkpoint is satisfied. We propose that the interplay between transcription, protein stability, and phosphorylation is important to regulate the level and activity of proteins involved in the highly dynamic chain of events that characterizes the checkpoint response and its resolution, opening a new perspective for future research.
maintaining transcription of a large number of genes during DNA replication might result in genome instability. In addition, checkpoint and replication control proteins might cause genomic instability if their activity is not restrained when the cell resumes replication.

RS is a common feature of cells with activated oncogenes or absent tumor suppressors that accelerate the rate of S phase entry and disrupt the DNA replication schedule (Hills and Diffley, 2014). The ensuing RS-induced DNA damage triggers activation of the checkpoint kinases (ATM and Chk2). This induces senescence or apoptosis, serving as an initial barrier to inhibit tumor development in its early stages by preventing proliferation. It is hypothesized that persistent RS creates an environment that selects for mutations that bypass checkpoint functions (most notably p53) and rescues proliferation. This allows tumor progression, which generates DNA damage contributing to rapid evolution and heterogeneity in Oncogenes, such as Ras, c-Myc, and cyclin E, induce E2F transcription to drive passage into S phase and cell proliferation. The generally accepted view is that this unscheduled S phase entry is at the basis of oncogene-induced RS; suggesting a direct link between E2F induction and oncogene-induced RS. However, our data show that sustained E2F transcription is a key mechanism of the checkpoint response to prevent RS-induced DNA damage. Our work supports a model where an increase in E2F activity promotes oncogene-driven cellular proliferation causing RS but also provides the mechanism of RS tolerance, which allows the cells to cope with the increased rates of replication. The increased reliance on sustained E2F transcription creates a potentially large therapeutic window for damaging cancer cells experiencing RS without affecting normal cells.

EXPERIMENTAL PROCEDURES
Cell Culture and Transfection
Cell lines used were T98G, RPE-1 hTERT, and HEK293 T-Rex E2F6 (previously described; Bertoli et al., 2013a). RPE1 TetON E2F6 and RPE1 TetON E2F6 c-Myc-ER were created for this study. See the Supplemental Information for details of maintenance and creation of cell lines. Transfections were performed with Lipofectamine 2000 (Invitrogen) according to manufacturer instructions. See the Supplemental Information for siRNA sequences. Transfected cells were split 24 hr after transfection and then used 24 hr later for the experiments. Typically, one hundred to two hundred fibers were measured for each.

Toledo et al. (2013a). Images were obtained with Leica TCS SP5 or SPE2 63x/3.0 NA objective lens, processed in Fiji. See the Supplemental Information for full details.

Flow cytometry of DAPI/RPA2/γH2AX was performed as previously described (Fornent et al., 2013). See the Supplemental Information for full details.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.04.036.

AUTHOR CONTRIBUTIONS

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Western Blot
Cell extracts were prepared in RIPA buffer, run on Novex 4%-12% Bis-Tris protein gels, probed for the antibodies indicated, and then secondary horseradish peroxidase (HRP) used (described fully in the Supplemental Information). Tubulin, GAPDH, and actin were loading controls; histone H3 was used for chromatin preparation loading controls.

Fibero Analysis
HEK293 T-Rex E2F6 or T98G were labeled with 25 μM CldU and then 250 μM IdU; see schematics. Fiber spreading and labeling were as in Petermann et al. (2010). Images were taken by confocal microscopy and analyzed with ImageJ.

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Sustained E2F-Dependent Transcription Is a Key Mechanism to Prevent Replication-Stress-Induced DNA Damage

Cosetta Bertoli, Anna E. Herlihy, Betheney R. Pennycook, Janos Kriston-Vizi, and Robertus A.M. de Bruin
Figure S1, related to Figure 1. Active protein synthesis is required to prevent RS-induced DNA damage. A) Right, scatter plot of mean intensity of RPA2 (green, x-axis) against γH2AX (red, y-axis) of single nuclei, 2 hr HU /+ Cycloheximide (Chx) in HEK293 T-Rex E2F6 cells. The logarithmic scale is identical for all. P values show significant differences on both axes with the Wilcoxon test. Arrows show increase/decrease of mean on each axis. Left, Representative immunofluorescence images showing chromatin-bound protein. Scale bar represents 10 μm. B) Right, scatter plot of mean intensity of RPA2 (green, x-axis) against γH2AX (red, y-axis) of single nuclei. Treatments shown, 7h, RPE1 cells. The logarithmic scale is identical for all. P values show significant differences (except for RPA2 between no treatment and HU + Chx) on both axes with the Wilcoxon test compared to control (-). Arrows show increase/decrease of mean on each axis. Left, Representative immunofluorescence images showing chromatin bound proteins. Scale bar represents 20 μm. C) RNA expression of HEK293 T-Rex E2F6 cells after HU treatment /+ E2F6 overexpression (Doxy 2 μg/ml, 2 hour pretreat) for the time points shown. RNA was extracted and quantified by RT qPCR, normalised to GAPDH and normalised to HU time point 0. n=3, error bars show SEM. *** P<0.001, ** P<0.01 with ANOVA and Tukey’s.
Figure S2, related to Figure 1. Active protein synthesis and E2F transcription is required to prevent RS-induced DNA damage. **A** Left, scatter plot of mean intensity of RPA2 (green, x-axis) against γH2AX (red, y-axis) of single nuclei. Treatments shown, 2 hr HU, HEK293 T-Rex E2F6. Black dots show non-S phase cells (RPA2<10 a.u.), orange and red dots show low and high levels of γH2AX respectively (arbitrary threshold γH2AX=30 a.u.). P values show differences of S phase cells on both axes with the Wilcoxon test compared to control (- or HU treated as appropriate). Arrows show increase/decrease of mean on each axis. Right, representative images. Scale bar represents 20 μm. **B** Right, scatter plot of mean intensity of RPA2 (green, x-axis) against γH2AX (red, y-axis) of single nuclei. Treatments shown, 2 hr HU, HEK293 T-Rex E2F6 cells. Doxy 1.5 hr pretreat. The logarithmic scale is identical for all. P values show significant differences on both axes with the Wilcoxon test compared to control (HU). Arrows show increase/decrease of mean on each axis. Left, Representative immunofluorescence images showing chromatin bound proteins. Scale bar represents 10 μm. **C** Western blot of WCE, HEK293 T-Rex E2F6 cells, treatments shown, 2h HU. E2F6 (Doxy, 2 μg/ml, 2 hr pretreat).
Figure S3, related to Figure 2 and 3. Protein synthesis and E2F transcription are required to maintain the levels of checkpoint proteins during RS. A) Western blot of WCE, T98G cells, treatments and times shown. B) Western blot of WCE, RPE1 cells, treatments shown, 4 h. C) Western blot of WCE, HEK293 T-Rex E2F6 cells, treatments and times shown. ActinoD is transcriptional inhibitor Actinomycin D. D) DNA fiber analysis in HEK293 T-Rex E2F6 cells. Bar graphs of green track lengths. Cells were treated for 3.5 hr -/+ Doxycycline at 2 μg/ml or 4 μg/ml as indicated (timing equivalent to 1.5 hr pretreat then 2 hr HU treatment with Doxy in Figure 3A). Cells were pulse labelled with CldU (red) for 20 min, then IdU (green) for 20 min and the lengths of green tracks measured. P values show no significant difference in track length with E2F6 overexpression with the Student’s T test.
Figure S4, related to Figure 4. E2F activity is required and sufficient to reduce RS-induced DNA damage in a compromised checkpoint response. A) Western blot WCE of serum starvation-synchronized T98G cells transfected with siRNA shown, and released in complete medium with HU for 30 hours. B) Western blot of WCE, RPE1 cells, transfected with the reported siRNA, 4h HU. C) Scatter plot of mean intensity of RPA2 (x-axis) against γH2AX (y-axis) of single nuclei transfected with the reported siRNA -/+ 4 hr HU, RPE1 cells. Black dots show non S phase cells (RPA2<20 a.u.), orange and red dots show low and high levels of γH2AX respectively (arbitrary threshold γH2AX=50 a.u.). P values show differences on both axes of S phase cells (RPA2 > 20 a.u.) with the Wilcoxon test compared to the non-siE2F6 equivalent. Arrows show increase/decrease of mean.}

**D**) Scatter plot of mean intensity of RPA2 (x-axis) against γH2AX (y-axis) of single nuclei transfected with the reported siRNA -/+ 4 hr HU, RPE1 cells. Black dots show non S phase cells (RPA2<20 a.u.), orange and red dots show low and high levels of γH2AX respectively (arbitrary threshold γH2AX=50 a.u.). P values show differences on both axes with the Wilcoxon test compared to the non siE2F6 equivalent. Arrows show increase/decrease of mean. E) Western blot of WCE, RPE1 cells transfected with the reported siRNA -/+ 4 hr HU. F) Western blot of WCE, RPE1 cells transfected with the reported siRNA.
Figure S5, related to Figure 4 and Figure 5. E2F activity is required for tolerance to oncogene-induced RS.

A) Experimental design as in Figure 4E, T98G cells. Left, bar graphs of length of DNA tracks before and after HU treatment with a second siRNA targeting E2F6 (E2F6-2) +/- UCN01. P values show difference with the Wilcoxon test. Right, western blot showing the silencing of E2F6 with the two siRNA compared to control siRNA.

B) Western blot showing the expression of c-Myc-ER in RPE1 TetON E2F6 c-Myc-ER cells compared to non-infected RPE1. Tubulin is the loading control.

C) Western blot of WCE, RPE1 TetON E2F6 c-Myc-ER cells treated with different RS-causing agents – c-Myc overexpression (times shown) or HU 7 hr (concentrations shown).

D) Colony formation assays showing the survival and proliferation of RPE1 TetON E2F6 c-Myc-ER cells exposed to c-Myc-ER induction (4OH-T, 100nM), E2F6 overexpression (Doxy 2 μg/ml), or a combination as shown, for the reported time, then diluted in normal medium and allowed to grow for 10 days before being fixed and stained.

E) Western blot of WCE, RPE1 TetON E2F6 c-Myc-ER treated with the shown siRNA, samples from Figure 5E.
Supplemental Experimental Procedures

Cell culture
HEK293 T-Rex E2F6 and T98G cells were maintained in DMEM (Gibco, 41965) 10% FBS (Sigma, F7524) supplemented with Penicillin/Streptomycin (Gibco, 15140). RPE-1 hTERT and RPE1 TetON E2F6 were maintained in DMEM or DMEM/F12 (Gibco, 31331) supplemented with Sodium Carbonate (Gibco, 25080) and Penicillin/Streptomycin, as recommended by ATCC. HEK293 T-Rex E2F6 cells were previously described (Bertoli et al., 2013) and were maintained in 5 µg/ml Blasticidin and 100 µg/ml Zeocin. RPE1 TetON E2F6 were created for this study. Cells were transfected with Lipofectamine 2000 (Invitrogen) following manufacturers instructions using the plasmid pcDNA4/T0 E2F6. Transformed cells were selected in 5 µg/ml Blasticidin and 200 µg/ml Zeocin and colonies tested for Doxycycline dependent E2F6 expression. RPE1 TetON E2F6 were maintained in 5 µg/ml Blasticidin and 100 µg/ml Zeocin. RPE1 TetON E2F6 c-Myc-ER was created for this study. Cells were obtained by retroviral infection of RPE1 TetON E2F6 using the pBABE c-Myc-ER plasmid (Addgene plasmid 19128) (Ricci et al., 2004). Infected cells were then selected in puromycin 5 µg/ml and the surviving cells used for the assays. The RPE1 TetON E2F6 c-MycER cells were maintained in phenol-free DMEM/F12 10% charcoal-treated bovine serum with the supplements described above and in 5 µg/ml Blasticidin, 100 µg/ml Zeocin and 2 µg/ml Puromycin.

siRNA Transfection
The sequences used were: siCont (LacZ) AAGCUACCGGAUAUCUUGA, siE2F6 AAGGAUUGUGUCAGCAGCU (Figures 4ABCDF, 5E, S4ADE, S5E), siE2F6-2 AAAAGUUGCAACGAAAUU (Figures S4B, S4C, S5A), siChk1 GAAGCAGUCCGAGAGAAUU (Bertoli et al., 2013) (Figures 4D, S4A), SMARTpool: siGENOME CHEK1 siRNA (M-003255-04-005, Dharmacon GE Life Sciences) (Figures 4ABC, S4B,S4C), siATR UUGUAGAAAUGGAUACUGA (validated Silencer Select s536, Thermo Fisher Scientific).

Immunofluorescence
Figures 1A, B, 4B, C, 5D, E, and Figures S1A, B, S2A, B, S4C,D : Cells were pre-extracted for 1 min in ice cold PBS 0.2% Triton-X100 (or 0.15% for HEK293 T-Rex cells), then fixed with 4% formaldehyde for 20 min and processed similarly to (Toledo et al., 2013). Briefly, coverslips were blocked in 1% BSA for 1 hr and incubated in primary antibodies overnight at 4°C – RPA2 (RPA2) (1:500, Ms, MABE285, Millipore) and Phospho-Histone H2A.X (γH2AX) (Ser139) (1:400, Rb, 20E3, Cell Signaling Technology). Coverslips were incubated in secondary antibodies for 1 hour at RT – anti-mouse Alexa Fluor 488, and anti-rabbit Alexa Fluor 647 both 1:2000 (LifeTechnologies), incubated 5 minutes in Hoechst 1:10,000, (Invitrogen), and mounted with Fluoroshield (Sigma). Images were obtained with a Leica TCS SP5, or a Leica TCS SPE2, 63x objective lens. Images were processed in Fiji – the Hoechst channel was used – segment nuclei with a median filter and Otsu thresholding. The mean gray value of each channel was then measured for each nucleus. The signals for individual cells were then plotted using Spotfire or Prism software.

Figure 3C,D: Cells were fixed with 4% formaldehyde for 20 min and permeabilised for 5 min in PBS 0.2% Triton-X100. Coverslips were then processed as above with RPA32 (RPA2) antibody (1:1000, Ms, MABE285, Millipore). The percentage of cells containing RPA2 foci was manually counted in Fiji.

Statistics
Figure 1A,B, 5D,E and Figure S2A, S4C: Statistical significance was analyzed using the Wilcoxon sum rank test; two sided, using the R function Wilcox.test. Only S phase cells were analyzed, defined as the portion of cells where RPA2 > 10 a.u. (Figure 1B, 5E, S2A), RPA2 > 20 a.u. (Figure 5D, S4C), or where RS occurs, as seen by an increase in γH2AX intensity during HU treatment (Figure 1A). Figures 4B and Figures S1A,B, S2B, S4D: Statistical significance was analyzed for both RPA2 and γH2AX against control conditions using the Wilcoxon sum rank test, two sided, using the R function Wilcox.test. Figures 1C, 3A, B and Figure S3D: Statistical significance was analyzed by Student’s T test. Figure 3C: Statistical significance was analyzed by two-way ANOVA (using the aov() function of R), the multiple comparisons of the means was performed by Tukey’s honest significant differences, using the R function TukeyHSD().*** P<0.001, * P<0.05. Figure 4F and Figure
SSA: Statistical significance was analyzed for differences between mean track length after – mean track length before HU treatment using the Wilcoxon sum rank test, two sided, using the R function Wilcox.test.

**Figure S1C:** Statistical significance was analyzed by two-way ANOVA, the multiple comparisons of the means was performed by Tukey’s honest significant differences, using Prism software. ***P<0.001, **P<0.01.

**Western blot**

Cell extracts were prepared in RIPA buffer (Tris-HCl pH7.5 20mM, NaCl 150mM, EDTA 1mM, EGTA 1mM, NP40 1%, NaDoc 1%) containing phosphatase inhibitor cocktails 2 and 3, 1:1000 (Sigma P5726 and P0044) and protease inhibitor cocktail, 1:1000 (Sigma, P8340). Run on Novex 4-12% Bis-Tris protein gels. The following antibodies were used for Western blot analysis, all were used at 1:1000 unless otherwise stated: Claspin (Rb, ab3720), CtIP (Rb, ab70163), Mre11 (Ms, ab214), Rad54 (Ms, ab11055), Histone H3 (Rb, ab1791) from Abcam, Chk1 (Ms, DCS310, sc56291), Cyclin E (Ms, DCS241, sc25280), ATR (Gt, N-19, sc-1887), Myc 9E10 (sc-40) from Santa Cruz, GAPDH (Ms, GT239) from GeneTex, Tublin (1:2000, Ms, MAB83408), RPA34-20 (Ms, MABE285), E2F6 (Ms, MABE57) from Millipore, FANCD2 (1:200, Rb, NB100-182) from Novus biological, RPA32 (RPA2) (S4/S8) (Rb, A300-245A-3) from Bethyl laboratories, Phospho-Histone H2A.X (γH2AX) (Ser139) (Rb, 20E3), Phospho-Chk1 (ser345) (Rb, 2341), p53 (Ser15) (Rb) from Cell Signaling Technology, Actin (Rb, A2066) from Sigma, RRM2 (Ms, M01, 1E1), E2F6 (Ms) a kind gift of Dr Jacky Lees. Secondary antibodies used were Goat anti-mouse and anti-rabbit HRP conjugated (Fisher Scientific, PA1-74421 Mouse, PI-31460 Rabbit).

**Fiber analysis**

HEK-293 T-Rex E2F6 or T98G were labeled with 25 μM CldU followed by 250 μM IdU, as in the schematics. Fiber spreading and labeling was performed as in (Petermann et al., 2010). Images were taken by confocal microscopy and analyzed with ImageJ. 150-200 fibers were measured for each experiment.

**RNA expression**

RNA was extracted with the Qiagen RNeasy Mini kit following manufacturers protocol. RT qPCR SYBR assay was carried out with Mesa Blue following manufacturers instructions, 80 ng RNA per well, experimental triplicates, BioRad CTX Connect qPCR machine. RNA levels were normalized to GAPDH. Experiment was done in biological triplicate. Primers used were: GAPDH Fw 5'- GAAATCCCATCACCATCTTCCAGG 3', Rv 5'- GAGCCCCAGCCTTCTCCATG-3', Cyclin E Fw 5'-TGTCCTGGATGTGACTGCCTTGA-3', Rv 5'-TGTCGCACCACTGATACCCTGAAA-3', CtIP, Fw 5'- CTGCTTGGCACACGTGTAAG-3', Rv 5'-TGGAATGTAGCGGAATCGGTGTCT-3'.

**Chromatin Preparation**

Buffer A (Hepes pH 7.9 10 mM; KCl 10 mM; MgCl2 1.5 mM; Sucrose 0.34 M; Glycerol 10 %; DTT 1 mM; and protease and phosphatase inhibitors as recommended by the supplier). Buffer B (3 mM EDTA; 0.2 mM EGTA; protease and phosphatase inhibitors). The nuclear fraction was pelleted at 1300 g, 5 min, 4°C. The chromatin fraction was pelleted at 1700 g, 5 min, 4°C. The sample was spun at 9600 g, 5 min, 4°C before use.

**FACS**

Flow cytometry was performed as described previously for DAPI/RPA2/γH2AX in (Forment et al., 2012). Phospho-Histone H2A.X (γH2AX) (Ser139) (Rb, 20E3) from Cell Signaling Technology used 1:200 and RPA2 (Ms, 9H8, ab2175) from Abcam used 1:250. Samples were measured on a BD LSR II flow cytometer using DIVA software (BD) and analyzed using FlowJo software.

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Survival Assay

RPE1 TetON E2F6 c-MycER were treated as shown in Figure S5D for 48 or 72 hours, they were then diluted and left to grow in 10cm dishes until colonies were visible. Cells were fixed and stained as in Bertoli et al, 2013.

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