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Investigating the functions of Tof1/Timeless in *Saccharomyces cerevisiae* and human cells

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University of Sussex
Doctor of Philosophy in Genome Stability
August 2020

Statement

I hereby declare that this thesis has not been and will not be, submitted in whole or in part to another University for the award of any other degree.

Rose Westhorpe

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I wish to thank every member of the Baxter lab that I have had the pleasure of working with during my PhD. First and foremost, I would like to thank my supervisor Jon for giving me the opportunity to carry out my PhD in his group, and for supporting me throughout it. I am hugely grateful for all of the advice and encouragement, for the knowledge that you have shared with me and for all of the Friday morning pastries. I also want to want to thank the other members of the Baxter lab- Andrea for her hugs and for putting up with my annoying questions, Nicola for making me laugh every day and for being the best pub companion and friend, and Issy for always being so positive and wholesome.

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Thesis Summary

University of Sussex

Rose Westhorpe

Doctor of Philosophy in Genome Stability

Investigating the functions of Tof1/Timeless in *Saccharomyces cerevisiae* and human cells

Aberrant DNA replication is a major source of genome instability in cells. The evolutionarily conserved Fork Protection Complex (FPC) consists of Tof1/Csm3 and Mrc1 in *S. cerevisiae* or Timeless/Tipin and Claspin in higher eukaryotes. FPC proteins travel with replication forks to mediate intra-S-checkpoint signalling and replisome stability in response to DNA replication stress (RS). However, the exact mechanisms by which the FPC mediates these functions remain unclear.

This thesis has addressed this problem in the budding yeast *S. cerevisiae* through the generation and characterisation of a series of Tof1 mutants. Specifically, using C-terminal Tof1 truncation mutants this thesis provides novel insights into the structure-function relationship of Tof1 in *S. cerevisiae* and the various roles this protein plays in responding to RS. I have demonstrated that Tof1 plays a specific role in responding to topological stress that is distinct from its other functions, and identified a second domain within the C-terminus of the protein that is required for suppression of camptothecin (CPT) induced lethality of *tof1Δ* cells, for replication fork pausing and for coupling helicase and polymerase activities in the presence of hydroxyurea (HU). I have also shown that the aforementioned functions of Tof1 are not dependent on intra-S-checkpoint activation, as an N-terminal segment alone of Tof1 is capable of checkpoint activation in response to HU. Together these results give a novel insight into how the Tof1 protein responds to different types of RS, and show that it operates through distinct domains capable of dealing with different obstacles to DNA replication.

Lastly, in addition to this work in *S. cerevisiae* I have targeted the endogenous *TIMELESS* gene in human cells with an inducible degron tag, in attempt to generate conditional Timeless cell lines. This work has further supported the evidence for an essential role for Timeless in mammals.

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1. Introduction

1.1. Eukaryotic DNA replication

For all life forms, the faithful duplication of cellular chromosomes is a prerequisite to genetic inheritance. In eukaryotes this duplication process, termed DNA replication, is carried out during the S-phase of the cell cycle by a multi-protein complex known as the replisome (Figure 1.2). Through a combination of genetic analyses and more recently using *in vitro* studies, the core enzymatic functions of the eukaryotic replisome have been elucidated in detail and are remarkably conserved across species. Generally, DNA replication is divided into three distinct stages. The first of these is termed initiation, where the core replisome machinery is assembled and the replicative helicase is activated to begin unwinding of the DNA duplex. Next is elongation, where DNA polymerases make copies of the parental DNA strands, and finally termination, where two replisomes converge upon each other to finish replication.

1.1.1. DNA Replication Initiation

Due to the large size of eukaryotic genomes, replication is initiated at multiple origins along cellular chromosomes in each S-phase (Figure 1.1). In the budding yeast *Saccharomyces cerevisiae* these origins, known as autonomously replicating sequences (ARS elements) are sequence specific and have been extensively characterised (Bell and Labib, 2016). In humans, however, origin DNA is less well defined and appears to have less of a sequence specificity (Vashee et al., 2003). Initiation begins when inactive, pre-replicative complexes (pre-RCs) are assembled at these origin sequences in G1 phase. Specifically, origin recognition complexes (ORCs), made up of ORC proteins 1-6, and Cdc6 first bind origin DNA in an ATP-dependent manner (Bell and Stillman, 1992, Liang et al., 1995). These factors then act as a platform for assembly of two minichromosome maintenance hexamers (Mcm2-7), which form the core of the replicative helicase, on the DNA duplex by Cdt1 (Figure 1.1) (Remus et al., 2009, Randell et al., 2006). The second step of initiation is the conversion of these inactive pre-replicative complexes into active replicative helicases, and this requires multiple firing factors and phosphorylation events. By using *in vitro* reconstitution of yeast replisomes, the minimal set of proteins required

for origin activation have been identified as Cyclin-Dependent Kinase (CDK), Dbf4-Dependent Kinase (DDK), Sld3/7, Dpb11, Sld2, Cdc45, polymerase ϵ , the GINS complex and Mcm10 (Yeeles et al., 2017, Yeeles et al., 2015).

The kinase activities of DDK and S-CDK are essential for replisome initiation, by recruiting the CMG components Cdc45 and GINS to the inactive Mcm2-7 double hexamers (reviewed in Labib, 2010). DDK consists of two subunits, the Cdc7 kinase and a second regulatory subunit, Dbf4 which relieves autoinhibition of Cdc7 (Kitada et al., 1992, Jackson et al., 1993, Dowell et al., 1994). DDK-dependent phosphorylation of Mcm4 and Mcm6 drives Sld3- and Sld7-dependent recruitment of Cdc45 to the replisome (Tanaka et al., 2011, Yabuuchi et al., 2006, Masai et al., 2006, Sheu and Stillman, 2006). Recruitment of Cdc45 appears to be dependent solely on DDK phosphorylation, as in the absence of DDK no downstream initiation factors are recruited to the replisome, whilst in the absence of S-CDK activity Sld3, Sld7 and Cdc45 are still recruited efficiently (Yeeles et al., 2015, Heller et al., 2011). GINS is recruited to the replisome via S-CDK-dependent phosphorylation. Specifically, S-CDK (Cdc28/Cdk1 in complex with S-phase regulatory cyclin Clb5/Clb6) phosphorylates Sld2 and Sld3, which in turn stimulates their binding to Dpb11 and facilitates GINS recruitment to the growing replisome (Tanaka et al., 2007, Zegerman and Diffley, 2007, Yeeles et al., 2015). These events result in the formation of two active Cdc45-MCM-GINS (CMG) helicases (Figure 1.1) upon which additional replisome factors are assembled.

As well as promoting replication initiation, high levels of CDK activity in S, G2 and M phases are crucial for preventing the further assembly and activation of additional helicases after the onset of S-phase. This ensures that each base in the genome is replicated once and only once per cell cycle. CDK-dependent phosphorylation of Cdc6 and Mcm2-7 results in their degradation and nuclear export, respectively, inhibiting pre-RC formation (Drury et al., 2000, Labib et al., 1999, Nguyen et al., 2001). Consequently, inhibition of CDK outside of G1 phase results in helicase re-loading and subsequent re-replication upon restoration of CDK activity (Dahmann et al., 1995). The importance of this temporal separation of pre-RC loading and helicase activation by fluctuating CDK activity is highlighted by the fact that re-replication has the potential to lead to increased genome instability and DNA damage (Green and Li, 2005).

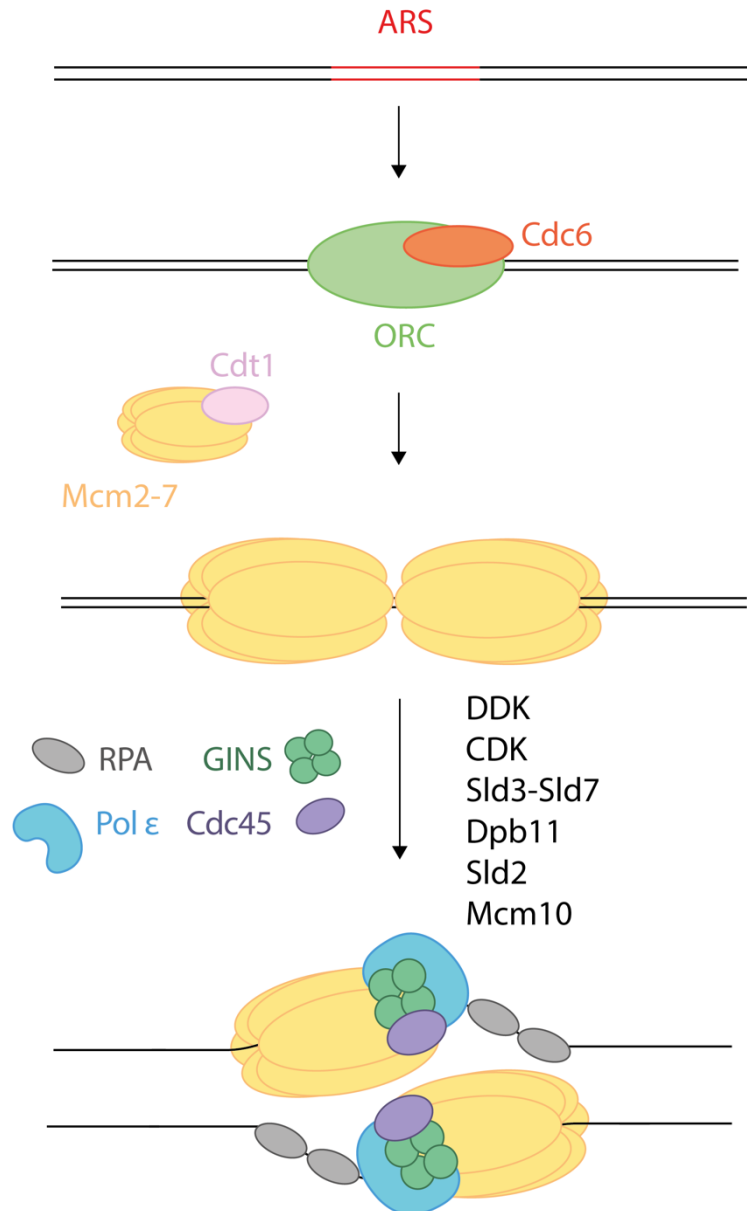


Figure 1.1: Schematic of eukaryotic replication initiation

DNA replication is initiated at specific origin sequences along chromosomes (known as autonomously replicating sequences/ARS in *S. cerevisiae*, shown in red). ORC proteins and Cdc6 first bind ARS elements, which is then followed by loading of two inactive Mcm2-7 hexamers by Cdt1. This forms the inactive “pre-replicative complex”, or pre-RC. Additional factors (Sld3-Sld7, Dpb11, Sld2, Mcm10) and both DDK- and CDK-dependent phosphorylation events are required for conversion of these pre-RCs into the active Cdc45-Mcm2-7-GINS (CMG) helicase capable of unwinding duplex DNA.

1.1.2. DNA Replication Elongation

Following their activation, the two CMG helicases formed at each origin are able to unwind dsDNA in a 3' to 5' fashion, forming the basis of two divergent replication forks at which unwinding and nascent strand synthesis takes place simultaneously. Unwinding of the DNA duplex provides the template for synthesis of the nascent strands. DNA synthesis is carried out by the replicative DNA polymerases Pol α , Pol ϵ and Pol δ , a group of B-family polymerases that synthesise nascent DNA strands in a 5' to 3' manner (Figure 1.2) (Johansson and Dixon, 2013). Both of the newly unwound strands first have to be primed before bulk synthesis. This function is carried out by the Pri1 and Pol1 subunits of the replicative primase Pol α which lay down 30-35 nucleotide primers consisting of both RNA and DNA (Muzi-Falconi et al., 2003). The primers laid down by pol α are then elongated by Pol ϵ and Pol δ , which both must synthesise DNA in the 5' to 3' direction. Due to the antiparallel nature of duplex DNA, this means that the two nascent strands at each replication fork are synthesised differently.

The leading strand is synthesised in the same direction as it emerges from the progressing CMG, in a continuous fashion by the highly processive Pol ϵ (Figure 1.2). Several studies have shown that Pol ϵ maintains contacts with the progressing CMG during elongation and thus is thought to stay recruited to the replisome during DNA replication. This was first shown by the interaction between Dpb2, a regulatory subunit of Pol ϵ , and the Psf2 subunit of GINS (Sengupta et al., 2013). Furthermore, cross-linking experiments have mapped interactions between the Pol2 subunit of Pol ϵ and Mcm2, Mcm6 and Cdc45 (Sun et al., 2015).

Lagging strand synthesis is carried out discontinuously, in short fragments by Pol α and Pol δ (Figure 1.2). These fragments are named Okazaki fragments, after Reji Okazaki & Tuneko Okazaki who originally proposed such a mechanism for lagging strand synthesis in 1968 (Okazaki et al., 1968). Whilst Pol α is maintained at the replisome via its interaction with the heterotrimeric Ctf4 complex (Zhu et al., 2007, Gambus et al., 2009, Tanaka et al., 2009a), it is still not clear whether Pol δ is maintained at the fork. Until recently there was no direct evidence for Pol δ recruitment to the replisome. However, recent single molecule data suggests that one molecule of Pol δ remains associated with

progressing replication forks rather than continuous recruitment of Pol δ to synthesise each Okazaki fragment (Lewis et al., 2019).

Once synthesised, Okazaki fragments then undergo maturation, in which the strand displacement activity of Pol δ removes 1-2 nucleotides at a time of the RNA primer and these small flaps are removed by Fen1 (Garg et al., 2004, Stith et al., 2008). This cycle continues until all RNA has been removed, leaving DNA-DNA ends that can be ligated by DNA Ligase 1.

Given the discontinuous manner in which it is synthesised, the presence of small amounts of single-stranded DNA (ssDNA) are inevitable on the lagging strand following CMG unwinding. In eukaryotes this is bound by ssDNA binding protein RPA, a complex of three proteins: RPA70, RPA32, and RPA14 that were first identified for their role in initiating SV40 replication (Wobbe et al., 1987, Fairman and Stillman, 1988, Wold and Kelly, 1988). RPA binding is thought to prevent re-annealing or nucleolytic degradation of the exposed ssDNA.

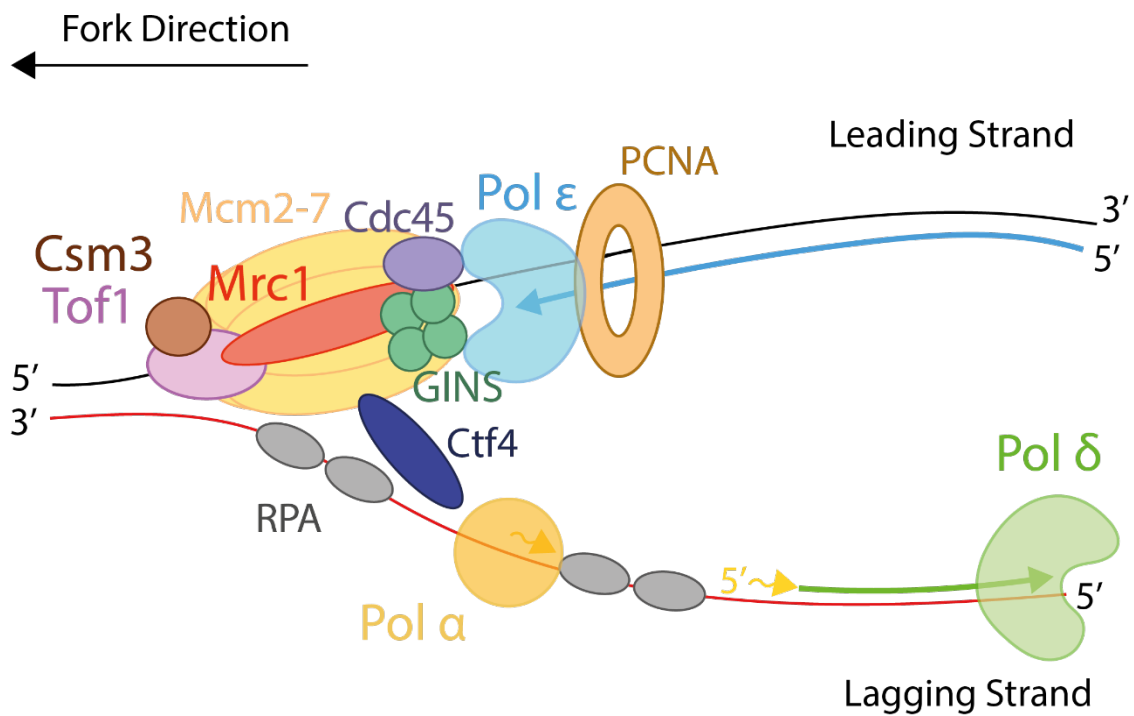


Figure 1.2: Schematic of a progressing eukaryotic replisome

Cdc45, Mcm2-7 and GINS form the CMG helicase which unwinds dsDNA in a 3' to 5' direction. The leading strand (light blue) is synthesised by Pol ε continuously in a 5' to 3' direction and has enhanced processivity due to the processivity factor PCNA. The lagging strand (green) is synthesised discontinuously in Okazaki fragments (light green) by the combined action of Pol α and Pol δ. Pol α first synthesises a short RNA primer (shown in red), which is then extended by Pol δ. RPA coats and protects exposed ssDNA. Additional replisome factors shown include the fork protection complex consisting of Tof1/Csm3 and Mrc1, and Ctf4 which plays a key role in Pol α recruitment to the replisome.

1.1.3. DNA Replication Termination

The final step of DNA replication, known as termination, occurs when two replication forks converge upon each other. For termination to occur, several steps need to be carried out: CMG must completely unwind the last stretch of dsDNA, replication of all bases must be completed, the replisomes disassembled and removed from the chromatin, and the newly replicated sister chromatids must be resolved from one another. Of all the replication stages, termination is the least well understood in eukaryotic cells and the mechanisms by which these termination steps are carried out are still being defined. However, several studies using cell-free systems have uncovered some of the mechanisms by which these different stages occur. Work in *Xenopus laevis* egg extracts has shown that at termination two CMGs do not slow or stall during convergence but pass each other and are only removed from the DNA following the final ligation step of one leading strand to the adjacent Okazaki fragment (Dewar et al., 2015). Fork convergence is stimulated by the Pif1 and Rrm3 helicases *in vitro* and *in vivo* (Deegan et al., 2019). CMG disassembly is then driven by polyubiquitylation of Mcm7 by cullin family ubiquitin ligases and subsequent disassembly by the protein remodelling protein p97 (ScCdc48) (Maric et al., 2014, Moreno et al., 2014). DNA topoisomerase enzymes also play a key role in terminating DNA replication (discussed in section 1.2.1.1).

1.1.4. Replisome Progression Complex (RPC)

The replisome proteins described above serve to establish the basic functions of separating the DNA duplex and synthesising nascent strands (Figure 1.2). The recent reconstitution of DNA replication *in vitro* has demonstrated that regulated origin firing and nascent DNA synthesis requires as little as 16 proteins to take place, representing only the factors needed for CMG activation as well as primase and polymerases (Yeeles et al., 2015, Yeeles et al., 2017). However, to establish *in vivo* rates of DNA synthesis using reconstituted replisomes, additional factors are required (Yeeles et al., 2017). These additional factors are recruited to and maintained at the replisome via their interaction with GINS, and form large complexes known as replisome progression complexes (RPCs) (Gambus et al., 2006). RPC components have broad spanning roles but generally are involved in processes that ensure replication is carried out as efficiently as possible and to help link chromosome duplication to other processes such as sister chromatid cohesion and chromatin assembly. RPCs contain many factors, including Cdc45, FACT, Ctf4, MCM10, Top1 and the fork protection complex consisting of Tof1/Csm3 and Mrc1 (Gambus et al., 2006).

1.1.5. Fork Protection Complex (FPC)

The FPC was first described by Noguchi and colleagues (2004) as consisting of the evolutionarily conserved replisome factors Swi1 and Swi3 in *Schizosaccharomyces pombe* (ScTof1/Csm3, hTimeless/hTipin). However, given the physical and functional interaction of these proteins with a third factor, Mrc1 (hClaspin), the term FPC is now generally used to refer to Tof1/Csm3 and Mrc1 (Katou et al., 2003, Bando et al., 2009). FPC proteins have a broad role in modulating the stability and progression of replication forks, both in endogenous contexts and in response to replication stress. For example, mutants lacking certain FPC components are sensitive to DNA damaging agents, cannot fully activate the intra-S-phase checkpoint, are unable to pause the replisome at barriers to CMG progression and cannot couple nascent DNA synthesis to CMG unwinding. In addition, the FPC has been shown to be required for normal rates of DNA replication both *in vivo* and *in vitro*. A detailed discussion of FPC proteins, in particular Tof1/Timeless, is included in section 1.3.

1.2. DNA Replication Stress (RS)

The process of DNA replication is seldom as straight-forward as earlier described. Genome duplication takes place on an already crowded DNA template due to other cellular processes such as transcription, DNA repair and chromatin remodelling. In addition, replisomes must face a range of endogenous and exogenous impediments to progression, including collisions of the replication machinery with stable protein-DNA complexes and DNA-protein crosslinks (DPCs), depletion of dNTP pools and topological stress (Figure 1.3). As a result, genomic DNA is particularly vulnerable during the process of chromosomal duplication.

Any event that can pause or stall replisome progression can be classed as replication stress (Zeman and Cimprich, 2014). Importantly, the inability to correctly respond to replication stress is commonly associated with decreased genomic stability and increased mutagenesis, and is now recognised as a promoting factor in tumour development in humans (Gaillard et al., Macheret and Halazonetis, 2015). Therefore, to ensure cellular viability and genome stability in the presence of replication stress events, a number of highly conserved pathways have evolved in eukaryotes. These involve checkpoint pathways capable of pausing the cell cycle to allow time to resolve the problem, pathways responsible for stabilisation and restart of stalled forks, and repair pathways to resolve DNA damage.

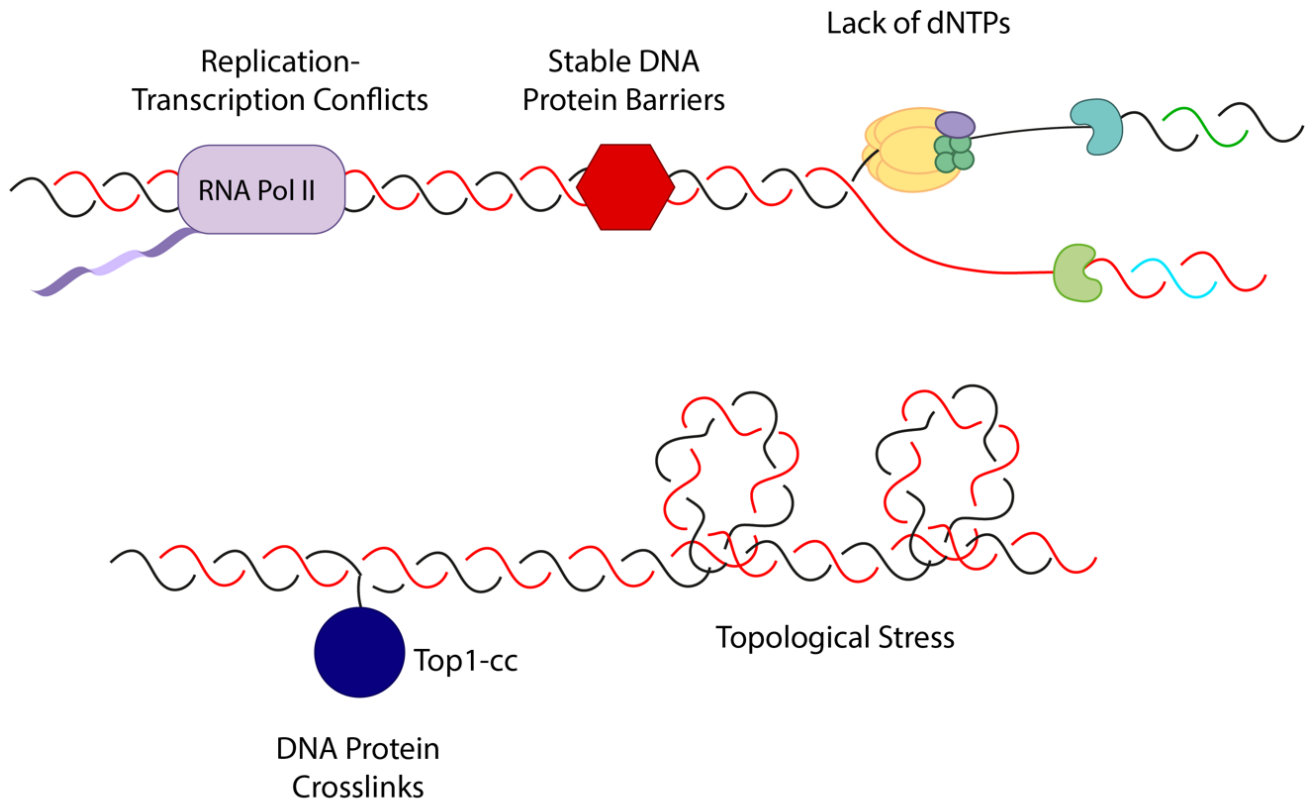


Figure 1.3: Schematic depicting common sources of DNA replication stress

During DNA replication the replisome is frequently challenged by numerous types of replication stress. Many of these represent physical blocks to CMG progression. Messenger RNA (mRNA) synthesis by RNA Pol II (top left) requires usage of the same template as DNA replication and this process can clash with the CMG helicase. Stable DNA binding proteins (top middle) can also physically clash with CMG progression. Topological/torsional stress (bottom right) results from overwinding of the template DNA and can impede on dsDNA unwinding by CMG. DNA protein crosslinks, for example a Top1-DNA crosslink (bottom left), result in a proteinacious block to CMG as well as DNA damage in the form of a single-stranded break (SSB). In addition to these physical barriers, replisomes can be slowed or stalled when cellular dNTP pools are low (top right), which can result in the absence of nascent strand synthesis by the replicative polymerases and the consequent decoupling of these enzymes from CMG progression.

1.2.1. Common Sources of Replication Stress

1.2.1.1. DNA Topological Stress and Topoisomerases

When Watson and Crick first described the structure of the DNA double helix in 1953, they recognised that separation of the two DNA strands could result in topological problems due to the helical nature of the molecule (Watson and Crick, 1953a, Watson and Crick, 1953b). Physiological B-form DNA is ordered as an antiparallel helix with each strand intertwined every 10.4 base pairs (Wang, 1979). During S-phase, the inability of CMG to remove these intertwinings between the two DNA strands as it unwinds them results in overwinding and positive supercoiling ahead of the fork (Figure 1.4A) (Keszthelyi et al., 2016). Due to the length of eukaryotic chromosomes, this problem is particularly acute as topological stress cannot diffuse off of DNA ends. In addition, the presence of stable protein-DNA complexes, nucleosomes, and other physical barriers can prevent diffusion of this torsion off of DNA ends, effectively trapping topological stress. If left unresolved, this build-up of helical stress can impede on progression of the replication machinery and is therefore a potential cause of significant replication stress (Keszthelyi et al., 2016).

Across all domains of life, cells have overcome this problem by evolving highly specialised enzymes known as topoisomerases. These enzymes are able to make transient breaks in the DNA phosphate backbone to mediate topological changes in the duplex (Wang, 2002). Type I topoisomerases generate single stranded breaks in the DNA whereas type II enzymes introduce breaks into both strands of the duplex, before passing another intact DNA molecule through the break (Champoux, 2001, Vos et al., 2011). In eukaryotes, type IB (ScTop1) and IIA (ScTop2) are utilised during DNA replication to relax overwinding ahead of replication forks (Figure 1.4A).

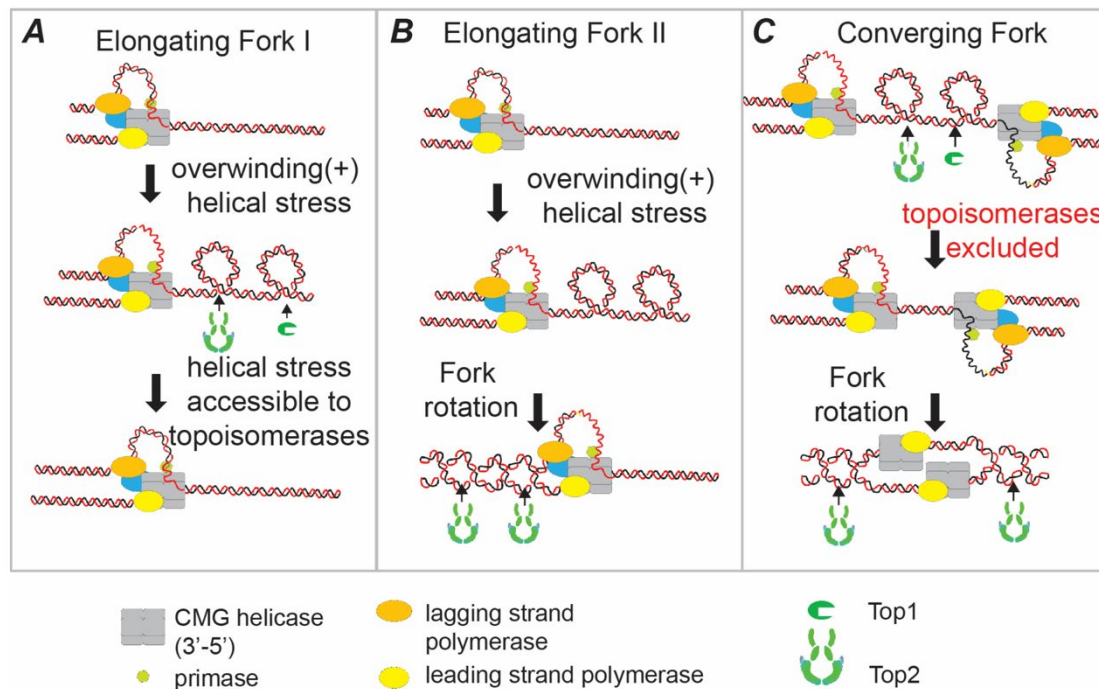


Figure 1.4: How the replisome resolves topological stress during DNA replication (taken from Keszthelyi et al., 2016).

A) During DNA replication, unwinding of the duplex by the CMG helicase results in overwinding of the template DNA ahead of the fork. Such helical stress is normally resolved by type IB and type IIA topoisomerases which generate transient nicks or double-stranded breaks in the DNA duplex, respectively. This results in relaxation of the DNA and permits further CMG progression.

B) As a backup pathway to resolve torsional stress the replisome can rotate on its axis (fork rotation) to diffuse overwinding ahead of the fork to behind the fork, where it manifests as intertwinings in the newly replicated sister chromatids. These intertwinings, or pre-catenanes, must be resolved by Top2 prior to chromosome segregation.

C) The convergence of two replisomes at the termination of DNA replication presents a topological challenge to genome duplication. A short stretch (100-150bp) of DNA between the converging replisomes, too small to be accessed by topoisomerases, can only be relaxed through rotation of the two replication forks. Top2 then must resolve pre-catenanes before mitotic chromosome segregation.

In specific contexts topoisomerase action is unable to be directed ahead of progressing replication forks. In such instances, topoisomerases may be spatially excluded from the short stretch of DNA between the elongating replisome and the impediment to progression, resulting in a local increase of torsional stress not accessible to topoisomerase action. In these cases, cells utilise a backup pathway known as fork rotation to resolve topological stress, where replication forks can rotate on their axis to assist with resolving overwinding ahead of the fork (Figure 1.4B). This is at the expense of generating intertwinings between the newly replicated sister chromatids behind the fork, known as pre-catenanes (Keszthelyi et al., 2016). Fork rotation is known to be particularly important during replication termination, when two replisomes converge upon each other (Figure 1.4C). Early studies using simian virus 40 (SV40) demonstrated that replication termination requires fork rotation to allow replication of the last 100-150 base pairs (Sundin and Varshavsky, 1980, Sundin and Varshavsky, 1981). Other known examples of contexts that require fork rotation to relax topological stress include at protein-DNA complexes such as sites of Cohesin loading and at replication-transcription collisions (Minchell et al., 2020).

Fork rotation presents an additional topological problem associated with DNA replication—the generation of catenated DNA molecules behind the fork (Figure 1.4B). Prior to mitosis, all catenanes between the sister chromatids must be resolved, an activity that can only be performed by type II topoisomerases (Holm et al., 1985). This function in resolving intertwinings is crucial for the completion of DNA replication and for proper segregation of the sister chromatids to daughter cells at anaphase, and failure to do so results in increased chromosome breakage during segregation (Baxter and Diffley, 2008).

1.2.1.2. Stable protein-DNA barriers to replication

DNA binding proteins are crucial for many cellular processes including replication, transcription and chromosomal organisation, amongst others. However, the presence of protein complexes on the DNA represents a physical impediment to the progression of replication forks, as they may be too large or too stably bound to allow passage of the CMG helicase. As a consequence, protein-DNA barriers are therefore a potential cause of replication stress and fork stalling, which can result in toxic genomic rearrangements (Carr and Lambert, 2013, Mirkin and Mirkin, 2007).

Many proteinaceous structures bound to the DNA duplex are known to pose a challenge to CMG progression (Ivessa et al., 2003). Some of these include proteinaceous genomic loci such as centromeres and telomeres, as well as pre-RCs that have not been converted into activated replisomes. At these sites the 5' to 3' helicase Rrm3 is thought to assist the replisome in removing such roadblocks. Rrm3 was first recognised for its role in preventing recombination at the rDNA (Keil and McWilliams, 1993) but has since been implicated in assisting the replisome to bypass a vast range of protein blocks including the telomeres, centromeres and dormant origins (Ivessa et al., 2002, Ivessa et al., 2003). The importance of this pathway in aiding the replisome to overcome proteinaceous barriers to replication is highlighted by the fact that *rrm3Δ* mutants frequently experience increased replication fork breakage and checkpoint activation (Ivessa et al., 2003).

1.2.1.3. Transcription-replication collisions

Cells are continuously synthesising new proteins, a process that initially requires transcription of the DNA to produce a messenger RNA (mRNA). Transcription can impede on replisome progression in multiple ways, including physical collisions between the replication and transcription machineries, the presence of RNA:DNA hybrids on the template DNA, and the topological stress generated by transcription. The many causes and consequences of transcription-replication conflicts have been reviewed extensively (Helmrich et al., 2013). This section focuses on the impact of direct collisions between the two processes and how these are avoided.

The physical collision of the replication and transcription machinery appears to have negative consequences for genome instability. In particular, the organisation of several prokaryotic genomes suggests that head-on collisions between the two processes is particularly problematic. For example, 75% of the predicted genes in *Bacillus subtilis* are arranged in a manner that ensures they will be transcribed in the same orientation as replication (Kunst et al., 1997). Reversing this bias results in replication slowing, increased recombination events, and genome instability (Wang et al., 2007, Srivatsan et al., 2010).

In both prokaryotes and eukaryotes, DNA replication and transcription are physically separated in certain genomic contexts, at sites of programmed replication fork pausing. Early studies in *Escherichia coli* led to the discovery of genomic 'Ter' sequences which were required for the polar arrest of each replication fork originating from the single origin OriC (Hill et al., 1987, Massy et al., 1987). It was later discovered that these sequences are bound by 'Ter' protein, which is required for the polar replication fork arrest to ensure that head-on collisions between replication and transcription machineries are minimised (Hill et al., 1989). In *S. cerevisiae*, the most well characterised of these programmed replication pause sites is the "replication fork barrier"/RFB located within the NTS1 region of the rDNA on chromosome XII (Figure 1.5) (Brewer and Fangman, 1988). This RFB site is bound by the Fork Blocking Less 1 protein (Fob1), which serves to pause replication forks in a unidirectional manner, ensuring that replication and transcription of the highly transcribed rDNA occurs in the same direction (Kobayashi and Horiuchi, 1996, Kobayashi, 2003, Ward et al., 2000). Similar RFB elements have been identified in the rDNA of higher eukaryotes, including

in *X. laevis* (Maric et al., 1999) mice (López-Estraño et al., 1998), and humans (Pütter and Grummt, 2002), suggesting that programmed fork pausing is a conserved mechanism to minimise collisions between replication and transcription.

Although they may be minimised in certain contexts, in general collisions between the transcription and replication machinery are inevitable. In mammalian cells, the longest of genes can take hours to transcribe- the dystrophin gene for example can take up to 16 hours (Tennyson et al., 1995). Inevitably, then, collisions between the replication and transcription are impossible to avoid completely. Such genomic loci have been described as common fragile sites due to their difficulty to replicate and their susceptibility to breakage and recombination, suggesting that active transcription is a hindrance to faithful DNA duplication (Helmrich et al., 2011).

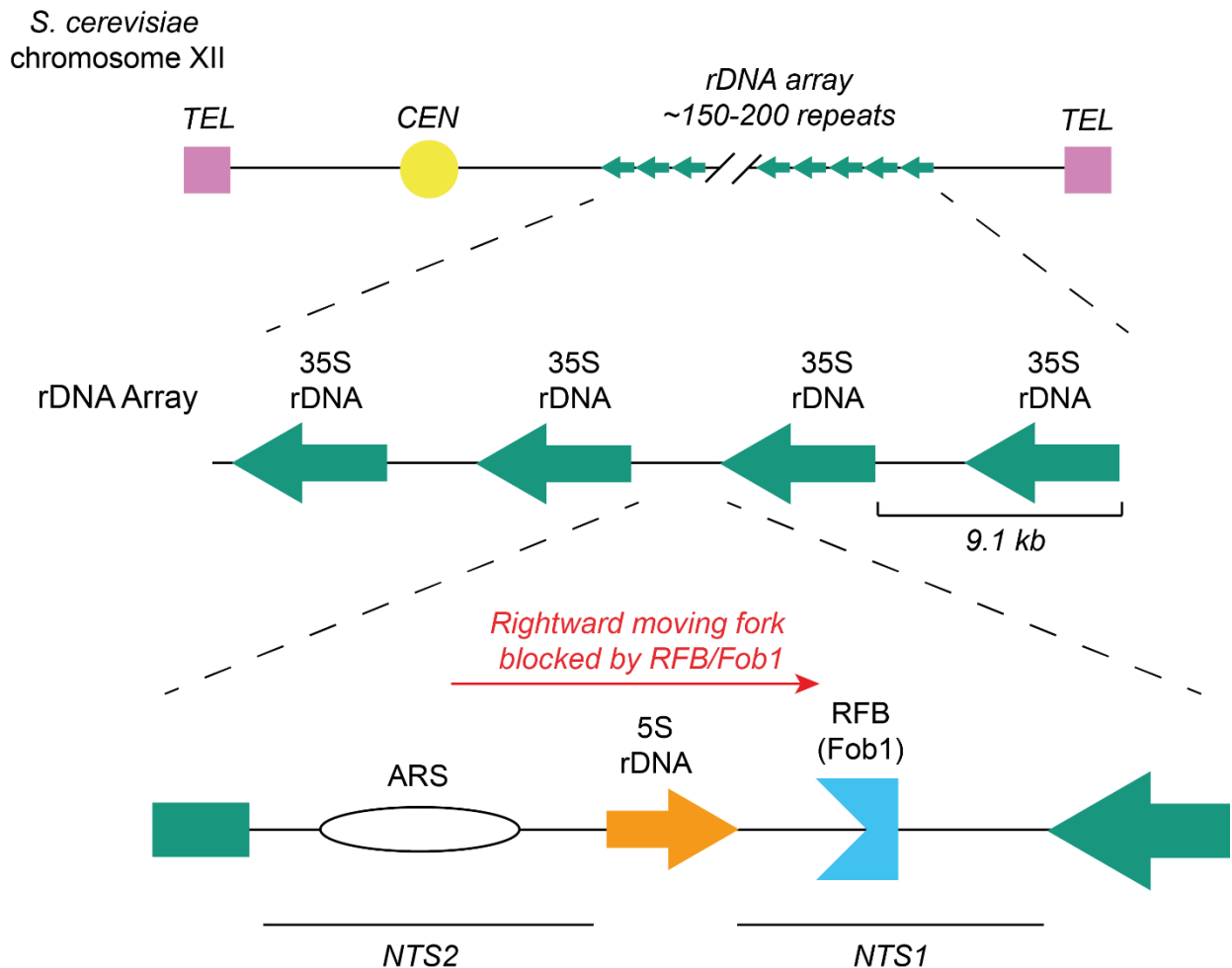


Figure 1.5: Schematic diagram of the rDNA locus on chromosome XII in *S. cerevisiae*

The rDNA is arranged as an array of repetitive sequences (estimated 150-200 repeats) located on chromosome XII in budding yeast. These repeats consist of 35S (green) and 5S (orange) rDNA genes that are transcribed in opposite directions. Between the 35S and 5S rDNA genes is one origin of replication (ARS) which, when activated, gives rise to two replication forks travelling in opposite directions. As depicted the leftward moving fork will travel in the same direction as transcription of the 35S rDNA and the rightward moving fork in the same direction as the 5S rDNA. The rightward moving fork is blocked in a polar fashion at the RFB sequence, which is tightly bound by Fob1 protein (blue). This block ensures that rightward moving forks do not enter the 35S rDNA as this could lead to head-on collisions between the replication and transcription machinery, a potential cause of replication stress.

1.2.1.4. DNA-protein crosslinks (DPCs)

As well as stably binding the DNA, proteins can become chemically crosslinked to the duplex, forming lesions known as DPCs. These differ from the DNA-binding proteins described earlier in that they are covalently linked to the DNA and are therefore trapped on the duplex. In contrast to proteins that bind to the DNA physiologically, the crosslinking of proteins to the DNA does not serve a physiological purpose, is highly toxic to cells and requires specialised repair pathways to remove the lesion (Stingele et al., 2017). In theory, any protein can become crosslinked to the DNA, as reactive aldehydes produced during normal cellular metabolism have the capability to generate DPCs in a non-specific manner (Barker et al., 2005). In addition, several widely used chemotherapeutic agents work by non-specifically crosslinking proteins to the DNA, including cisplatin (Chválová et al., 2007). Such DPCs are commonly referred to as 'non-enzymatic' DPCs, to distinguish them from 'enzymatic' DPCs, which are instead formed when an enzyme that forms a covalent intermediate with DNA as part of its normal enzymatic cycle becomes trapped (Barker et al., 2005).

A frequent and toxic source of enzymatic DPCs within cells arises from trapped topoisomerase enzymes on the DNA duplex. These lesions occur when the re-ligation step carried out by topoisomerase enzymes does not occur, leaving the enzyme covalently linked to the broken DNA end(s) and forming lesions known as topoisomerase "covalent complexes" (Topo-cc's) (Sun et al., 2020). Interestingly, cells lacking enzymes required for removal of trapped topoisomerases are extremely sick even in the absence of topoisomerase poisons that promote the formation of Topo-cc's, suggesting that these lesions occur relatively frequently in the genome (Stingele et al., 2014). Topo-cc's can arise endogenously, for example near sites of DNA damage that distort the DNA duplex and interfere with the re-aligning of broken DNA ends, or can be induced by small molecules (Pommier et al., 2016). The presence of Topo-cc's creates several problems for the cell: the bulky enzyme acts as an extremely stable physical barrier to replisome progression, and in addition the prevention of re-ligation results in DNA damage in the form of either a single (in the case of Top1) or a double-stranded (Top2) break in the DNA duplex (Pommier et al., 2016).

The impact of DPCs on the replisome has been demonstrated *in vitro* where it was shown using 2D gel electrophoresis that cisplatin-stabilised protein adducts induce replisome

stalling (Chválová et al., 2007). *In vivo* data paints a similar picture. In both prokaryotes and eukaryotes, it has been shown that DPCs can stall CMG progression when on the leading strand or polymerase extension when on the lagging strand (Figure 1.6) (Fu et al., 2011, Nakano et al., 2013). Furthermore, work in *X. laevis* egg extracts suggests that DPC repair is coupled to replication, demonstrating the need for effective DPC removal during DNA replication (Duxin et al., 2014).

As a consequence, cells have evolved highly specialised repair pathways to resolve DPCs (Figure 1.6) (reviewed in Stingeles et al., 2017). As the protein component of DPCs is the major hindrance to replication and repair, this is primarily targeted by the protease Wss1 (SPRTN in higher eukaryotes (Stingeles et al., 2016)). Wss1 cleaves the crosslinked protein down to a smaller adduct which can then be bypassed during replication, most likely by translesion synthesis (TLS) polymerases (Stingeles et al., 2014). In addition to proteolytic processing of DPCs, nuclease activity is also important for repair of these lesions: it has been demonstrated that the endonuclease activity of Mre11 (a component of the MRX/MRN complex) is crucial for nuclease-dependent processing of DPCs (Hoa et al., 2016). This then generates a 'clean' double-stranded break (DSB) that can be repaired via the canonical DSB repair pathways. Lastly, in the case of Top1- and Top2-cc's, specific enzymes known as tyrosyl-DNA phosphodiesterases (TDPs) can directly cleave the phosphotyrosyl bond that is generated between trapped Top1 and Top2 enzymes and DNA (Pommier et al., 2014).

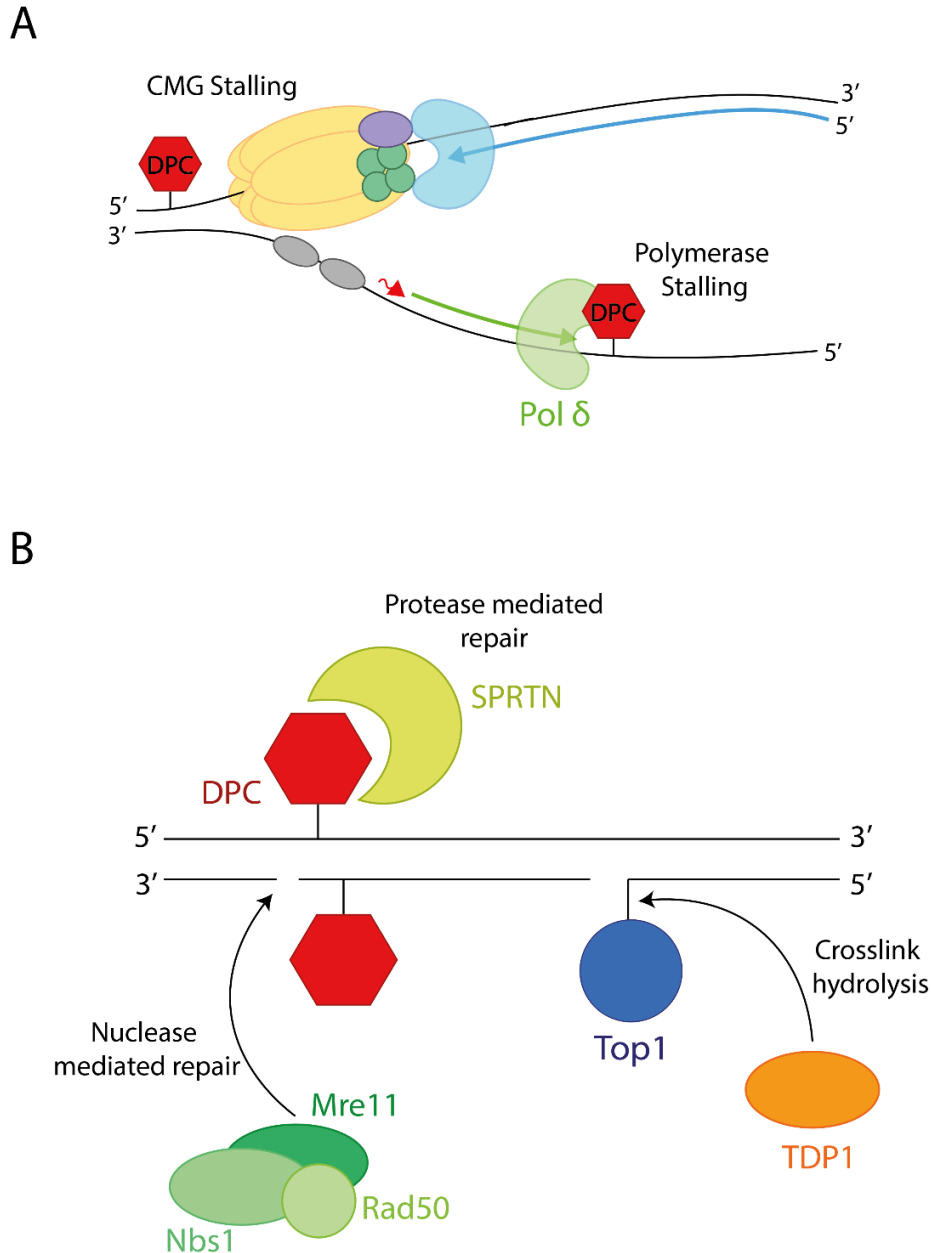


Figure 1.6: Schematic representation of replisome stalling by DPCs and DPC repair

A) DPCs represent physical barriers to replisome progression. As the protein component of DPCs is too large to fit through the central channel of CMG, DPCs on the leading strand will stall CMG unwinding at the replication fork. On the other hand, lagging-strand lesions may block polymerase extension as the active site of the replicative polymerases cannot accommodate bulky lesions.

B) DPCs can be repaired by various mechanisms. Cells have dedicated DPC repair pathways such as proteolytic degradation of the crosslinked protein by SPRTN (ScWss1). Trapped Top1 and Top2 enzymes can be removed by cleavage of the phosphotyrosine link between the active site tyrosyl and the DNA phosphate backbone; these reactions are catalysed by TDP1 (for Top1-ccs) and TDP2 (for Top2-cc's). Lastly, nucleases can repair DPC lesions by cleavage of the surrounding DNA and removal of the crosslinked DNA bases. This then leaves a 'clean' DSB which can be repaired by the canonical DSB repair pathways.

1.2.1.5. Misregulation of dNTP Pools

In addition to the physical hindrances to replisome progression described above, replication forks are highly sensitive to changes in deoxyribonucleoside triphosphate (dNTP) pools, which serve as the building blocks for nascent strand synthesis. dNTP production requires the activity of specialised enzymes known as ribonucleotide reductases (RNRs), which catalyse the reduction of ribonucleosides to deoxyribonucleosides by replacing the 2'-OH of ribonucleotide di- or triphosphates with a hydrogen atom (Figure 1.7) (Nordlund and Reichard, 2006). Normally, RNR activity is tightly regulated within cells to ensure an appropriate balance of all four dNTPs, and has been shown to be upregulated before and throughout S-phase to maintain sufficient dNTP pools in both *S. cerevisiae* and mammals (Chabes et al., 2003, Kumar et al., 2010). Insufficient amounts of cellular dNTPs during DNA replication can lead to fork stalling, as the replicative polymerases cannot catalyse synthesis of nascent DNA strands without these precursor molecules. Alternatively, constitutively high levels of dNTPs may also have toxic consequences for the cell and can be a source of mutagenesis (Kumar et al., 2010).

Studies of nucleotide limitation *in vivo* have been largely conducted with the use of the drug hydroxyurea (HU), a potent inhibitor of RNRs (Figure 1.7). Treatment with HU can lead to replication fork slowing (Alvino et al., 2007, Poli et al., 2012) and/or complete stalling (Bianchi et al., 1986, Petermann et al., 2010). Timely and robust activation of the S-phase checkpoint is vital for cellular viability in response to HU-induced fork slowing and stalling (Singh and Xu, 2016, Ciccia and Elledge, 2010).

In addition to this direct impact on dNTP synthesis, HU-mediated disruption of RNR activity has recently been shown to modulate S-phase dynamics and genome stability through the generation of increased cellular levels of reactive oxygen species (ROS) (Somyajit et al., 2017). Somyajit and colleagues postulated that HU-mediated disruption of electron transfer between RNR subunits could lead to the formation of elevated ROS within cells. Indeed, treatment with a low dose of HU was shown to result in elevated cellular levels of ROS without inhibiting bulk dNTP levels (Somyajit et al., 2017). Interestingly, the same study found that the FPC factor Timeless dissociates from the replisome in response to increased ROS levels, suggesting that such metabolic

imbalances are sensed by the FPC to alter replisome dynamics and maintain genome stability (discussed in section 1.3.2.4).

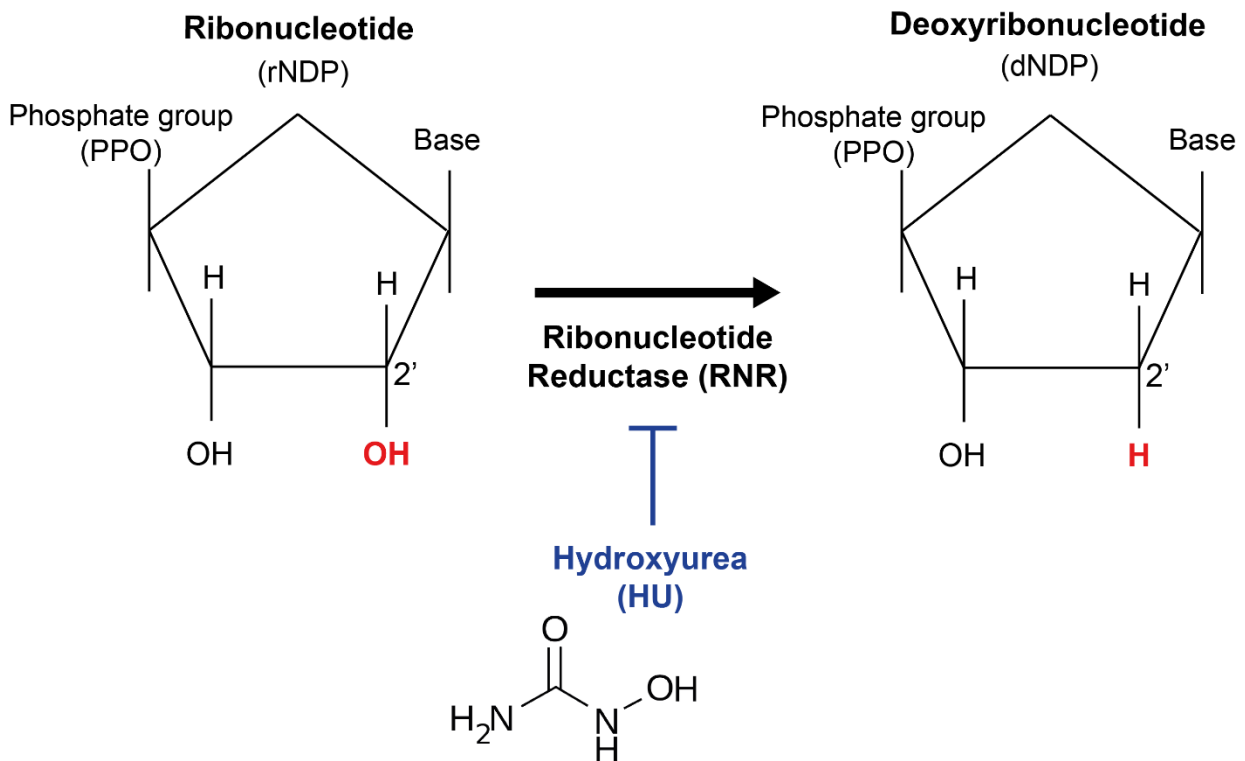


Figure 1.7: Ribonucleotide reductase (RNR) mechanism of action and inhibition

RNR catalyses the reduction of ribonucleotides (specifically ribonucleotide diphosphates/rNDPs) into deoxyribonucleotide diphosphates (dNDPs) by removal of the 2'-OH group. dNDPs are subsequently converted to deoxyribonucleotide triphosphates (dNTPs), the precursor molecule for DNA synthesis (not shown). Hydroxyurea (HU) is a potent inhibitor of RNR and consequently leads to low dNTP cellular pools.

1.2.2. The DNA Replication Checkpoint (DRC)

In response to replication stress and damaged DNA templates, cells can delay cell cycle progression by activating the DRC, a cascade of DNA damage-dependent signalling events primarily occurring through protein phosphorylation. This allows the initial detection of stress or damage to be transmitted through to effector kinase proteins which phosphorylate a number of targets including those responsible for delaying further replication origin firing and cell cycle progression, and upregulating repair genes (Paulsen and Cimprich, 2007). The replication fork is highly sensitive to DNA damage, since it is likely to be the first structure to encounter many lesions during S-phase. In *S. cerevisiae*, temperature-sensitive *cdc6* and *cdc45* mutants that do not initiate replication will still enter mitosis even when treated with low doses of MMS, suggesting that certain DNA lesions require a replication fork to sense them (Tercero et al., 2003). Similarly, in human cells treated with UV, replication is required for DRC activation (Ward et al., 2004). Over the years, a vast amount of work has uncovered the mechanisms by which the DRC is activated and how it functions to safeguard genome stability. These findings are summarised below.

1.2.2.1. ATR Activation

In eukaryotes, the central component in the DRC is the Ataxia Telangiectasia and Rad3-Related (ATR) kinase (Mec1 in *S. cerevisiae*), which is activated in response to a wide range of replication stressing agents including dNTP depletion, DNA cross-linkers and topoisomerase poisons amongst others (Saldivar et al., 2017). Rather than being activated by the different lesions themselves, ATR-type kinases are activated by a common substrate that accumulates at stalled forks, ssDNA. Specifically, RPA-coated ssDNA acts as the substrate for ATR recruitment and subsequent activation, which is thought to accumulate at forks when the replicative polymerases and helicase become uncoupled from one another by a DNA lesion (Longhese et al., 1996, Garvik et al., 1995, Kim and Brill, 2001, Byun et al., 2005). Alternatively, RPA-ssDNA can be generated when lesions are processed by repair pathways (Paulsen and Cimprich, 2007). The recruitment of ATR to RPA-ssDNA is mediated by its association with ATR-Interacting Protein (ATRIP) (Sc Ddc2) (Figure 1.8) (Cortez et al., 2001, Costanzo et al., 2003, Zou and Elledge, 2003, Paciotti et al., 2000). Once recruited to RPA-ssDNA, additional factors are required to fully activate ATR. These include the 9-1-1 complex, consisting of Rad9, Hus1

and Rad1 (*Sc* Ddc1, Mec3, Rad17) which is loaded by the Rad17-RFC complex (*Sc* RFC-Rad24), as well as TopBP1 (*Sc* Dpb11) and Dna2 (Awasthi et al., 2016, Paulsen and Cimprich, 2007). In addition, the replisome components Claspin and Timeless-Tipin are required for full activation of the DRC (Figure 1.8) (discussed in subsection 1.3.2.1) (Alcasabas et al., 2001, Osborn and Elledge, 2003, Ünsal-Kaçmaz et al., 2007, Yoshizawa-Sugata and Masai, 2007).

Once activated, ATR phosphorylates and activates downstream effector kinases that can mediate cellular changes to enact the full checkpoint response. In mammals, these are the checkpoint kinases Chk1 and Chk2 which diffuse freely in the cell and transmit the checkpoint signal to a range of substrates to halt cell cycle progression and further origin firing (Figure 1.8) (Bartek and Lukas, 2003). Whilst Chk1 is thought to be the primary target for ATR-mediated checkpoint activation, there appears to be cross-talk between the two kinases. In *S. cerevisiae*, Rad53 is the homolog of mammalian Chk2, although it is more functionally related to Chk1 and is the main effector kinase in budding yeast, acting as the effector kinase both for the DRC and the DDC (Pardo et al., 2016).

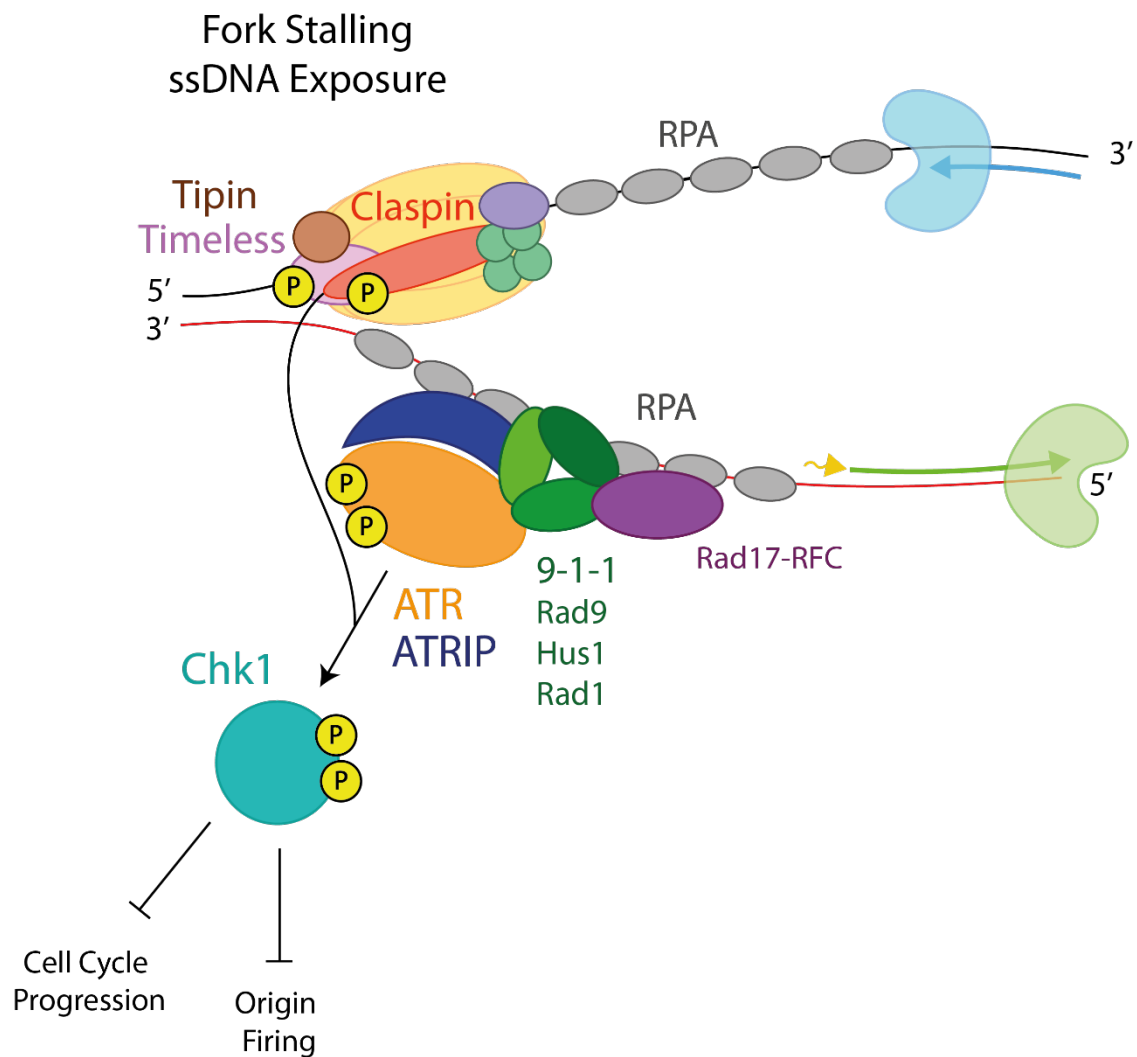


Figure 1.8: Schematic representation of the DNA replication checkpoint

Replication stress generates stretches of ssDNA at replication forks, which are coated with single-stranded DNA binding protein RPA. These long ssDNA-RPA tracks are the signal for ATR activation, as ATR is recruited to and activated at these stretches by ATR interacting protein/ATRIP. ATR requires several other factors for its full activation including the 9-1-1 complex (consisting of Rad9-Hus1-Rad1) and the Rad17-RFC complex, amongst others. Once activated ATR phosphorylates a number of substrates, including itself, components of the replisome and the effector checkpoint kinase Chk1. Full activation of Chk1 requires the checkpoint mediator function of the fork protection complex proteins Tim/Tipin and Claspin. Once fully active, Chk1 is able to phosphorylate numerous substrates and regulate their activity, to halt cell cycle progression and new origin firing under conditions of replication stress.

Activation of the effector kinases by ATR results in the phosphorylation of many downstream substrates, modulating replisome progression and stability in a number of ways. In *S. cerevisiae*, initiation factors Sld3 and Dbf4 are phosphorylated in a Rad53-dependent manner to inhibit origin firing following checkpoint activation (Zegerman and Diffley, 2010, Lopez-Mosqueda et al., 2010). Similar studies in human cells have shown that Treslin, the human homolog of Sld3, is regulated in a similar fashion to prevent Cdc45 loading, a key step in replication initiation (Guo et al., 2014). In both *S. cerevisiae* and in mammalian cells, RNR activity is upregulated in a checkpoint dependent manner, ensuring cells have a plentiful supply of dNTPs available for DNA synthesis (Zhang et al., 2009, Huang et al., 1998).

Full activation of the effector checkpoint kinases requires the checkpoint mediator function of the FPC proteins Tim/Tipin and Claspin (Figure 1.8), which is discussed further in section 1.3.2.1.

1.2.2.2. Replication fork stabilisation

As well as repressing origin firing and upregulating genes required for faithful S-phase progression, there is evidence that replication forks themselves are regulated by the DRC. The separation-of-function *mec1-100* allele cannot repress origin firing but is not hypersensitive to MMS like *mec1Δ* cells (Paciotti et al., 2001). In addition, phosphorylation mutations in the HEAT repeats of human ATR results in cells that can delay the G2/M transition but cannot resume replication following HU-induced arrest, and are inviable as a consequence (Nam et al., 2011). These studies suggest that DRC activation has an essential role in protecting and stabilising replication forks in response to replication stress outside of its role in regulating cell cycle progression. Failure to maintain the stability and eventual restart of stalled replication forks is often described as “fork collapse”, a poorly understood phenomenon thought to result in the generation of DSBs (Cortez, 2015).

Replication fork collapse could result from a number of problems associated with a loss of ATR function. It was initially thought that the DRC may work to maintain the physical association of replisome components at the fork (Lopes et al., 2001), although more genome-wide analyses do not support this argument (De Piccoli et al., 2012). Another

way in which the DRC may protect replication forks from collapse is by regulating the way in which stalled forks are processed into pathogenic structures by enzymes such as nucleases and helicases. For example, the sensitivity of *rad53Δ* cells to genotoxic agents such as UV irradiation can be rescued by deletion of *EXO1*, and it has been shown that Rad53-dependent phosphorylation results in inhibition of this nuclease (Morin et al., 2008, Segurado and Diffley, 2008). In *S. cerevisiae*, regression of stalled replication forks is associated with loss of the DRC and this is considered a pathogenic outcome of fork stalling (Lopes et al., 2006, Sogo et al., 2002, Lopes et al., 2001). In contrast, in higher eukaryotes it appears that reversed forks are more frequent and are actually intermediates of forks undergoing remodelling and repair. However, the failure to regulate fork reversal properly by the DRC is associated with improper processing, increased DNA damage and genome instability (Neelsen and Lopes, 2015, Quinet et al., 2017). There is also evidence that accumulation of unprotected ssDNA as a result of RPA exhaustion following unsuppressed origin firing may result in terminal fork arrest (Toledo 2013). It is likely that the role of the DRC in protecting stalled forks is not limited to one of these activities but instead involves a combination of all of them, and may be dependent both on the genomic context within which a replication fork stalls, and the lesion responsible for stalling the fork.

1.2.2.3. Replication fork restart

After removal of the replisome impediment, if a replication fork has been sufficiently protected from collapsing, it may be restarted by a number of mechanisms. Restart mechanisms will vary depending on which strand the replisome-stalling lesion is encountered. It has long been thought that lagging-strand lesions should be easily bypassed due to the discontinuous nature of Okazaki fragment synthesis on this strand, a hypothesis that has been confirmed with reconstituted replisomes *in vitro* (Taylor and Yeeles, 2018). Replication forks stalled by leading strand lesions, however, require active mechanisms to restart replication.

In bacteria, which do not have an excess of origins able to rescue stalled forks, restart can be mediated by re-assembly of the replisome and priming downstream of the lesion (Heller and Marians, 2006, Yeeles and Marians, 2011). In eukaryotic cells, however, replisomes cannot be re-assembled after initiation due to CDK activity. It is unclear how

often stalled forks are restarted in the absence of genotoxic lesions, as the excess of origins in eukaryotic cells ensures that fork stalling events can likely be rescued by a fork approaching in the opposite direction. However, there is evidence that replication fork restart mechanisms do operate in eukaryotes and become particularly important in response to replication stress. DNA combing and fibre analysis experiments have identified many key players involved in replication fork restart mechanisms *in vivo*. For example, in mammalian cells, the primase-polymerase PrimPol is able to re-prime on the leading strand downstream of polymerase-blocking lesions to restart DNA replication (Bianchi et al., 2013, Mourón et al., 2013). Whilst the yeasts lack PrimPol, reconstitution of the replisome using budding yeast proteins has recently revealed that Pol α -dependent re-priming downstream of leading strand lesions takes place in yeast after CMG progression is uncoupled from DNA synthesis, although RPA accumulation negatively regulates this process (Taylor and Yeeles, 2018).

In addition to directly restarting stalled replication forks, cells utilise different mechanisms to bypass lesions and allow stalled forks to progress. Translesion synthesis (TLS) and template switching are regulated by ubiquitination of the replisome component PCNA, with monoubiquitination mediated by Rad18 and Rad6 favouring recruitment of TLS polymerases, and polyubiquitination mediated by Rad5 and Mms-Ubc13 directing restart towards template switching (Hoege et al., 2002, Parker and Ulrich, 2009, Lee and Myung, 2008). TLS polymerases have a larger active site than the replicative polymerases, allowing them to replicate over damaged bases and thus restart replication forks stalled by a range of lesions (Zhao and Washington, 2017). However, as a consequence, they are generally error-prone and can introduce mutations into the DNA. In contrast, template switching utilises the undamaged sister chromatid as a template to restart stalled DNA synthesis and is thus thought to be a less mutagenic process. The mechanisms governing which restart pathway is chosen are yet to be understood, but it is likely to involve factors such as the type of lesion faced by the replisome and the genomic context within which the replication fork is stalled.

1.3. The Timeless protein family and their diverse functions

The primary focus of this thesis is an in-depth analysis of the Tof1/Timeless protein in both *S. cerevisiae* and human cells. Timeless family proteins are structurally conserved across eukaryotic organisms and have a wide range of diverse roles. The first of these family members to be functionally characterised was *Schizosaccharomyces pombe* Swi1 in 1984 where *swi1* mutants defective in mating type switching were isolated (Egel et al., 1984). However, the mapping of the *TIMELESS* gene in *Drosophila melanogaster* 10 years later and the discovery that it plays a crucial role in circadian rhythm regulation in the fruit fly gave rise to the family name (Sehgal et al., 1994).

Since these initial studies, Timeless orthologues have been identified in other eukaryotes including *S. cerevisiae*, *S. pombe*, and humans. A BLAST search of the *D. melanogaster* N-terminal Timeless domain (Tim-N) demonstrates that this region is conserved in all of these orthologues. Interestingly, however, the role of Tim proteins in other organisms appears to be different to that in *D. melanogaster*. Instead, in these organisms Timeless exists as part of the fork protection complex (FPC). The FPC associates and travels with replication forks to perform numerous functions in preserving replication fork stability and genome integrity during S-phase (Leman and Noguchi, 2014).

This section aims to review the many known functions of Timeless proteins in eukaryotic organisms. I focus on their S-phase roles in fork stabilisation and maintaining genome integrity in yeast and higher eukaryotes. In addition, this section discusses the recently published structures of Tim proteins in yeast and humans.

1.3.1. Structure of Timeless proteins

Until recently, very little was known about the structures and domain architectures of proteins in the Timeless family. Timeless proteins in all organisms share a conserved N-terminal 'Timeless' domain, which defines the Timeless family (Mazzocchi et al., 2016). However, the human Tim orthologue is more structurally and functionally related to a second Timeless protein found in *D. melanogaster*, known as Tim2 or Timeout (Benna et al., 2000). Phylogenetic analyses have led to the speculation that *D. melanogaster* Tim1 arose from a duplication of Tim2, and adopted a circadian-specific function in the fly (Mazzocchi et al., 2016).

To date, there are three structures for Timeless orthologues deposited in the protein data bank (PDB). The first available structure of a Timeless family protein was the crystal structure of an N-terminal fragment of human Timeless, spanning amino acids 1-463 (Holzer et al., 2017). This structure revealed a horseshoe-like conformation for the Tim N-terminus, and suggested a protein-protein interaction domain due to the presence of a concave groove with structural similarity to other ligand-binding proteins such as HspBP1 (Holzer et al., 2017). Potentially, this region acts as a docking site for specific proteins or other molecules, although this is yet to be determined in human cells.

Two recent structures of Tof1/Csm3 show that the N-terminal half of Tof1 is comprised of repeated alpha helices which form a crescent/horseshoe like shape, and is highly structurally similar to the N-terminal Tim structure previously determined (Baretić et al., 2020, Grabarczyk, 2020). Csm3 is also comprised of alpha-helices, forming a five-helix bundle that binds to the C-terminal half of Tof1 (Baretić et al., 2020, Grabarczyk, 2020). Csm3 recruitment to the replisome appears to be almost entirely mediated by its interaction with Tof1 (Baretić et al., 2020).

The recent cryo-EM structure of Tof1/Csm3, Mrc1 and Ctf4 in complex with CMG reveals that in budding yeast, Tof1/Csm3 are maintained ahead of the replisome on the N tier face of MCM, and make contact with duplex DNA head of CMG (Baretić et al., 2020). Interestingly, in the same study very little density could be assigned to Mrc1 in the structure, although cross-linking mass spectrometry experiments found contacts between the N-terminus of Mrc1 to Tof1, and the C-terminus to Cdc45, indicating that Mrc1 may

span the entire CMG to make contacts with multiple replisome components (Baretić et al., 2020). These findings have important implications for the roles of these proteins in their known functions at the replisome, as their positions in this molecular machine explains why they are capable of stabilising replisomes in response to virtually all types of replication stress. Further characterisation of the DNA-binding activity of Tof1/Csm3 and its role in replisome pausing and stabilisation will provide interesting insights into the molecular details of Tof1/Csm3 function at the replication fork.

1.3.2. S-phase functions of Timeless proteins

DNA replication is a potential source of major genome instability in cells, as the replisome frequently encounters numerous different types of replication stress that must be continually resolved (reviewed in section 1.2). However, cells accomplish this task without major problems every cell cycle, due to the presence of specific factors that have evolved to preserve genome stability during DNA replication.

Specifically, the ‘fork protection complex’ (FPC) is a conserved complex of proteins that perform a wide range of roles in replisome stability at the replication fork. The FPC was originally defined as consisting of Tim (ScTof1/SpSwi1) and Tipin (ScCsm3/SpSwi3) (Noguchi et al., 2004), but these proteins interact both physically and often functionally with a third protein, Claspin (ScMrc1/SpMrc1). The physical association of FPC proteins has been well established; the interaction between Tim and Tipin appears to be crucial for the stable expression of each protein, suggesting that these proteins exist as a constitutive heterodimer (Gotter, 2003, Noguchi et al., 2004, Chou and Elledge, 2006). The interaction between Tof1/Csm3 and Mrc1 has also been demonstrated using biochemical analyses (Bando et al., 2009). In budding yeast, Tof1/Csm3 and Mrc1 have been shown to physically associate and travel with the replisome (Katou et al., 2003, Gambus et al., 2006). Such close association with the core replication machinery places the FPC proteins in the ideal location to assist with both normal replisome progression and to protect stalled forks. Given this, it is unsurprising that FPC proteins have important numerous roles during genome duplication. These roles are summarised below.

1.3.2.1. Mediating the DNA Replication Checkpoint (DRC)

In response to treatment with genotoxic agents, cells rapidly activate a cascade of signalling events commonly referred to as “checkpoint” responses, to halt progression through the cell cycle until the checkpoint-activating event(s) have been resolved (see section 1.2.2). FPC proteins have been shown to play a key role in mediation of the DRC from the initial ATR-mediated activation to activation of effector kinases that then limit S-phase progression and protect stalled forks until they can be safely restarted. This function was initially characterised in the yeasts: Tof1 was initially identified as a mediator of the S-phase checkpoint in a screen designed to find mutants that were more reliant on the DRC when missing the Rad9-dependent DNA damage checkpoint (DDC). In this screen, deletion of Tof1 in a Rad9-deficient background led to hypersensitivity of cells to the alkylating agent MMS, leading to the conclusion that Tof1 acts to preserve cell viability in response to genotoxic agents specifically in the S-phase branch of the checkpoint (Foss, 2001). In addition, cells lacking both Tof1 and Rad9 were unable to elicit phosphorylation and activation of the effector kinase Rad53 in response to HU, despite both single mutants showing a normal level of activation under the same conditions (Figure 1.9) (Foss, 2001). Soon after, it was found that *S. pombe* cells lacking Swi1 or Swi3 and the ScRad9 orthologue Chk1 were more sensitive to UV irradiation and HU than either single mutant, and were unable to phosphorylate Cds1, the effector checkpoint kinase in fission yeast (Noguchi et al., 2003, Noguchi et al., 2004).

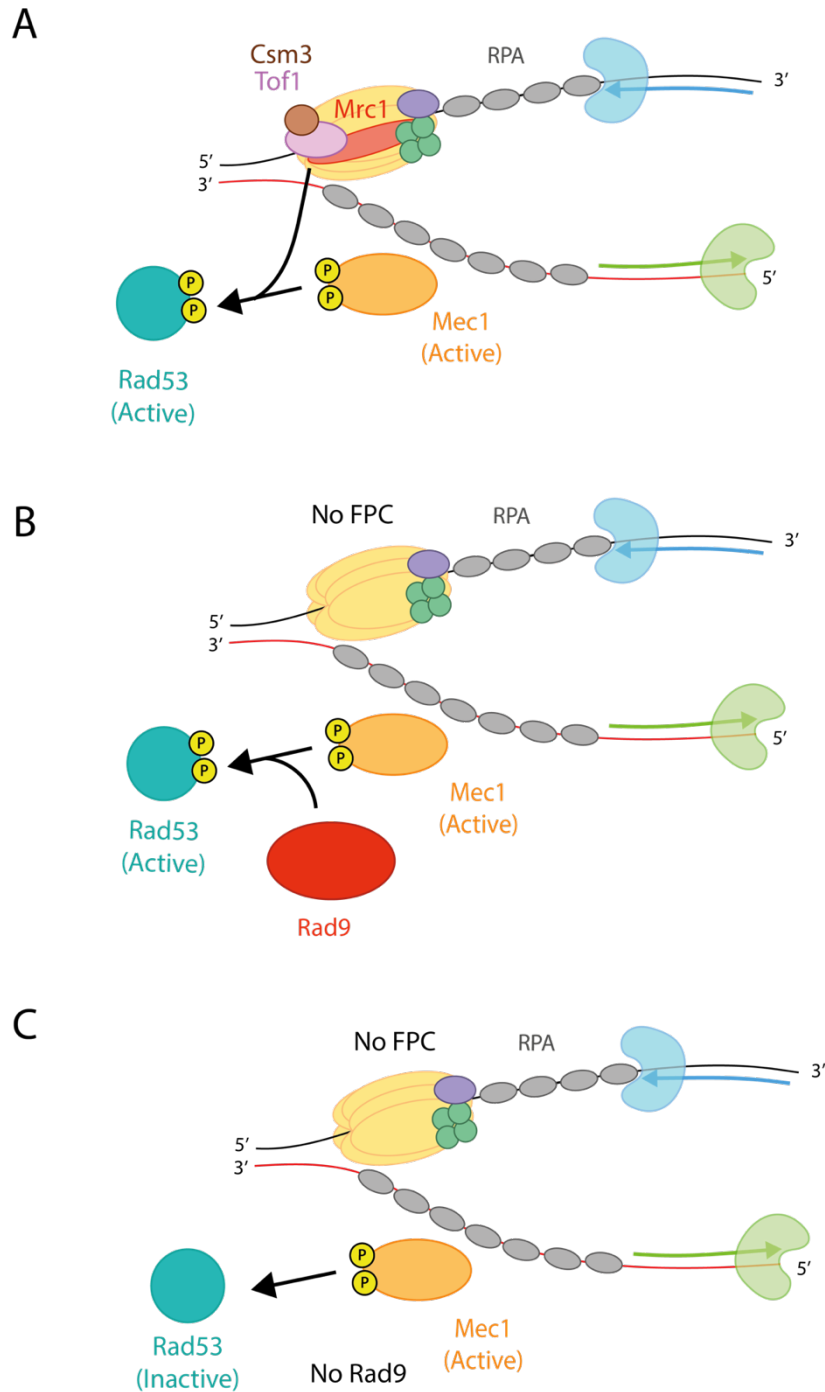


Figure 1.9: Schematic of DRC and DDC-dependent mechanisms of Rad53 (hChk1) activation in *S. cerevisiae*

A) Replication stress events result in the accumulation of RPA-coated ssDNA, the trigger for Mec1 (hATR) activation. Mrc1 and Tof1 act as mediators of Mec1 activation to result in activation of the checkpoint effector kinase Rad53.

B) In the absence of Tof1 and Mrc1, the DDC protein Rad9 is able to mediate activation of Rad53 to lead to robust checkpoint activation.

C) In the absence of *tof1* Δ and *rad9* Δ , Rad53 is not activated leading to a loss of checkpoint function (Foss, 2001).

The role of FPC proteins in DRC mediation is conserved in metazoa. Shortly after the discovery that the yeast Tim/Tipin orthologues acted as checkpoint mediators, a physical interaction between Tim and Chk1 was found in human cells, and it was shown that Tim depletion led to decreased Chk1 phosphorylation in response to HU treatment and decreased fork slowing after UV irradiation (Ünsal-Kaçmaz et al., 2005, Ünsal-Kaçmaz et al., 2007). Similar reductions of Chk1 phosphorylation following treatment with replication stressing agents have been visualised following Tipin depletion in human and *X. laevis* model systems (Yoshizawa-Sugata and Masai, 2007, Errico et al., 2007). In addition, depletion of Tim or Tipin in human cells leads to spontaneous γ H2AX formation, consistent with increased endogenous DNA damage occurring due to impaired checkpoint signalling (Chou and Elledge, 2006).

These findings lead to the question of how exactly Tim/Tipin act as checkpoint mediators. As earlier discussed, Tim/Tipin physically interact with a third protein, Claspin, which has a long-established and conserved role in DRC activation (Smits et al., 2019). Claspin was initially discovered in *X. laevis* egg extracts where it was found to interact with and activate the checkpoint mediator Chk1 in response to synthetic oligonucleotides containing double-stranded DNA ends (and thus mimicking DSBs) as well as the DNA polymerase inhibitor aphidicolin (Kumagai and Dunphy, 2000). Later orthologues were identified in budding and fission yeasts (ScMrc1/SpMrc1) (Alcasabas et al., 2001, Tanaka and Russell, 2001), as well as in humans (hClaspin) (Chini and Chen, 2003), where the role of these proteins in mediating checkpoint activation appears to be conserved across species. In budding yeast, *mrc1 Δ* mutants are more sensitive to replication stressing agent HU than *tof1 Δ* or *csm3 Δ* mutants (Redon et al., 2006). Potentially, the roles of Tim/Tipin in DRC activation are to support Claspin's key function of Chk1 activation at stalled replication forks. Consistent with this, siRNA-mediated depletion of Tim or Tipin in human cells leads to a partial reduction in the nuclear localisation of Claspin (Yoshizawa-Sugata and Masai, 2007). In addition, Tipin has been shown to interact with RPA via an N-terminal region (Ünsal-Kaçmaz et al., 2007, Gotter et al., 2007). Specifically, cryo-EM analyses have found that Tim/Tipin form a complex of 1:1:1 stoichiometry with RPA and that in this complex RPA adopts a more compact conformation, normally associated with DNA-binding activity at longer (≥ 30 nt) stretches of DNA (Witosch et al., 2014). This interaction has been shown to stabilise both Tipin and Claspin on RPA-coated ssDNA and a Tipin mutant that cannot bind RPA (Tipin-L195A) is unable to mediate Chk1 phosphorylation in response to replication stress (Kemp et al., 2010). Potentially,

Tim/Tipin may be required to promote Claspin function at RPA-coated ssDNA generated at stalled forks, enabling it to carry out its role in mediating DRC activation. However, it is still not clear whether Tim/Tipin may only function to support Claspin-mediated DRC activities at the fork, or whether they may have a Claspin-independent role in DRC activation.

1.3.2.2. Normal replisome progression

Even in the absence of DNA damaging agents or replication stress, FPC proteins appear to have a role in normal S-phase progression. In budding yeast, *tof1Δ* cells complete DNA replication with a 15 minute delay compared to wild-type cells (Tourrière et al., 2005). Cells containing a checkpoint defective Mrc1 mutants, *mrc1^{AQ}* cells do not show impaired fork speeds (Alcasabas et al., 2001), indicating that the role of Tim/Tipin in fork progression is separate from its function in DRC activation. Similarly, in human cells Tim and Tipin depletion using siRNA results in a reduction in fork speed as visualised by DNA combing, in the absence of any DNA damage (Ünsal-Kaçmaz et al., 2007), and Tipin downregulation has been shown to decrease normal S-phase progression (Yoshizawa-Sugata and Masai, 2007).

More recently, reconstitution of the replisome *in vitro* with budding yeast proteins has supported the evidence for a role for FPC proteins in normal replisome progression. These experiments demonstrated that a 'minimal replisome' could not reach maximal rates of DNA replication *in vitro* without FPC proteins Tof1/Csm3 and Mrc1 (Yeeles et al., 2017). Interestingly, in these experiments omitting Mrc1 from the reaction led to reduced elongation compared to omitting Tof1/Csm3, and adding concentrations of just 5 nM Mrc1 to reactions containing Tof1/Csm3 allowed maximum rates of synthesis (Yeeles et al., 2017). This is consistent with *in vivo* data where *mrc1Δ* cells show a more severe delay in S-phase progression than *tof1Δ* cells in unchallenged conditions (Hodgson et al., 2007). Taken together this suggests that Mrc1 is the key driver in normal replisome progression, and that Tof1/Csm3 act to enhance Mrc1 activity, potentially through its stabilisation and/or recruitment to the replisome.

1.3.2.3. Replisome pausing at programmed replication fork barriers

Chromosomal DNA is coated with numerous stably bound proteins, many of which represent sites of programmed fork arrest. At such sites, FPC proteins have been shown to have a key role in stably pausing replisome progression. The first evidence of a role for Timeless proteins in programmed replisome pausing came from the observation that, as visualised using 2D gels, *swi1Δ* and *swi3Δ* mutants lose the ability to pause replication forks at the mating type locus of *S. pombe* (Dalgaard and Klar, 2000). This finding was consistent with the argument that this programmed pause was required for mating type switching, as several Swi mutants had previously been reported to have mating type switching defects (Egel et al., 1984, Gutz and Schmidt, 1985).

In addition, Tim/Tipin and their orthologues have been shown to be required for programmed fork pausing at the rDNA loci in several organisms. As discussed in section 1.2.1.3 of this thesis, unidirectional pausing of replication forks at the rDNA ensures that replication and transcription of the highly transcribed rDNA genes occurs in the same direction to prevent head-on collisions between the replisome and transcription machinery. These pauses are enacted by a proteinaceous component, such as the budding yeast RFB which is bound by the Fob1 protein (Figure 1.10) (Kobayashi and Horiuchi, 1996, Kobayashi, 2003, Ward et al., 2000). Budding yeast Tof1 and Csm3 are required for fork arrest at the Fob1-bound RFB (Figure 1.10) (Calzada et al., 2005, Mohanty et al., 2006). Interestingly, *tof1Δ rrm3Δ* or *csm3Δ rrm3Δ* double mutants are able to pause, indicating that Tof1/Csm3 may act to counteract the “sweepase” activity of the Rrm3 helicase at this locus, preventing Fob1 removal (Figure 1.10) (Mohanty et al., 2006).

Interestingly, there is evidence that DDK-dependent phosphorylation of Tof1 promotes replication fork pausing at the rDNA RFB by promoting its retention at the replisome (Bastia et al., 2016). Specifically, temperature sensitive *cdc7-1* or *dbf4-1* mutants fail to retain Tof1 in chromatin fractions and are unable to support fork pausing at the RFB as a result (Bastia et al., 2016).

A recent study focused on the role of topoisomerases in Tof1-dependent replisome pausing at the rDNA (Shyian et al., 2019). This work identified a C-terminal Tof1

truncation unable to mediate the previously characterised interaction between this region of Tof1 and Top1. Tof1 mutants unable to bind Top1 were found to have a small decrease in pausing efficiency at the rDNA when depleted for Top2, leading to the conclusion that topoisomerase activity is required for replisome pausing at the rDNA genes.

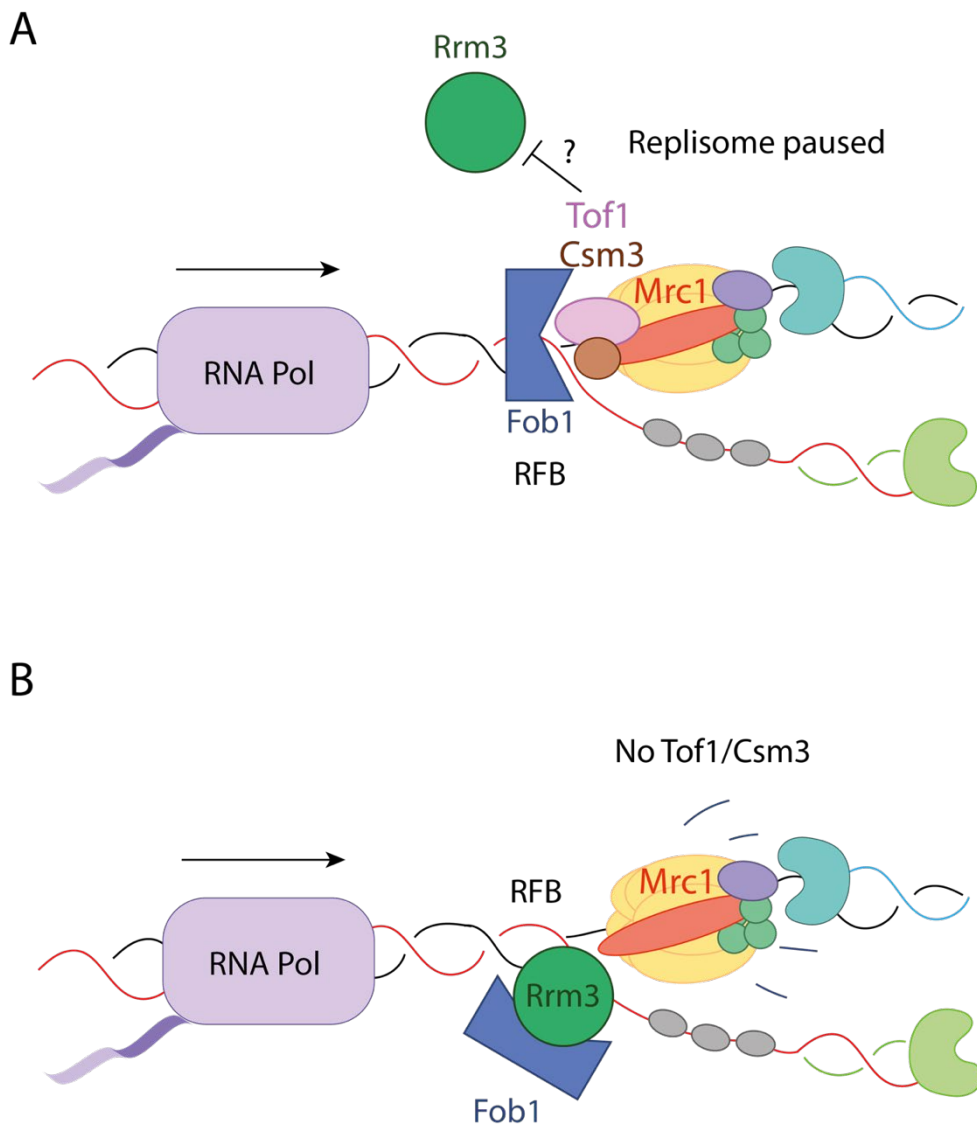


Figure 1.10: Schematic of Tof1/Csm3 mediating replisome pausing at the rDNA RFB

A) In the presence of Tof1/Csm3, CMG is able to stably pause at the Fob1-bound RFB sequence within the rDNA repeats. This prevents head-on collisions between the replisome and RNA polymerase transcribing rDNA genes in the opposite direction. Some genetic evidence suggests that Tof1/Csm3 act to counteract the action of the “sweepase” helicase Rrm3 to prevent removal of Fob1 at the RFB (Mohanty et al., 2006), although how this occurs mechanistically is unclear.

B) In the absence of Tof1/Csm3, CMG continues elongating through the RFB without pausing, which is thought to result in head-on collisions between the replication machinery and RNA polymerase transcribing in the opposite direction. Such head-on collisions are thought to lead to increased genome instability and recombination. Rrm3 has been shown to remove protein blocks including Fob1, suggesting that its action to remove Fob1 at the rDNA RFB results in CMG progression without pausing.

FPC-mediated fork pausing has been shown to be a conserved mechanism to avoid replication-transcription collisions at the rDNA of several other organisms. In *S. pombe*, the rDNA contains three specific sequences that induce polar fork arrest in a Swi1- and Swi3-dependent manner (Krings and Bastia, 2004). Interestingly, two of these sequences, *Ter2* and *Ter3*, are bound by Reb1, which contains a conserved 'Myb' domain known to bind DNA (Sánchez-Gorostiaga et al., 2004). In human cells, the Reb1 orthologue TTF-1, which also contains a Myb domain, has been demonstrated as the factor responsible for fork arrest at the rDNA in human cells (Gerber et al., 1997). Together these suggest that FPC proteins may act in a similar fashion to pause replication forks at the TTF-1-bound rDNA locus in mammals, although evidence for this is currently lacking.

1.3.2.4. *Replisome stability*

In addition to programmed pause sites, Timeless-dependent replisome stabilisation also has a key role in protecting forks threatened by replication stress, for example in conditions of dNTP limitation and at difficult-to-replicate regions. In these situations, the replisome is stably maintained until the challenge in question has been resolved and elongation can continue. An inability to stabilise the replisome in response to replication stress is strongly associated with a loss of genomic stability and a decrease in cellular viability. This most likely results from collapsed forks which can result in DNA breaks and toxic recombination intermediates (Zeman and Cimprich, 2014). Several contexts, both endogenous and exogenous, have been shown to require FPC-mediated replisome stabilisation, as discussed below.

Hydroxyurea (HU)

As discussed in section 1.2.1.5, dNTP synthesis is inhibited in the presence of HU, resulting in insufficient dNTP pools for nascent strand synthesis and impaired replisome progression. This leads to replication fork slowing, stalling, and eventually fork collapse if dNTP synthesis is not resumed (Singh and Xu, 2016). Cells lacking Tof1 or Csm3 are sensitive to HU (Redon et al., 2006), suggesting a role in protecting replication forks from HU-mediated damage. Katou and colleagues (2003) investigated the role of FPC proteins in replisome stability by following nascent DNA synthesis and CMG progression side by side. In HU-treated *tof1Δ*, *csm3Δ* or *mrc1Δ* cells, Cdc45 can be isolated at genomic loci 2.5-3kb further away from replication origins than BrdU, indicating an uncoupling of CMG progression and DNA synthesis in these mutants (Figure 1.11). This would potentially result in the exposure of large amounts of ssDNA due to helicase unwinding in the absence of nascent DNA synthesis, likely resulting in RPA-mediated ATR activation. However, in the same study checkpoint-deficient *mec1Δ tel1Δ* mutants did not have a coupling defect, suggesting that the FPC has a non-DRC related function in replisome stabilisation. Consistent with this, human fibroblasts depleted for Timeless and Claspin show more chromosomal aberrations than cells depleted for either protein individually (Smith-Roe et al., 2014). However, the role of FPC proteins in replisome coupling and general replisome stabilisation is still poorly understood.

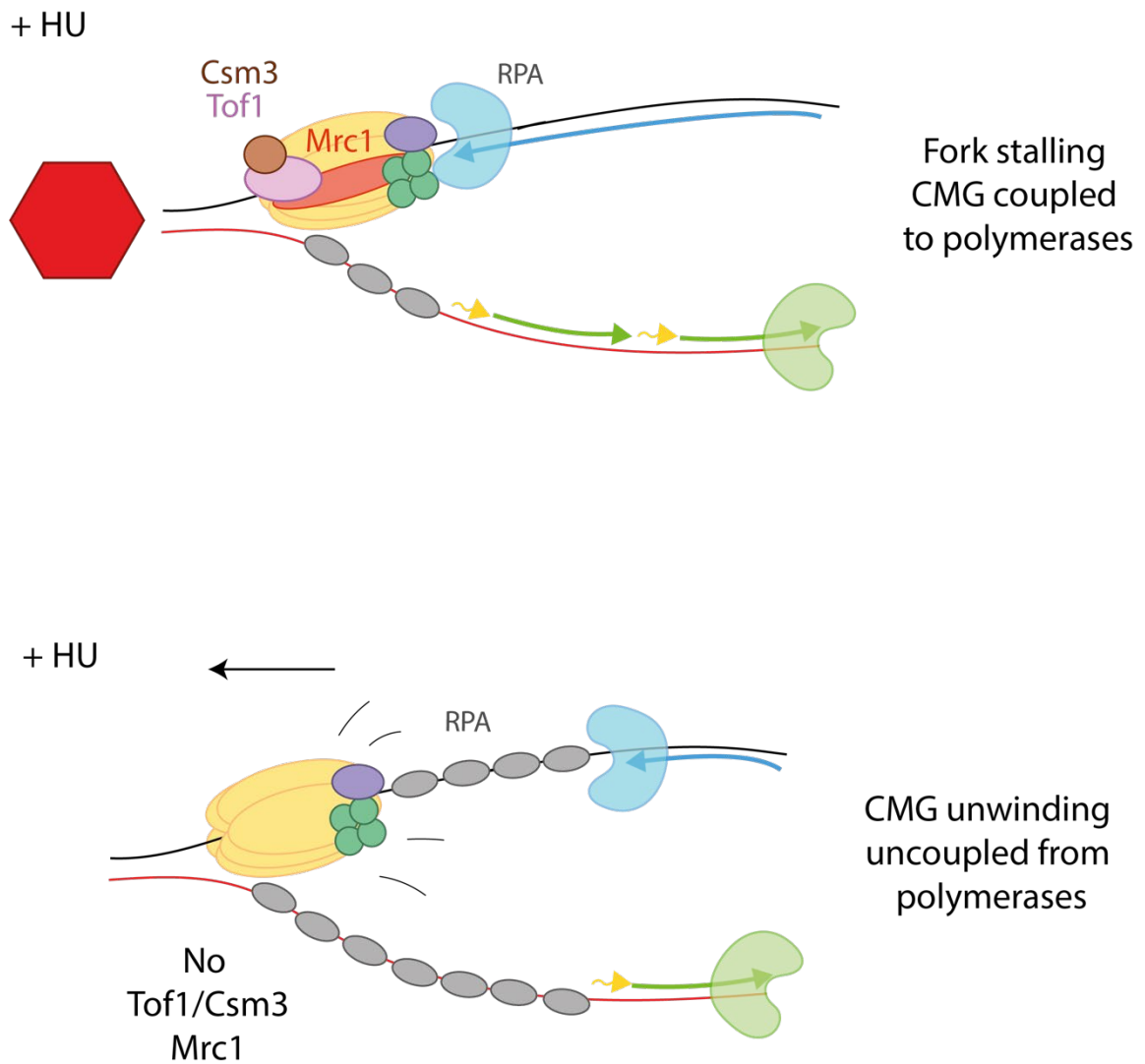


Figure 1.11: Schematic of Tof1/Csm3 and Mrc1-dependent replisome coupling in the presence of HU.

A) After treatment with HU, the absence of nascent strand synthesis results in replication fork stalling. In wild-type cells this completely halts replisome progression close to the site of fork arrest. B) In cells lacking FPC proteins Tof1/Csm3 and Mrc1, HU treatment results in uncoupling of CMG progression and nascent strand synthesis (Katou et al., 2003). CMG continues to unwind duplex DNA ahead of the fork in the absence of DNA polymerisation, which is predicted to generate large amounts of ssDNA at the fork (coated by RPA).

A more recent study by Somyajit and colleagues (2017) has suggested a novel and intriguing function of human Timeless in the response to reactive oxygen species (ROS) generated by HU treatment. Short treatments with low doses of HU slowed fork speeds without drastically altering dNTP levels, leading to the hypothesis that Tim plays an additional role in response to HU treatment, specifically in response to HU-induced ROS. Interestingly, peroxiredoxin 2 (PRDX2) was detected at replisomes by iPOND and found to interact with Tim. Elevated ROS levels led to PRDX2 dissociation from chromatin and subsequent dissociation of Tim from the replisome, suggesting that Tim can act as an indirect sensor of ROS to slow replication forks under conditions of stress. Such a mechanism could explain why *TIM* is frequently overexpressed in several cancer types (Yoshida et al., 2013), as Tim may be required to overcome the elevated levels of ROS that are frequently associated with cancer cells.

Difficult-to-replicate regions

Some genomic loci are inherently more difficult to replicate than others. Many of these include highly transcribed regions, such as the rDNA repeats discussed earlier, as well as repetitive sequences and specific chromosomal regions such as the centromeres and telomeres. Such regions are classified as 'difficult to replicate' due to increased incidences of fork stalling and breakage at these loci. Loss of FPC proteins often exacerbates these phenotypes highlighting the importance of Tim/Tipin and their orthologues in replisome stabilisation through such regions.

A unique genomic context in which DRC-mediated replisome stabilisation appears to be important is at the telomeres. Telomeres are inherently difficult to replicate due to their repetitive nature and the presence of many proteins that bind these telomeric repeats (Gilson and Géli, 2007). In several organisms, Timeless has been linked to a role in maintaining the stability of replication forks through the telomeres. In *S. pombe* and mammalian cells, telomere binding proteins SpTaz1 and hTRF1/TRF2 contain Myb DNA-binding domains, similarly to rDNA binding proteins SpReb1 and hTTF-1 that have been shown to require FPC proteins for efficient replisome pausing. Consistent with this, FPC mutants do show defects in telomere replication. In fission yeast, *swi1Δ* mutants show telomere shortening and increased markers of recombination at the telomeres (Gadaleta et al., 2016). Interestingly, in budding yeast loss of Tof1 results in telomere lengthening (Grandin and Charbonneau, 2007). However, the lack of a Myb-domain containing

Taz1/TRF orthologue in *S. cerevisiae* may explain these differences in telomere maintenance and the role of FPC proteins in this process. In human cells, Tim has been shown to interact with shelterin complex components TRF1 and TRF2, and Tim depletion results in telomere shortening and increased markers of DSBs and telomere aberrations (Leman et al., 2012). In the same study, depletion of Timeless also slowed replication of “naked” telomeric DNA *in vitro*, suggesting that telomeric DNA itself, known to be highly repetitive, requires FPC proteins for efficient duplication (Leman et al., 2012).

Other repetitive DNA sequences have been shown to require Tim/Tipin for their efficient and faithful replication. Repetitive elements are present throughout the genome and are thought to stall replication forks by forming non-B form secondary structures such as hairpins, cruciforms and G4 quadruplexes that are inhibitory to replisome progression (Wang and Vasquez, 2014). Interestingly, the role of FPC proteins in replication through repetitive sequences appears to be different to their role in programmed fork pausing at protein barriers. In contrast to the loss of fork pausing observed at various genomic loci (such as at the rDNA), budding yeast *tof1Δ* and *mrclΔ* mutants showed increased fork stalling at inverted *Alu* repeat sequences (Voineagu et al., 2008) and repeat instability at trinucleotide repeats (Razidlo and Lahue, 2008). Consistently, both Tof1 and Mrc1 have recently been shown to play a key role in preventing instability and chromosomal breakage at extended CAG repeats (Gellon et al., 2018). However, in this study Mrc1 was required to prevent fork breakage at repeat sequences of all lengths, including those that were not seen to stall replication in wild-type cells. In contrast, Tof1 appears to have a particular role in fork stabilisation at longer repeats (>85 repeats) which would be predicted to form secondary structures (Gellon et al., 2018). Recent work in mammalian cells suggests that a Timeless C-terminal DNA binding domain preferentially binds G4-forming DNA, and is required for processive replication past these structures via an interaction with DDX11 (Lerner et al., 2020).

Trapped topoisomerase enzymes

As discussed earlier (see section 1.2.1.4), cross-linked topoisomerase-DNA complexes, or Topo-cc's, represent a physical barrier to replisome progression and induce DNA damage and genome instability (Figure 1.12A). Although these lesions can arise endogenously, our understanding of how these lesions and their consequences for

genome stability have been facilitated by the use of small molecules that 'trap' topoisomerases on the DNA. One such example is camptothecin (CPT), a drug specifically used to trap Top1 covalent complexes on the DNA. Top1 normally resolves positive supercoils by a 'free rotation' mechanism, whereby an active site tyrosyl oxygen of the enzyme attacks the DNA phosphate backbone, generating a covalent phosphotyrosine link between the enzyme and the 3' end of the broken DNA. This allows the DNA to freely rotate to diffuse torsional stress, before the break is re-sealed by essentially the reverse reaction to the first step. CPT binds to both the Top1 enzyme and the DNA during the intermediate step, preventing re-ligation of the nicked DNA and therefore stabilisation of the Top1-DNA crosslink (Figure 1.12A) (Pommier, 2009). CPT-induced Top1-cc's are thought to generate toxic structures such as DSBs through replication fork run-off (Strumberg et al., 2000) or reversed replication forks that may be misprocessed (Regairaz et al., 2011). In budding and fission yeast *tof1Δ/csm3Δ* and *swi3Δ* mutants are hypersensitive to CPT, respectively (Redon et al., 2006, Rapp et al., 2010), indicating that they play a key role in replisome stabilisation at these lesions. Similarly, Tipin knockout DT40 cells display sensitivity to CPT (Hosono et al., 2014) suggesting that the role of FPC proteins in response to CPT-induced DNA damage is conserved.

What is currently unclear is how FPC proteins Tof1/Csm3 protect cells from Top1-cc lesions. Top1-cc lesions are thought to generate DNA damage when replication forks encounter them. Considering that CMG unwinds DNA in the 3' to 5' direction and translocates on the leading strand, it is thought that the nick generated by Top1, leaving a 5' free DNA end, could lead to replication fork run-off (Figure 1.12B). Potentially, the fork-pausing function of Tim/Tipin is required to stably inhibit fork progression at Top1-cc's and inhibit fork run-off. However, data to support this model is currently lacking.

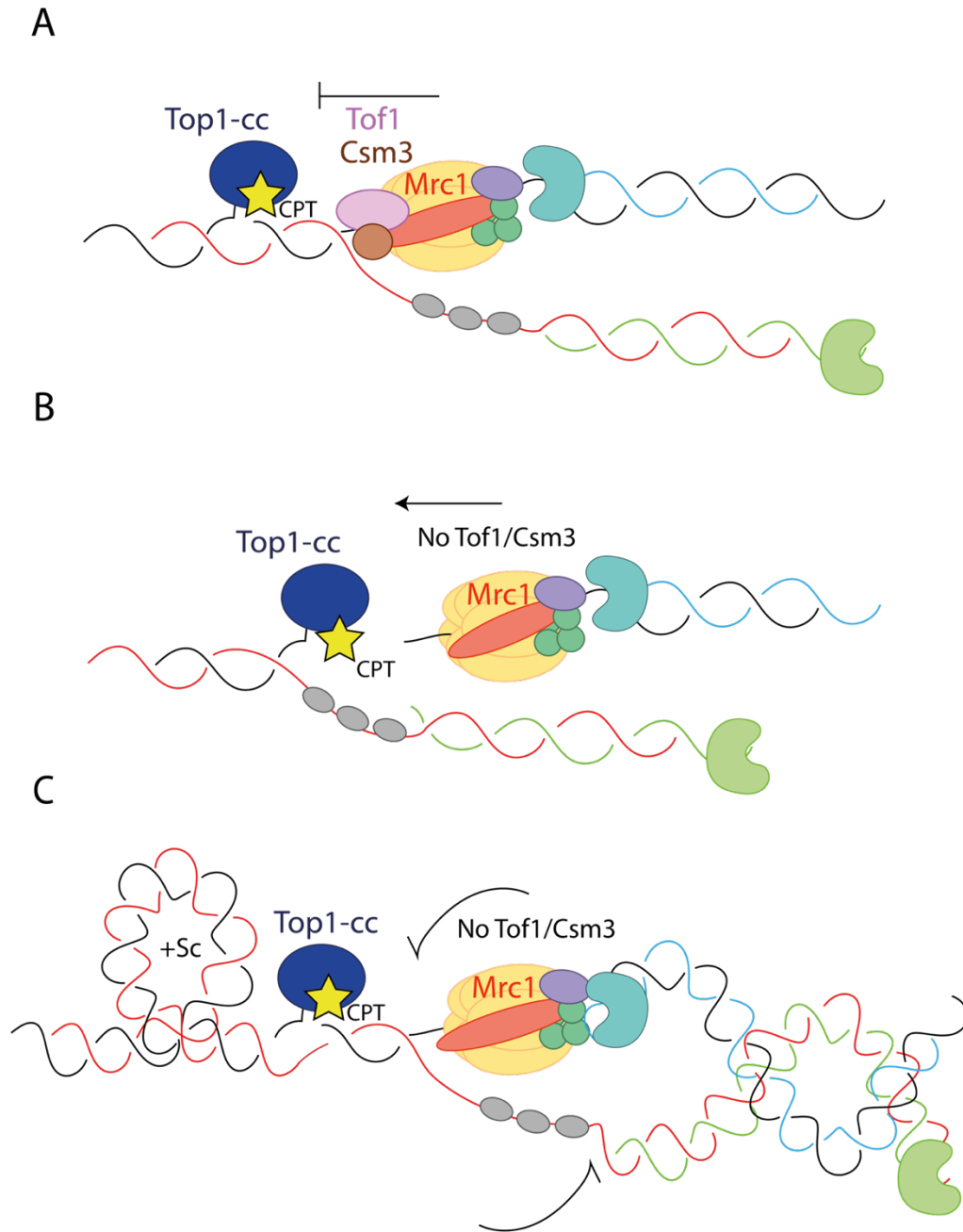


Figure 1.12: Possible mechanisms for CPT-induced toxicity in *tof1Δ* and *csm3Δ* cells

A) Treatment of cells with CPT (yellow star) stabilises Top1-cc's on the DNA. Tof1/Csm3 prevent CPT-induced toxicity, through an unknown mechanism.

B) Cells lacking Tof1/Csm3 may be unable to pause the replisome at stabilised Top1-cc's, leading to replication fork run-off as the CMG encounters the nick introduced by Top1. This is thought to lead to the generation of one-ended DSBs which are toxic to the cell.

C) CPT leads to local increases of topological stress, which would be predicted to lead to increased levels of replication fork rotation in cells lacking Tof1/Csm3. The unrestrained generation of precatenanes between the newly replicated sister chromatids could lead to chromosome segregation defects and cell death.

Alternatively, CPT-induced increases in topological stress on the DNA may be responsible for the toxicity of Top1-trapping in *tof1Δ* cells (Figure 1.12C). Local increases in topological stress resulting from CPT treatment would be more problematic for cells lacking Top1/Csm3 as these proteins play a key role in restricting high levels of fork rotation and subsequent DNA damage (Schalbetter et al., 2015). Recently it was shown in budding yeast that deletion of *SIR2*, *SIR3* or *SIR4* genes, normally responsible for histone deacetylation and heterochromatin maintenance, can suppress CPT sensitivity of *tof1Δ* and *csm3Δ* cells (Puddu et al., 2017). Potentially, a more relaxed or 'open' chromatin template in Sir mutants would be less likely to accumulate CPT-induced topological stress, which would then be less toxic to cells lacking Top1.

1.3.2.5. Inhibition of DNA replication fork rotation

As discussed in section 1.2.1.1, changes in DNA topology readily occur during many cellular processes, including DNA replication. During DNA replication, in the absence of topoisomerase activity, or at contexts where topoisomerase activity may be sterically hindered (e.g. at termination) the fork may rotate on its axis to diffuse topological stress ahead of the fork to behind the fork, where it manifests as intertwinings between the newly replicated sister chromatids (Keszthelyi et al., 2016). Generally fork rotation is restricted during DNA replication. In budding yeast, this depends on Top1/Csm3 but not Mrc1 (Figure 1.13B) (Schalbetter et al., 2015). In cells lacking Top1/Csm3 and partially depleted for Top2, episomal plasmids extracted from post-replicative cells are highly intertangled, suggesting that Top1/Csm3 play an important role in restricting the rotation of the replisome as a way of alleviating high levels of topological stress (Schalbetter et al., 2015). As already mentioned, Top1 binds Top1 *in vivo* (Park and Sternglanz, 1999, Shyian et al., 2019). Potentially, Top1-mediated recruitment of Top1 is required for resolution of topological stress ahead of the replisome, reducing the requirement for fork rotation to resolve stress (Figure 1.13A). However, direct evidence for increased fork rotation in Top1 mutants unable to bind Top1 is currently lacking.

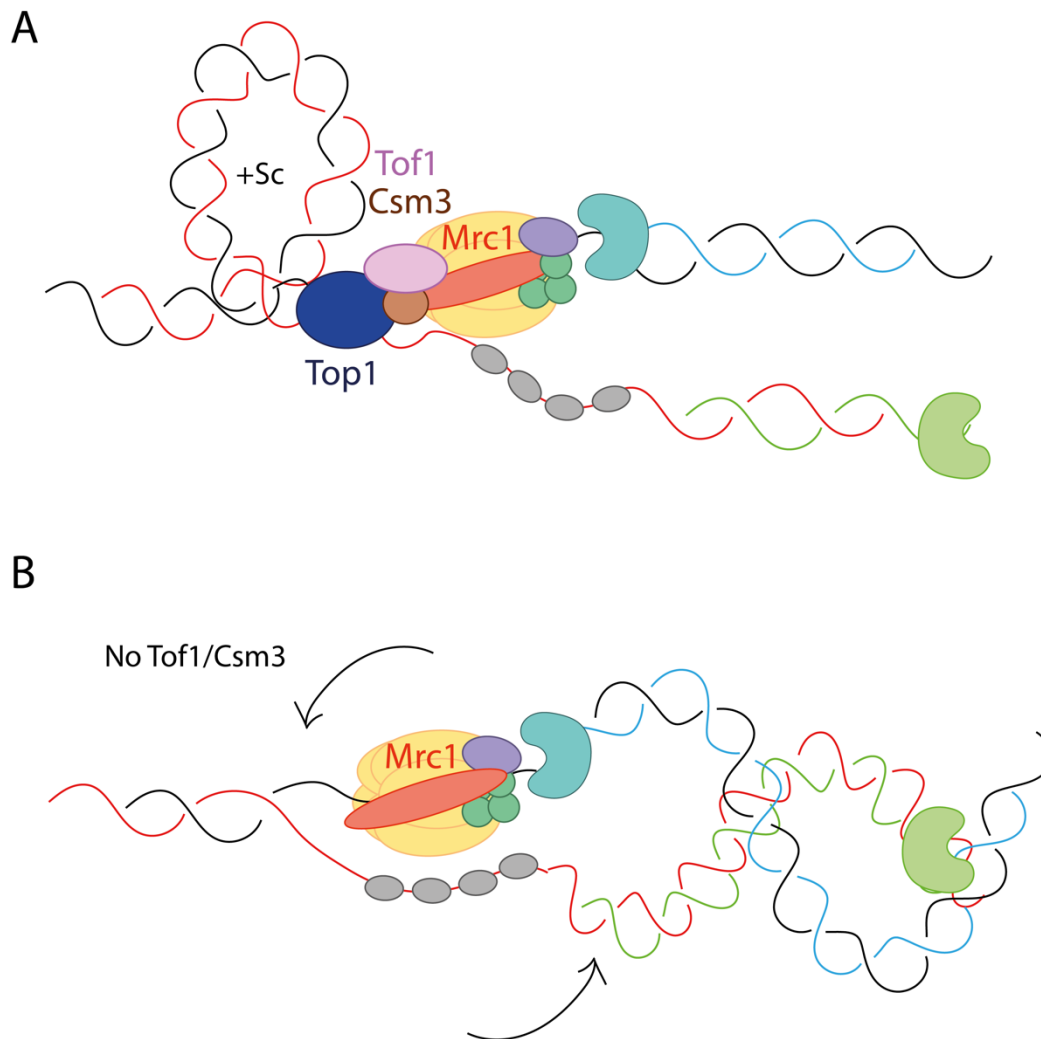


Figure 1.13: Schematic of DNA replication fork rotation restriction by Tof1/Csm3

A) During normal elongation, replication fork rotation is generally restricted by FPC proteins Tof1 and Csm3. Although this has not been definitively answered, the well characterised Tof1-Top1 interaction could provide a way of direct recruitment of Top1 to the progressing replisome, reducing the frequency of fork rotation events.

B) In the absence of Tof1 or Csm3 (but not Mrc1), replication fork rotation is not restricted, resulting in the generation of intertwinings (pre-catenanes) between the newly replicated sister chromatids.

1.3.2.6. Establishment of sister chromatid cohesion

During DNA replication, newly synthesised sister chromatids are held together in a process known as sister chromatid cohesion (SCC), by the conserved cohesin protein complex. Cohesin consists of two long arm proteins, Smc1 and Smc3, along with a kleisin subunit Scc1, which together form a ring-shaped structure that is bound by additional accessory proteins Scc3, Pds5 and Wpl1 (Makrantonis and Marston, 2018). Cohesin complexes are loaded on the DNA before and during DNA replication and remain associated with the DNA until mitosis when Scc1 is cleaved by separase, allowing the sister chromatids to segregate to the daughter cells (Makrantonis and Marston, 2018). Failure to properly establish and maintain SCC is associated with increased chromosome missegregation and chromosomal instability. Tof1/Csm3 and Mrc1 have all been implicated in promoting SCC, and are known as 'nonessential cohesion establishment factors', to distinguish them from factors that are essential for SCC and thus cellular viability (i.e. cohesin subunits).

FPC proteins were first associated with a role in SCC due to early screens that led to the identification of budding yeast Csm3 (chromosome segregation in meiosis 3) (Rabitsch et al., 2001). In this study, cells lacking Csm3 exhibited chromosome segregation defects and reduced spore viability in meiosis, suggesting a defect in chromosome cohesion. Several years later, Csm3 and Tof1 were identified in a screen looking for mutants that showed increased chromosome segregation defects in a strain lacking Ctf8, a component of the Ctf18-RFC complex that normally promotes efficient SCC (Mayer et al., 2004). In higher eukaryotes the role of Tim/Tipin in SCC appears to be conserved, with cohesion defects due to Tim/Tipin depletion being observed in *C. elegans* (Chan et al., 2003), *X. laevis* (Tanaka et al., 2009b, Errico et al., 2009) and human cells (Leman et al., 2010).

These findings give rise to the question: how do FPC proteins regulate SCC to maintain genome stability? Work in budding yeast suggests that Tof1/Csm3 and Mrc1 may play a role in promoting acetylation of Smc3 during DNA replication. During S-phase, the acetyltransferase Eco1 acetylates Smc3, preventing removal of cohesion complexes associated with the DNA by Wapl1 (Rolef Ben-Shahar et al., 2008, Zhang et al., 2008). Cells lacking Tof1, Csm3 or Mrc1 show reduced Smc3 acetylation, suggesting that they contribute to SCC by promoting Eco1 function (Borges et al., 2013). A more recent study using yeast genetics suggests that Tof1/Csm3 play a role specifically in conversion of

pre-loaded cohesin complexes ahead of progressing replisomes to behind the fork where they can perform SCC. Specifically, this work used assays to visualise conversion of pre-loaded cohesion complexes into cohesive structures and found that this pathway of SCC establishment is dependent on Tof1/Csm3 but not on Mrc1 (Srinivasan et al., 2020). In contrast, Tof1/Csm3 do not contribute to *de novo* loading of cohesin complexes during replisome progression. However it is still unclear exactly how Tof1/Csm3 support establishment of SCC. FPC proteins may have a functional role in establishing SCC, or their presence may be required to support the structure or function of other SCC-promoting factors at the replisome.

1.3.3. Non-S-phase functions of Timeless proteins

1.3.3.1. *Timeless proteins and circadian rhythm*

Circadian rhythms are the internal, autonomous 24-hour cycles present in virtually all organisms including bacteria, insects, plants and humans (Patke et al., 2019). These 24-hour rhythms control many aspects of an organisms behaviour and physiology, and are influenced by external cues, the most prominent of which being light (Patke et al., 2019). Over the years, *D. melanogaster* has served as a popular model organism for studying these rhythms at the molecular level. These studies have identified a network of 'clock genes', the cyclical expression of which forms a feedback loop that controls expression of other genes, including their own. The *timeless (tim)* gene was first cloned and identified as a clock gene by Sehgal and colleagues (1994), as mutations in *tim* were found to disrupt the normal circadian behaviours of eclosion and locomotor activity and altered the normal circadian expression pattern of *per* mRNA. Both *tim* and *per* mRNA levels oscillate (Sehgal et al., 1994, Hardin et al., 1990), and the protein products of these genes (dTIM and dPER) physically interact (Gekakis et al., 1995, Saez and Young, 1996). Together, dTIM and dPER form the basis of a circadian rhythm at the cellular level, as they form a negative feedback loop that cycles with 24 hour rhythmicity. dTIM and dPER accumulate in the night and interact, which stabilises the proteins and allows them to enter the nucleus. Once in the nucleus dTIM/dPER heterodimers negatively regulate their own expression to close the feedback loop, as well regulating the expression of other genes that oscillate with the circadian clock (Peschel and Helfrich-Förster, 2011).

Mammalian Tim was first identified and cloned based on its weak sequence similarity with dTIM, and was found to be expressed in the core of the mammalian circadian clock, the suprachiasmatic nucleus (SCN) (Koike et al., 1998, Sangoram et al., 1998, Takumi et al., 1999, Zylka et al., 1998). However, as previously discussed it was later found that human Tim is more similar in sequence to a second Timeless paralog in *D. melanogaster*, Timeout (dTim2) (Benna et al., 2000), suggesting that dTIM is not the true orthologue of human Tim.

Despite this, since the discovery of dTIM as a circadian regulator, researchers have been trying to determine whether this function may be conserved in mammals. This has been largely hindered by the inability to generate *TIM* knockout mice due to early embryonic lethality (Gotter et al., 2000). Therefore, studies have focused on the role of Tim in the molecular clock, although the resulting data is often conflicting. For example, whilst many studies have not detected circadian oscillation of Tim mRNA and protein levels (Koike et al., 1998, Sangoram et al., 1998, Takumi et al., 1999, Zylka et al., 1998), others have reported small but noticeable differences in Tim expression over time (Tischkau et al., 1999, Barnes et al., 2003, Engelen et al., 2013). Potentially, these differences could be explained by the generation of multiple transcripts from the *TIM* gene, producing different gene products with varied functions. Indeed, a shorter (~2.5kb) Tim isoform has been identified in mice, corresponding to the last 13 exons of the gene (Li et al., 2000). Interestingly, studies reporting no circadian oscillations of Tim mRNA and/or protein have generally used materials designed to probe for the longer Tim isoform, i.e. at the N-terminus, whereas materials used to visualise the C-terminal regions have not observed rhythmic changes in Tim levels. However, disrupting specifically the longer Tim isoform with antisense oligonucleotides still disrupts its essential, non-circadian functions in development (Xiao et al., 2003). Therefore, it appears that the 'long' Tim isoform is the functionally relevant transcript for Tim's well-characterised roles in genome maintenance.

1.3.3.2. *Timeless proteins and DNA repair*

Mammalian cells depleted for Timeless using siRNA show spontaneous γ H2AX formation, increased Rad52 and Rad51 foci, and increased chromosomal breakage events (Chou and Elledge, 2006, Urtishak et al., 2009). These phenotypes are usually attributed to the S-phase functions of Tim in safeguarding the genome during genome

duplication. However, recent data has suggested that these phenotypes could also be partially attributed to a repair function of Timeless.

Two back-to-back studies published in recent years observed a robust interaction between a C-terminal domain of human Timeless and the catalytic domain of PARP-1 (Xie et al., 2015, Young et al., 2015). Both studies found that this interaction is required to efficiently recruit Timeless to sites of DNA damage independently of the catalytic function of PARP-1, and that both homologous recombination (HR) and non-homologous end joining (NHEJ)-mediated repair of breaks is impaired if this interaction is disrupted. Interestingly, recruitment of Timeless to DNA lesions by PARP-1 was found to occur outside of S-phase, highlighting a potential role of Timeless outside of DNA replication. The exact role of Timeless in promoting DNA repair is not completely understood from these studies, but Young and colleagues (2015) reported a reduction in recruitment of several repair factors to break sites such as KU80 and DNA-PKcs. These data suggest that the PARP-1/TIM interaction may be required for efficient recruitment of repair factors to break sites, an observation that is likely to be followed up in more detail in future studies.

1.4. Aims of this thesis

Despite advances in our understanding of the many functions of Timeless family proteins, there is still a limited understanding of the detail underpinning how these pleiotropic functions are carried out and how they may relate to one another. This is in part a consequence of limited information about how the structure of these proteins relates to their functions.

The aim of this work is to understand in more detail the various S-phase functions of Tof1 and Timeless in *S. cerevisiae* and in human cells, respectively. This can be divided into two core aims: first, to carry out an in-depth structure function analysis of the Tof1 protein in budding yeast cells. Second, to investigate the functions of human Timeless during DNA replication. I have approached these aims by utilising a wide range of molecular and cellular techniques.

First, I have generated, characterised and utilised multiple Tof1 truncation mutants to separate out the roles of this protein in responding to different types of replication stress in budding yeast. Specifically, I have investigated the role of the Tof1 C-terminus in responding to topological stress, protein-DNA barriers and Top1-ccs, and to nucleotide limitation. I have also investigated in more detail the poorly understood role of Tof1 in the DNA replication checkpoint and replication fork restart following arrest.

Second, I have attempted to develop human cell lines in which Timeless protein can be degraded in a regulated fashion. This has been achieved by using CRISPR-Cas9 genome editing to knock-in inducible degron tags into the Timeless C-terminus. This data further supports an essential role for Timeless in mammals.

2. Materials and Methods

2.1. Cell Culture

2.1.1. Media for yeast cell culture

2.1.1.1. Yeast Peptone (YP) rich media

1.8% w/v (10.8 g/l) Yeast extract (Melford Laboratories Y1333)

3.6% w/v (36 g/l) Peptone (Melford Laboratories P1328)

pH 5.5

Autoclave sterilised

2% w/v (20 g/l) of carbon source added as indicated for each experiment (see 2.3.2.1):

- Glucose (Fisher Scientific 10395940) (**YPD**)
- Raffinose pentahydrate (Merck R0250) (**YP Raff**)
- Galactose (Merck G0625) (**YP Gal**)

2.1.1.2. Yeast Peptone Agar plates (YP Agar)

As YP with 2% (20 g/l) Difco Bacto Agar (Fisher Scientific 11758223)

2.1.1.3. -URA dropout minimal media

0.192% w/v (1.92 g/l) Yeast synthetic drop-out medium supplements without uracil (Merck Y1501)

0.134% w/v (1.34 g/l) Yeast nitrogen base without amino acids (Melford Y2004)

Filter sterilised

2% w/v (20 g/l) Glucose

2.1.1.4. Dropout minimal media plates

0.134% w/v (1.34 g/l) Yeast nitrogen base without amino acids (Melford Y2004)

Autoclave sterilised

2% w/v (20 g/l) Glucose

0.004% w/v (0.04 g/l) each of:

- Adenine sulphate, Uracil, L-Histidine, L-Leucine & L-Tryptophan

N.B. For selection of strains containing auxotrophic selection markers e.g. *URA3* the appropriate amino acid was not included in the media.

2.1.1.5. Rapid sporulation media (RSM)

0.25% w/v (2.5 g/l) Yeast extract (Melford, Y1333)

1.5% w/v (15 g/l) Potassium acetate (Fisher 10522955)

0.1% w/v (1 g/l) Glucose (Fisher Scientific)

2% w/v (20 g/l) Difco Bacto agar

Autoclave sterilised

Amino acid mix added after sterilisation:

- 80 mg/l each of: Adenine sulphate, Uracil
- 40 mg/l each of: L-Histidine, L-Leucine, L-Lysine, L-Tryptophan, L-Methionine, L-Arginine
- 16 mg/l L-Tyrosine
- 200 mg/l L-Phenylalanine

2.1.2. Media for human cell culture

RPE-1 cells were maintained in culture at 37°C in DMEM/F-12 medium (Gibco™ 12-634) supplemented with 10% filter-sterilised fetal calf serum (FCS) and 1X Penicillin-Streptomycin-Glutamine (Gibco™ 10378016). Cells were split 1:5 every 2-3 days or as required (when growing at 70-80% confluency) by trypsinisation.

2.1.2.1. *Trypsinisation of human cells*

RPE-1 cells were first washed once in PBS pre-warmed to 37°C before addition of just enough of 0.5% Trypsin-EDTA (Gibco 15400054) to cover the bottom of the growth dish or flask. Cells were incubated at 37°C for 2-5 minutes or until cells had visibly detached, before quenching the trypsin with the same volume of DMEM/F12 media. Cells were spun down at 1500 RPM for 5 min and re-suspended in fresh DMEM/F12 media before counting and re-seeding into fresh flasks.

2.1.3. Human cell culture techniques

2.1.3.1. *Electroporation transfection of RPE-1 cells*

RPE-1 cells were transfected using the Neon transfection system (Thermo Fisher Scientific). For a single transfection, 10⁶ cells were washed and trypsinised as above (see 2.1.2.1) and pelleted before re-suspending in 30 µl 'R' buffer (supplied with Neon transfection kit). Cells were transferred to a second tube containing 1.5 µg plasmid DNA before being electroporated according to the manufacturer's instructions, with the following settings used for electroporation: 1350V, 20ms, 2 pulses. Immediately after electroporation cells in the Neon transfection pipette tip were dropped into 2 ml of pre-warmed (37°C) DMEM/F12 media in one well of a 6-well plate. Cells were left to recover for 48 hrs before re-plating into selection media. To select for cells with successful integration of gene targeting cassettes conferring resistance to Neomycin, cells from one transfection were washed and trypsinised as above (see 2.1.2.1) before re-suspension in 80ml DMEM/F12 media supplemented with 1 mg/ml G-418. Cells were then transferred to 4 x 96-well plates with 200 µl of cell suspension per well. After 3 weeks incubation at

37°C, individual wells were checked for colonies and successfully growing colonies were expanded in DMEM/F12 media supplemented with 1 mg/ml G-418.

2.1.3.2. DNA Extraction from human cells

Genomic DNA was extracted from RPE-1 cells using DNeasy Blood & Tissue Kits (QIAGEN 69504) according to manufacturer's instructions.

2.1.3.3. Preparation of protein samples for western blotting

RPE-1 cells growing in 6-well plates were trypsinised and pelleted as above (see 2.1.2.1) before being re-suspended in 30 µL ice-cold lysis buffer (150mM NaCl, 1% NP-40, 50mM Tris-HCl pH 8.0). 10 µL of 4X Sample Buffer (250mM Tris (pH 6.8), 20% Glycerol, 4mg/mL Bromophenol blue, 0.8mg/mL SDS) was added to the cell suspension before probe sonication for 5 seconds on 20% amplitude (sonication was repeated if mixture was still viscous). Samples were then boiled at 95°C for 5 minutes and stored at -80°C overnight before running on SDS-PAGE gels.

2.1.4. Commonly used drugs

Name	Supplier	Working Concentration
Nourseothrecin (NAT)	Werner BioAgents (clonNAT)	100 µg/ml for yeast selection
Geneticin disulphate (G418)	Melford (G0175)	200 µg/ml for yeast selection 1 mg/ml for RPE-1 OsTIR cell selection
Hygromycin B (HygB)	Invitrogen (1068701050)	300 µg/ml for yeast selection
Ampicillin sodium salt (Amp)	Merck (A9518)	100 µg/ml for bacterial selection
Camptothecin (CPT)	Selleckchem (S1288)	As indicated in text
Hydroxyurea (HU)	Merck (400046)	As indicated in text

Table 2.1: List of commonly used drugs in this thesis

2.2. Yeast strains, plasmids and primers

2.2.1. List of yeast strains

No.	Name	Genotype	Source
1991	<i>wt</i>	<i>MATa, ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100, UBR1::GAL1-10-Ubiquitin-M-LacI fragment-Myc-UBR1 (HIS3), leu2-3::pCM244 (CMVp-tetR'-SSN6, LEU2) x3</i>	(Tanaka and Diffley, 2002).
1993	<i>tof1Δ</i>	1991 + <i>TOF1::hphMX</i>	This thesis
JB2075	<i>mrc1Δ</i>	1991 + <i>MRC1::natNT2</i>	This thesis
1528	<i>wt TOF1</i>	1991 + <i>TOF1::tof1-codon-optimised-wildtype (NatNT2)</i>	This thesis
1549	<i>tof1-627</i>	1991 + <i>TOF1::tof1-codon-optimised-627-1238Δ (NatNT2)</i>	This thesis
1596	<i>tof1-762</i>	1991 + <i>TOF1::tof1-codon-optimised-762-1238Δ (NatNT2)</i>	This thesis
1589	<i>tof1-830</i>	1991 + <i>TOF1::tof1-codon-optimised-830-1238Δ (NatNT2)</i>	This thesis
1546	<i>tof1-997</i>	1991 + <i>TOF1::tof1-codon-optimised-997-1238Δ (NatNT2)</i>	This thesis
1537	<i>tof1-1182</i>	1991 + <i>TOF1::tof1-codon-optimised-1182-1238Δ (NatNT2)</i>	This thesis
2188	<i>TOF1-TAP</i>	1991 + <i>endogenous TOF1 tagged with TAP (KanMX)</i>	This thesis
2182	<i>TOF1 wt TAP</i>	1991 + <i>TOF1::tof1-codon-optimised-wildtype-TAP (KanMX)</i>	This thesis
2183	<i>tof1-627 TAP</i>	1991 + <i>TOF1::tof1-codon-optimised-627-1238Δ-TAP (KanMX)</i>	This thesis

2184	<i>tof1-762 TAP</i>	1991 + <i>TOF1::tof1-codon- optimised-762-1238Δ-TAP</i> (KanMX)	This thesis
2185	<i>tof1-830 TAP</i>	1991 + <i>TOF1::tof1-codon- optimised-830-1238Δ-TAP</i> (KanMX)	This thesis
2186	<i>tof1-997 TAP</i>	1991 + <i>TOF1::tof1-codon- optimised-997-1238Δ-TAP</i> (KanMX)	This thesis
2187	<i>tof1-1182 TAP</i>	1991 + <i>TOF1::tof1-codon- optimised-1182-1238Δ-TAP</i> (KanMX)	This thesis
2288	<i>TOF1 wt mrc1- 3FLAG</i>	1528 + <i>Mrc1-3FLAG (NatNT2)</i>	This thesis
2191	<i>TOF1 wt-TAP mrc1-3FLAG</i>	2182 + <i>Mrc1-3FLAG (NatNT2)</i>	This thesis
2192	<i>tof1-627-TAP mrc1-3FLAG</i>	2183 + <i>Mrc1-3FLAG (NatNT2)</i>	This thesis
2194	<i>tof1-762-TAP mrc1-3FLAG</i>	2184 + <i>Mrc1-3FLAG (NatNT2)</i>	This thesis
2195	<i>tof1-830-TAP mrc1-3FLAG</i>	2185 + <i>Mrc1-3FLAG (NatNT2)</i>	This thesis
2196	<i>tof1-997-TAP mrc1-3FLAG</i>	2186 + <i>Mrc1-3FLAG (NatNT2)</i>	This thesis
2197	<i>tof1-1182-TAP mrc1-3FLAG</i>	2187 + <i>Mrc1-3FLAG (NatNT2)</i>	This thesis
1988	<i>csm3Δ</i>	1991 + <i>CSM3:: NatNT2</i>	This thesis
2465	<i>GAL-HA-TOF1 wt</i>	1528 + <i>N-terminal GAL S - 3xHA</i> (TRP)	This thesis
2466	<i>GAL-HA-tof1-627</i>	1549 + <i>N-terminal GAL S - 3xHA</i> (TRP)	This thesis
2490	<i>GAL-HA-tof1-901</i>	1536 + <i>N-terminal GAL S - 3xHA</i> (TRP)	This thesis

2467	<i>GAL-HA-tof1-997</i>	1546 + N-terminal GAL S - 3xHA (TRP)	This thesis
2468	<i>GAL-HA-tof1-1182</i>	1537 + N-terminal GAL S - 3xHA (TRP)	This thesis
1536	<i>tof1-901</i>	1991 + TOF1::tof1-codon-optimised-901-1238Δ (NatNT2)	This thesis
1591	<i>tof1 810-950Δ</i>	1991 + TOF1::tof1-codon-optimised-810-950Δ (NatNT2)	This thesis
1649	<i>tof1 810-870Δ</i>	1991 + TOF1::tof1-codon-optimised-810-870Δ (NatNT2)	This thesis
1650	<i>tof1-871-950Δ</i>	1991 + TOF1::tof1-codon-optimised-871-950Δ (NatNT2)	This thesis
1478	<i>w303 ade3 met15</i>	Mat alpha <i>ade2-1 his3-11 leu2-3 trp1-1 ura3-1 can1-100 ade3Δ::hisG met15Δ::KANMX</i>	This thesis
2241	<i>tof1 840</i>	1478 + TOF1::tof1-codon-optimised-840-1238Δ (NatNT2)	This thesis
2242	<i>tof1 850</i>	1478 + TOF1::tof1-codon-optimised-850-1238Δ (NatNT2)	This thesis
2244	<i>tof1 860</i>	1478 + TOF1::tof1-codon-optimised-860-1238Δ (NatNT2)	This thesis
2245	<i>tof1 870</i>	1478 + TOF1::tof1-codon-optimised-870-1238Δ (NatNT2)	This thesis
2246	<i>tof1 880</i>	1478 + TOF1::tof1-codon-optimised-880-1238Δ (NatNT2)	This thesis
2247	<i>tof1 890</i>	1478 + TOF1::tof1-codon-optimised-890-1238Δ (NatNT2)	This thesis
684	<i>tof1Δ rad9Δ</i>	1993 + <i>rad9::NatNT2</i>	This thesis
1718	<i>TOF1 wt rad9Δ</i>	1528 + <i>rad9::KanMX</i>	This thesis
1719	<i>tof1-627 rad9Δ</i>	1549 + <i>rad9::KanMX</i>	This thesis
1777	<i>tof1-762 rad9Δ</i>	1596 + <i>rad9::KanMX</i>	This thesis

1767	<i>tof1-830 rad9Δ</i>	1589 + <i>rad9::KanMX</i>	This thesis
1722	<i>tof1-997 rad9Δ</i>	1546 + <i>rad9::KanMX</i>	This thesis
1769	<i>tof1-1182 rad9Δ</i>	1537 + <i>rad9::KanMX</i>	This thesis
1762	<i>TOF1 wt top2-4</i> <i>pRS316</i>	1528 + <i>top2-4</i> , <i>pRS316</i> (URA)	This thesis
1610	<i>tof1-627 top2-4</i> <i>pRS316</i>	1549 + <i>top2-4</i> , <i>pRS316</i> (URA)	This thesis
2290	<i>tof1-762 top2-4</i> <i>pRS316</i>	1596 + <i>top2-4</i> , <i>pRS316</i> (URA)	This thesis
1717	<i>tof1-830 top2-4</i> <i>pRS316</i>	1589 + <i>top2-4</i> , <i>pRS316</i> (URA)	This thesis
1763	<i>tof1-997 top2-4</i> <i>pRS316</i>	1546 + <i>top2-4</i> , <i>pRS316</i> (URA)	This thesis
1611	<i>tof1-1182 top2-4</i> <i>pRS316</i>	1537 + <i>top2-4</i> , <i>pRS316</i> (URA)	This thesis
1731	<i>wt</i> <i>pRS426-RFB</i>	1991 + <i>pRS426-1xRFB</i> (URA)	This thesis
1730	<i>tof1Δ pRS426-</i> <i>RFB</i>	1993 + <i>pRS426-1xRFB</i> (URA)	This thesis
1732	<i>TOF1 wt</i> <i>pRS426-RFB</i>	1528 + <i>pRS426-1xRFB</i> (URA)	This thesis
1739	<i>tof1-627</i> <i>pRS426-RFB</i>	1549 + <i>pRS426-1xRFB</i> (URA)	This thesis
2333	<i>tof1-762</i> <i>pRS426-RFB</i>	1596 + <i>pRS426-1xRFB</i> (URA)	This thesis
1740	<i>tof1-830</i> <i>pRS426-RFB</i>	1589 + <i>pRS426-1xRFB</i> (URA)	This thesis
1733	<i>tof1-997</i> <i>pRS426-RFB</i>	1546 + <i>pRS426-1xRFB</i> (URA)	This thesis
1741	<i>tof1-1182</i> <i>pRS426-RFB</i>	1537 + <i>pRS426-1xRFB</i> (URA)	This thesis

Table 2.2: Strains generated and/or used in this thesis

2.2.2. List of plasmids

Plasmid Name	Source	Used for
pRS306/Tof1- Gal-CBP- Csm3	Yeeles et al., 2017	Generation of pBAK004 containing Tof1-codon optimised sequence upstream of NATNT2 selection marker
PBAK004 (<i>TOF1</i> -wt- NATNT2)	This thesis	Used as template for site-directed mutagenesis of <i>TOF1</i> codon-optimised gene
tof1-627-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-627-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1-762-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-762-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1-830-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-830-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1-997-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-997-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1-1182-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-1182-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1-901-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-901-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1 810-950Δ-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-810-950Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1 810-870Δ-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-810-870Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1-871-950Δ-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-871-950Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus

tof1 840-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-840-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1 850-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-850-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1 860-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-860-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1 870-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-870-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1 880-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-880-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
tof1 890-NATNT2	This thesis	Used as PCR template to generate fragment: tof1-codon-optimised-890-1238Δ-NATNT2 for yeast transformation into endogenous <i>TOF1</i> locus
pYM13	Janke et al. (2004)	Used for amplification of TAP-tag constructs to target the <i>TOF1</i> ORF
pYM-N32	Janke et al. (2004)	Construction of pYM-N32-TRP by replacing the NsrR gene with the <i>Kluyveromyces lactis TRP1</i> gene
pYM-N32-TRP1	This thesis	Amplification of TRP-GAL S-3xHA construct for N-terminal Tof1 HA-tagging
pRS316	Sikorski, R. S. and Hieter, P. (1989)	Transformed into <i>tof1</i> truncation <i>top2-4</i> strains for fork rotation/catenation assay
4XRFB	Luis Aragon Lab (unpublished)	Used for amplification of 1xRFB sequence for cloning into pRS426
pRS426	Christianson et al. (1992)	Backbone vector for insertion of RFB sequence (amplified from 4XRFB vector) via Gibson assembly to generate pRS426-RFB

pRS426-RFB	This thesis	Transformed into <i>tof1</i> truncation strains for fork pausing assay
pBluescript II KS (+)	Agilent Technologies	Cloning vector used for generation of plasmids pBS-C-TIM-LARA and pBS-N-TIM-LARA
pBS-C-TIM-LARA	This thesis	Intermediate vector used for generation of plasmid pBS-C-TIM-mAID-SMASH-Neo
mKate2_p2a_neo	Hochegger lab	Amplification of P2A and Neomycin resistance gene sequences for integration into pBS-C-TIM-LARA to generate pBS-CTIM-SMASH-mAID-Neo
hCCNB1_mAID_SMASH_T2A_Neo	Hochegger lab	Amplification of SMASH, mAID, T2A and Neo sequences for construction of pBS-CTIM-SMASH-mAID-Neo and pBS-N-TIM-Neo-SMASH-mAID
pBS-N-TIM-LARA	This thesis	Intermediate vector used for generation of plasmid pBS-N-TIM-Neo-SMASH-mAID
H2B-GFP	Hochegger lab	Used as a control to assess efficacy of transfection into RPE-1 OsTIR1 cells
pBS-NTIM-Neo-SMASH-mAID	This thesis	Transfected into RPE-1 OsTIR1 cells for targeting the N-terminus of the <i>TIM</i> gene with SMASH-mAID tag and Neo antibiotic selection marker
pBS-CTIM-mAID-SMASH-Neo	This thesis	Transfected into RPE-1 OsTIR1 cells for targeting the C-terminus of the <i>TIM</i> gene with SMASH-mAID tag and Neo antibiotic selection marker
SpCas9(BB)-2A-Puro (PX459)	(Slaymaker et al., 2015)	Vector used for insertion of <i>TIM</i> N- and C-terminal targeting gRNAs
SpCas9-CTIM	This thesis	Co-transfection with pBS-C-TIM-mAID-SMASH-Neo into RPE-1 OsTIR1 cells to co-express SpCas9 and C-terminal <i>TIM</i> targeting gRNA
SpCas9-NTIM	This thesis	Co-transfection with pBS_N_TIM_mAID_SMASH_Neo into RPE-1 OsTIR1 cells to co-express SpCas9 and N-terminal TIM targeting gRNA

Table 2.3: Plasmids generated and/or used in this thesis

2.2.3. List of oligonucleotides

Oligo	Sequence (5' to 3')	Used for
TOF1_insert_F	gttcgtaagtcgcctcacatatgataataccatc tagcttgtaggggttagtgtatctttaatataggag ggcgcacactatgtctgtgacttgcaaca	Amplification of mutagenised Tof1 codon-optimised sequences for insertion into the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1_insert_R2	tactttgaacttaaaaacagagtaaagaagtg gttctaaaattacacgtattaaagggattaatta ctacatattcattccagtatagcgaccagcattc	Amplification of mutagenised Tof1 codon-optimised sequences for insertion into the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1_627_stop_F w	taagaagggtttctcttctagcacagagtcttcgt ccaagc	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-627</i> truncated sequence
TOF1_627_stop_R	cttggaatgtcacctcaagaaatgtgaacttct ttgtcttctggac	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-627</i> truncated sequence
TOF1_762_stop_F	gaagtcctcagcttggtcgttagttgtgtgcc accattgcac	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-762</i> truncated sequence
TOF1_762_stop_R	gtgcaatggtgggaacaacaactaaacgaac caagctggagacttc	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-762</i> truncated sequence
TOF1_830_stop_F	tctacctgttgacgatggaagtaggaattgtt ggaccaattgtgaag	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-830</i> truncated sequence
TOF1_830_stop_R	cttcaacaattggtccaacaattcctacttaccat cgccaacaaggtaga	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-830</i> truncated sequence

TOF1_901_stop_F	ttggttactctattccaagaaagtagaacgaac catgtttcttgccagg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-901</i> truncated sequence
TOF1_901_stop_R	cctggcaagaacatggttcgttctactttcttg aatagagtaaccaa	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-901</i> truncated sequence
TOF1_997_stop_F	ggacttggataacaacgcttaggacaagttga agggtacca	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-997</i> truncated sequence
TOF1_997_stop_R	tgtacccttcaactgtcctaagcgtgttatcca agtcc	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-997</i> truncated sequence
TOF1_1182_stop_F	cagaaactcctgggttctctgaccatctaact ctcaaaacatg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-1182</i> truncated sequence
TOF1_1182_stop_R	catgttttgagagtagatggtcaggaagaacc caaggagtttctg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1-1182</i> truncated sequence
TOF1_del_810- 950_PAB_F	cgaagaagcttgccaccagattcttctctcac aacg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate internal deletion mutant of amino acids 810-950
TOF1_el_810- 950_PAB_R	cgttgtgagagaaagaatctggtggcaaagct tcttcg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate internal deletion mutant of amino acids 810-950
TOF1_810_870_del _F	cgaagaagcttgccaccattgactggtgttttg aaca	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to

		generate internal deletion mutant of amino acids 810-870
TOF1_810_870_del_R	tggtcaaaacaccagtcgaatggtggcaaagcttcttcg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate internal deletion mutant of amino acids 810-870
TOF1_871_950_del_F	caccaaacgaatacttcaccgattcttctctcaaacga	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate internal deletion mutant of amino acids 871-950
TOF1_871_950_del_R	tcgttgtagagagaaagaatcggtagaagtattctgttggtg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate internal deletion mutant of amino acids 871-950
TOF1_S1	cgtgaagtcgcctcacatatgataataccatctagcttggtgggttagtgatatcttaatataggaggcgcacactatgcgtacgctgcaggctcgac	Amplification of targeting constructs for insertion at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1_S2	ttgaacttaaaaacagagtaaagaagtggctctaaaattacacgtattaaagggttaattactacatattcattctcaatcgatgaattcgagctcg	Amplification of targeting constructs for insertion at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1_S3	aaagcgatgaagatgaagaggccatccgccttttggcaaaaagtctagagtggttttgagccaaaggatgatgatgatcgtagctgcaggctcgac	Amplification of C-terminal TAP-tag targeting construct from pYM13 for insertion at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1_S4	aacaaagcgattctagctctcaagacagtcaaggagaagtcagcagcgtagtagtaccttggtgcaagtcagcagacatcatcgatgaattctctgtcg	Amplification of N-terminal TRP-GAL-HA targeting construct for insertion at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TRP_HA_TOF1_CO_FW	gttcgtaagtcgcctcacatatgataataccatctagcttggtgggttagtgatatcttaatataggaggcgcacacttacgctgctcagtgctagg	Amplification of N-terminal TRP-GAL-HA targeting construct for insertion at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>

TOF1co_wt_S3	aatctgatgaagacgaagaagctatcagattg ttcggaagaagtcagagttgtttgtctcaag gtgactctgacgaccgtacgctgcaggtcgac	Amplification of C-terminal TAP-tag targeting construct from pYM13 for insertion at the <i>TOF1</i> codon optimised ORF at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1co_627_S3	aggtcttgaacaataactctgatgacaagacttt ggcattgaaggtaagtcagaagacaaaag aagttcaacatttctcgtacgctgcaggtcgac	Amplification of C-terminal TAP-tag targeting construct from pYM13 for insertion at aa 627 of the <i>TOF1</i> codon optimised ORF at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1co_762_S3	gaaagtacgtttctcaattctctgattactcttgg ctagattgaagaagagattgaagaagtctcca gcttggttcgttcgtacgctgcaggtcgac	Amplification of C-terminal TAP-tag targeting construct from pYM13 for insertion at aa 762 of the <i>TOF1</i> codon optimised ORF at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1co_830_S3	acgaagaagctttgccaccatctattttgttga catgaagtacggtgttttggctctacctgttga cgatggtaagcgtacgctgcaggtcgac	Amplification of C-terminal TAP-tag targeting construct from pYM13 for insertion at aa 830 of the <i>TOF1</i> codon optimised ORF at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1co_997_S3	acgatccatacattgttcagacgaccaaatttt gtccaagtctgatgctgcttactcaaggacttg gataacaacgctcgtacgctgcaggtcgac	Amplification of C-terminal TAP-tag targeting construct from pYM13 for insertion at aa 997 of the <i>TOF1</i> codon optimised ORF at the endogenous <i>TOF1</i> locus in <i>S. cerevisiae</i>
TOF1co_1182_S3	tcgacgtcaacaacaacaacaaccaatt gtccgacgatgacgtcaactctgaatccagaa actcctgggttcttccgtagcgtgcaggtcga c	Amplification of C-terminal TAP-tag targeting construct from pYM13 for insertion at aa 1182 of the <i>TOF1</i> codon optimised ORF

at the endogenous *TOF1* locus in
S. cerevisiae

Ascl_TRP_FW	aattggcgcgccttacgctgctcagtgctaggw	Amplification of <i>TRP1</i> gene from pYM22 with Ascl restriction site for cloning into pYM-N32
Mfel_TRP_RV	catacaattgcatcgatgaattcgagctcg	Amplification of <i>TRP1</i> gene from pYM22 with Mfel restriction site for cloning into pYM-N32
RAD9_S1	aacgccatagaaaagagcatagtgagaaaa tcttcaacatcagggtatgcgtacgctgcagg tcgac	Amplification of deletion constructs targeted to the endogenous <i>RAD9</i> gene in <i>S. cerevisiae</i>
RAD9_S2	ttaatcgccctttctatcaattatgagtttatattt ttataattcaatcgatgaattcgagctcg	Amplification of deletion constructs targeted to the endogenous <i>RAD9</i> gene in <i>S. cerevisiae</i>
MRC1_S1	ctaaggaagttcgttattcgctttgaacttatcac caaataatttagtgcgtacgctgcaggctcgac	Amplification of deletion constructs targeted to the endogenous <i>MRC1</i> gene in <i>S. cerevisiae</i>
MRC1_S2	aagacagcttctggagttcaatcaacttcttcg aaaagataaaaaaccaatcgatgaattcgag ctcg	Amplification of deletion constructs targeted to the endogenous <i>MRC1</i> gene in <i>S. cerevisiae</i>
TOF1_840_stop_F	aattgttggaaccaattgtgaagcactagactca caccttgatattttcaagtc	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1 840</i> truncated sequence
TOF1_840_stop_R	gacttgaaaatatccaaggtgtgagtctagtc ttcaacaattggtccaacaatt	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1 840</i> truncated sequence
TOF1_850_stop_F	ccttgatattttcaagtcttagtgactgttaacgt taacgc	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to

		generate <i>tof1</i> 850 truncated sequence
TOF1_850_stop_R	gcgttaacgttaacagtcaactaagacttgaaa atatccaagg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1</i> 850 truncated sequence
TOF1_860_stop_F	cgtaacgctggaagtaaaccgtcaaccac c	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1</i> 860 truncated sequence
TOF1_860_stop_R	ggtgggtgacggtttactaccagcgtaacg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1</i> 860 truncated sequence
TOF1_870_stop_F	caaccaccaaacgaatacttctagtgtgactg gtgtttgaacaacg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1</i> 870 truncated sequence
TOF1_870_stop_R	cgttgttcaaaacaccagtcactagaagtatt cgttggtgggttg	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1</i> 870 truncated sequence
TOF1_880_stop_F	ctggtgtttgaacaacgatccatagtcaagga caaggattacagagc	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1</i> 880 truncated sequence
TOF1_880_stop_R	gctctgtaatcctgtcctgaactatggatcggtg ttcaaaacaccag	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1</i> 880 truncated sequence
TOF1_890_stop_F	gacaaggattacagagctttagttagttgattg gttactctatt	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1</i> 890 truncated sequence

TOF1_890_stop_R	aatagagtaaccaatcaacaactacaaagct ctgtaatcctgtc	Mutagenesis of <i>TOF1</i> codon optimised ORF in pBAK004 to generate <i>tof1 890</i> truncated sequence
RFB1_pRS426_Gib son_FW	aacctctgacacatgcagctaggccttctgca gatttc	Amplification of RFB sequence from plasmid 4xRFB with homologous arms to vector pRS426 for Gibson assembly of RFB sequence into pRS426
RFB1_pRS426_Gib son_RV	cagacaagctgtgaccgtcttctgcagtttatt ctttctaag	Amplification of RFB sequence from plasmid 4xRFB with homologous arms to vector pRS426 for Gibson assembly of RFB sequence into pRS426
N_Tim_gRNA_Top	caccgttggttggtccactgatgta	Insertion of <i>TIMELESS</i> targeting sgRNA into SpCas9 plasmid for CRISPR/Cas9 genome editing of <i>TIMELESS</i> (N-terminal)
N_Tim_gRNA_Botto m	aaactacatcagtggaaccaaac	Insertion of <i>TIMELESS</i> targeting sgRNA into SpCas9 plasmid for CRISPR/Cas9 genome editing of <i>TIMELESS</i> (N-terminal)
N_Tim_RA	tgtgcaagtcaagcttacatcagtggaaccaac caac	Construction of plasmid pBS-N- TIM-LARA
N_Tim_Pbs_2	cgaggtcgacggtatcgataaaaagctaggg aggcaac	Construction of plasmid pBS-N- TIM-LARA
N_Tim_LA	ccactgatgtaagcttgactgcacatgatgaa ctg	Construction of plasmid pBS-N- TIM-LARA
N_Tim_Pbs_1	gctgcaggaattcgatatcagagcctcttggtt tgatc	Construction of plasmid pBS-N- TIM-LARA
N_Tim_pBS_3	gctgcaggaattcgatatcaatggattacaag gatgacgacgataagggc	Construction of plasmid pBS- NTIM-Neo-SMASH-mAID
N_Tim_mAID	atttctccttaggcgcgcccagagcccat	Construction of plasmid pBS- NTIM-Neo-SMASH-mAID

N_Tim_SMASh	gggcgcgcctaaggagaaatcagcctgc	Construction of plasmid pBS-NTIM-Neo-SMASh-mAID
N_Tim_pBS_4	cgaggtcgacgggatcgatatgctccggctccc ttatacatcctcaggtcgatc	Construction of plasmid pBS-NTIM-Neo-SMASh-mAID
N_Tim_pBS_LARA_1	ttggttggtccactgatgtatgattgaacaagat ggattgc	Construction of plasmid pBS-NTIM-Neo-SMASh-mAID
N_Tim_SMASH_mAID	tgtaatccattgggccaggattctcctc	Construction of plasmid pBS-NTIM-Neo-SMASh-mAID
N_Tim_Neo_T2A	tcctggcccaatggattacaaggatgacgac	Construction of plasmid pBS-NTIM-Neo-SMASh-mAID
N_Tim_pBS_LARA_2	cagttcatcatgtgcaagtctgctccggctccctt atac	Construction of plasmid pBS-NTIM-Neo-SMASh-mAID
C_Tim_gRNA_Top	caccgcagagttggaagggcatcac	Insertion of <i>TIMELESS</i> targeting sgRNA into SpCas9 plasmid for CRISPR/Cas9 genome editing of <i>TIMELESS</i> (C-terminal)
C_Tim_gRNA_Bottom	aaacgtatgaccctccaactctgc	Insertion of <i>TIMELESS</i> targeting sgRNA into SpCas9 plasmid for CRISPR/Cas9 genome editing of <i>TIMELESS</i> (C-terminal)
C_Tim_pBS_LA_ATT	ggctgcaggaattcgatatcggccctgtagg aaaccttc	Construction of plasmid pBS-C-TIM-LARA
C_Tim_RA_HIND_LA_ATT	tcttagctctaagcttgatcctcatcatcctcaa tctg	Construction of plasmid pBS-C-TIM-LARA
C_Tim_LA_HIND_RA_ATT	tgaggatgacaagcttagagctaagaagcct aggggtagagatagacacgtttttagatggtgc attcaagtcagagttggaagggcatcacattac ccagaagctcc	Construction of plasmid pBS-C-TIM-LARA
C_Tim_pBS_RA_ATT	tcgaggtcgacgggatcgatagggtgggga acttagac	Construction of plasmid pBS-C-TIM-LARA
pBS_C_Tim_LARA	ttgaggatgatgaggatgacggcgctcagcg gcatca	Construction of plasmid pBS-CTIM-mAID-SMASh-Neo
P2A_Neo	agttagtagcatcacaggacttctcggtcaggga tgatagc	Construction of plasmid pBS-CTIM-mAID-SMASh-Neo

mAID_SMASh	agtctgtatgctactaacttcagcctgc	Construction of plasmid pBS-CTIM-mAID-SMASh-Neo
pBS_C_Tim_LARA	ctaccctaggcttcttagctcttcacagaaga actcgtcaag	Construction of plasmid pBS-CTIM-mAID-SMASh-Neo

Table 2.4: Primers generated and used in this thesis

2.3. Yeast Techniques

2.3.1. Yeast Genetic Techniques

2.3.1.1. *Lithium acetate (LiOAc) yeast transformation*

For yeast transformation 10 ml of log phase yeast culture was first pelleted by centrifugation at 3500 rpm for 5 min, washed once in 2 ml of water and centrifuged again, this time at 3000 rpm for 5 min. The cells were washed once in 250 µl water and once in 250 µl LiOAc-TE (0.1 M LiOAc, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) with 1 min centrifugations at 8000 rpm in between. The resulting cells were resuspended in 50 µl LiOAc-TE before addition of 5 µl salmon sperm DNA (Invitrogen 15632011) (pre-boiled for 5 min and kept on ice), 1 µg of plasmid or linear DNA, and 300 µl LiOAc-TE in 40% PEG. Cells were incubated for 45 min at 25°C with shaking at 850 rpm before addition of 40 µl DMSO (Fisher scientific D/4121/PB08). Cells were heat shocked in a 42°C water bath for 15 minutes followed by an immediate 2 min incubation on ice. Cells were centrifuged at 8000 rpm for 1 min and resuspended in 1ml YPD, followed by either a 1 hr incubation (for transformation of auxotrophic markers e.g. *URA3*) or overnight (for transformation of antibiotic resistance selection markers e.g. NAT) before resuspension of the cells in 1 x TE and plating onto the appropriate selection plates. For plasmid transformations, 10% and 90% of the transformation mixture was plates onto two plates respectively. For linear transformations the entire transformation mixture was plated to one plate. Transformation plates were incubated at 25°C for 48 hrs.

2.3.1.2. *Yeast colony PCR*

For colony PCR a small amount of yeast colony was first inoculated in 50 µl of water and boiled for 10 min. Taq polymerase (Fisher) was used to amplify products, according to the manufacturer's instructions and using 5 µl of boiled colony as the template DNA. Unless otherwise stated two sets of primers were used for all colony PCR reactions to generate products overlapping with the 5' and the 3' insertion sites to check for site-specific integration of targeting constructs.

2.3.1.3. Genetic crosses

To cross yeast strains, a small amount each of the mating type a and α strains to be crossed were mixed on a YPD plate and incubated for 24 hrs at 25°C. Crosses were then streaked to give single colonies which were transferred to RSM plates. Sporulation was confirmed by visualisation of tetrads following 2-5 days of growth on RSM.

2.3.1.4. Tetrad dissection

Spores generated by genetic crosses were inoculated in 250 μ l water and treated with 1 μ l zymolase (AMS Biotech, 120493-1) at room temperature for 5 min. The tetrads were centrifuged at 2000 rpm for 2 min and resuspended in water before being plated to the edge of a YPD plate and dissected using a Singer tetrad dissector (Singer MSM400).

2.3.2. Yeast Cellular Biology Techniques

2.3.2.1. Cell-Cycle Synchronisation and Time Courses

For plasmid catenation experiments in *top2-4* pRS316-containing strains, cells were grown to mid-log phase in -URA dropout minimal media +2% glucose, before being re-suspended in YPD. Cells were arrested in G1 by addition of 10 μ g/ml alpha factor peptide (Genscript custom order: peptide sequence WHWLQLKPGQPMY) for 1.5 hrs, after which a second dose of alpha factor (5 μ g/ml) was added. When >90% of cells were unbudded, cultures were shifted to the restrictive temperature for *top2-4* (37°C), for one hour before release into S-phase by washing three times with YPD at 37°C. Time 0 indicates the time from addition of the first wash. 50 μ g/ml nocodazole was added to cultures at 45 minutes to prevent mitotic entry, and at 80 minutes from release 10 ml samples for 2D gel and Southern blotting analysis were collected by centrifugation and snap-freezing the pellets in liquid nitrogen.

For fork pausing experiments cells containing pRS426-RFB were grown to mid-log phase in -URA dropout minimal media +2% glucose. 10 ml cultures were collected by centrifugation and snap-freezing the pellets in liquid nitrogen.

For experiments involving treatment with HU, cells were grown in YPD to mid-log phase before either addition of 200 mM for 120 minutes (for Rad53 activation experiments in Chapter 5: Figure 5.1), or for experiments started with a G1 arrest, mid-log cells were first arrested in G1 in the same way as is described for catenation assays (see above). To release cultures into the cell cycle, cultures were washed 3 times in YPD and re-suspended after the third wash in YPD containing 200 mM HU. Time 0 was designated as the time from the first wash. Time points were taken as indicated. For release from HU experiments, HU-containing media was washed off of cells by washing 3 times with YPD before re-suspending in YPD. Time 0 is taken as the time from the first YPD wash.

2.3.2.2. FACS analysis of DNA content

For analysis of cell cycle progression, 0.5 ml of yeast culture was pelleted by centrifugation at 13000 rpm for 15 seconds before removal of all growth media. The pellets were re-suspended in 0.5 ml 70% ethanol and stored at 4°C before processing and analysis.

Fixed cells were washed in 50 mM Tris pH 8.0 and 10 mg/ml RNaseA (Sigma-Aldrich) was added. Cells were incubated overnight at 37°C, pelleted and re-suspended in freshly made 5 mg/ml pepsin in 5 mM HCl and incubated again at 37°C for 30 min. Fixed cells were washed once more in 50 mM Tris pH 8.0 and re-suspended in 0.5 mg/ml propidium iodide in 50mM Tris pH 8.0. Samples were sonicated for 5 seconds each on low power to reduce clumping before analysis using the BD Accuri™ C6 Plus (BD Biosciences).

2.3.2.3. Drug sensitivity assays

Yeast cells were grown to mid-log phase before being serially diluted 10-fold in YPD. 5 µl of each dilution was spotted onto YPD plates containing the indicated dose of drug or control reagent and incubated for 24-28 hr at 25°C or 30°C before imaging.

2.3.2.4. Colony Survival Assays

Yeast cells were grown to mid-log phase before being arrested in G1 by the addition of 10 µg/ml alpha factor peptide. When cells were >90% unbudded they were released into the cell cycle in the presence of 200 mM HU for 1 hr. Following the HU treatment cells were counted, diluted in YPD medium and plated onto YPD plates. Colonies were counted 48 hrs after plating and the viability was calculated as the percentage of plated cells able to form colonies. Statistical significance was calculated using a two-tailed unpaired Students *t-test*.

2.3.3. Yeast Molecular Biology Techniques

2.3.3.1. TCA Protein Extraction from Yeast Cells

10 ml of mid-log yeast cultures were centrifuged at 3500 rpm for 5 min and the resulting cell pellets were snap-frozen in liquid nitrogen before storing at -80°C. All further steps were carried out on ice, and centrifugation and homogenisation steps at 4°C. 200 µl of 20% TCA was added to thawed cell pellets and the cell suspension was transferred to screw-cap tubes containing 500 µl of 0.5mm zirconia/silica beads (BioSpec Products). Cells were homogenised using a FastPrep-24 (MP Biomedicals) on max speed (6.5 m/s) for 4 x 1 min pulses, with 1 minute on ice in between pulses. Beads were separated from the lysate by piercing the tubes and centrifugation of the mixtures into fresh tubes at 3000 rpm for 2 min. Beads were washed once with 600 µl 5% TCA and centrifuged again into tubes containing the cell lysate. Cell extract/TCA mixtures were then centrifuged at 13000 rpm for 5 min before removing all TCA from the resulting pellets. This step was repeated once to ensure complete removal of all TCA. To the pellets 200 µl of 1 X sample buffer was added before boiling samples for 5 min. Samples were spun at 13000 rpm for 5 min and the resulting supernatants were collected and stored at -20°C for SDS-PAGE and western blotting analysis.

2.3.3.2. TAP pulldowns

200 ml of mid-log yeast cultures were centrifuged for 5 min at 3500 rpm and washed once in PBS. The resulting cell pellet was snap-frozen in liquid nitrogen and stored at -80°C

before proceeding. Frozen pellets were re-suspended in 0.5 ml of ice-cold lysis buffer (50 mM HEPES pH 7.5, 300 mM KCl, 5 mM EDTA, 10% Glycerol, 80 mM Beta-glycerophosphate, 0.01% Triton X-100, 1 protease inhibitor tablet (cOmplete™, EDTA-free Protease Inhibitor Cocktail, Roche) and 1 phosphatase inhibitor tablet (PhosSTOP™, Roche)) in screw-cap tubes. 300 µl of zirconia/silica beads (BioSpec Products) were added and the cell suspensions were homogenised using the FastPrep-24 (MP Biomedicals) on max speed (6.5 m/s) for 4 pulses of 1 min each, with 3 min on ice in between each pulse. The resulting lysate was transferred to fresh tubes and cleared by centrifugation at 13000 rpm for 5 min. Cleared supernatants were then transferred to fresh tubes before starting the pulldown. Before addition of beads, 5% input sample was taken (typically 1% of the input was loaded for western blot analysis). 10 µl of IgG Sepharose 6 Fast Flow affinity resin (GE-Healthcare, #17096901) for each pulldown were pre-washed 3 times in cold lysis buffer before being added to the cleared cell lysates. The lysate/bead mixtures were incubated at 4°C on a rotating platform for 2 hrs and subsequently poured into Poly-Prep® Chromatography Columns (Bio-Rad #7311550). Unbound fractions were collected through the columns and stored at -20°C. The beads were washed 4 times in the columns with 1 ml of cold lysis buffer before the bound fraction was eluted in 0.5 ml of 0.2 M glycine pH 3.0. Input, unbound and eluted proteins were precipitated by TCA extraction before running on 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad #4561096) and western blotting was performed as described.

2.3.3.3. ChIP-Seq, Sync-Seq and Bioinformatic Analysis

For materials and methods relating to ChIP-Seq and bioinformatic analysis (carried out by Dr Andrea Keszthelyi) see Appendix I.

2.4. General Molecular Biology Techniques

2.4.1. *E. coli* transformation

For *E. coli* transformations, 5-alpha Competent Cells (New England Biolabs C2987H) were used and transformed according to the supplier's instructions. Transformed cells were plated onto LB plates + Amp and incubated at 37°C overnight.

2.4.2. DNA extraction from *E. coli*

To extract plasmid DNA from *E. coli* cells, single colonies were inoculated and grown overnight in 5 ml LB media + Amp at 37°C, before DNA extraction using QIAprep Spin Miniprep Kits (QIAGEN 27104) according to manufacturer's instructions.

2.4.3. Restriction digests

All restriction enzyme digestions were carried out according to manufacturer's instructions. Enzymes were supplied by New England Biolabs.

2.4.4. DNA Purification of PCR products

PCR products were cleaned-up either using QIAquick PCR Purification Kits (QIAGEN 28104), or for in-gel DNA extractions, Monarch® DNA Gel Extraction Kits (NEB T1020) were used. All PCR clean-up kits were used according to manufacturer's instructions.

2.4.5. Gibson Assembly

To construct the pRS426-RFB, pBS-C-TIM-SMASH-mAID and pBS-NTIM-Neo-SMASH-mAID vectors, NEBuilder® HiFi DNA Assembly Cloning Kits (New England Biolabs E5520S) were used according to manufacturer's instructions. The NEBuilder online primer design tool was used to design primers for amplification of the fragments to be assembled (New England Biolabs 2020).

2.4.6. Polymerase Chain Reaction (PCR)

All polymerases used according to manufacturer's instructions

Polymerase	Supplier/Product code	Application
Taq DNA Polymerase	Thermo Fisher Scientific EP0402	Yeast colony PCR and genotyping PCR in RPE-1 OsTIR1 cells
Phusion® High-Fidelity PCR Master Mix	New England Biolabs M0531	Amplification of products from plasmid and genomic DNA (from RPE-1 OsTIR1 cells) for use in Gibson Assembly reactions
KOD Hot Start Polymerase	Merck Millipore 71086	Amplification of linear products from plasmid DNA for yeast transformation

Table 2.5: Polymerases used for PCR reactions in this thesis

2.4.7. Site-Directed Mutagenesis of *TOF1* plasmids

The codon optimised *TOF1* sequence in vector pBAK004 was mutagenised using QuikChange Lightning Site Directed Mutagenesis kits (Agilent, 210518) according to manufacturer's instructions, with primer sets designed to incorporate premature stop codons into the *TOF1* open reading frame (see table 2.4 for primers). The Agilent online mutagenesis primer design tool was used to design mutagenic primers (Agilent n.d.).

2.4.8. Construction of vectors for CRISPR-Cas9 genome editing

Two types of vectors were generated for CRISPR-Cas9 genome editing at the endogenous *TIM* locus in RPE-1 cells. Vector SpCas9 (see table 2.2.2) containing the *Streptococcus pyogenes* Cas9 endonuclease was modified to incorporate guide RNA (gRNA) sequences which were chosen using the Benchling online CRISPR gRNA tool (Benchling [Biology Software]. (2020). Retrieved from <https://benchling.com>) (TTGGTTGGTCCACTGATGTA or CAGAGTTGGAAGGGTCATAC for N- and C-terminal *TIM* targeting, respectively). Oligos containing the desired gRNA sequences were annealed and cloned into vector SpCas9 as described by Ran et al. (2013).

To make the donor template for C-terminal *TIM* SMASh-mAID gene targeting (plasmid pBS-C-TIM-mAID-SMASh-Neo), Gibson assembly was used to assemble a homologous left arm (*Homo sapiens* chromosome XII 56417719 to 56418370), a linker sequence (Hégarat et al., 2020), mAID (Nishimura et al., 2009), SMASh (Chung et al., 2015), P2A, Neomycin resistance gene (P2A and Neomycin sequences were gifts from Hochegger lab) and a homologous right arm (*H. sapiens* chromosome XII 56417082 to 56417715) into the HindIII site of pBluescript. As the gRNA target sequence chosen for C-terminal CRISPR-Cas9 editing was situated in 'right arm' genomic region, the protospacer adjacent motif (PAM) site in the right arm was mutated as follows: 5' CAGAGTTGGAAGGGTCATACAGG'3 to 5' CAGAGTTGGAAGGGTCATACATT'3 to prevent re-cutting by SpCas9 after insertion.

To make the donor template for N-terminal *TIM* SMASh-mAID gene targeting (plasmid pBS-N-TIM-Neo-SMASh-mAID), Gibson assembly was used to assemble the left arm (*H. sapiens* chromosome XII 56434171-56434429), Neomycin resistance gene, T2A (Neomycin and T2A sequences were gifts from Hochegger lab) SMASh (Chung et al., 2015), mAID (Nishimura et al., 2009), a linker sequence (Hégarat et al., 2020) and the right arm (*H. sapiens* chromosome XII 56433755-56434167) into the HindIII site of pBluescript.

The left and right arm sequences for all gene targeting were Phusion PCR-amplified from genomic DNA extracted from RPE-1 OsTIR1 cells.

2.4.9. DNA Purification for Southern Blotting

Frozen pellets were re-suspended in 0.4 ml of DNA Extraction Buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 1% SDS) along with 40 Units of lyticase (Sigma-Aldrich, L2524) and 5 μ l 2-mercaptoethanol (Sigma Aldrich, 63689). Samples were incubated at 37°C for 5 min before addition of 450 μ l phenol/chloroform/isoamylalcohol (25:25:1, Sigma-Aldrich) and mixing by rotation. Phase lock tubes (5 Prime, 2302800) were used to collect the aqueous layer, by centrifugation for 5 min at 12000 rpm. DNA was ethanol precipitated by addition of roughly 2 x volume of 100% EtOH and washed once in 70% EtOH before air-drying and solubilisation in 10 mM Tris pH 8.0.

For plasmid DNA catenation analysis of pRS316, purified DNA was nicked with Nb.Bsm1 (New England Biolabs, R0706S) according to manufacturer's instructions.

For fork pausing analysis of pRS416-RFB, purified DNA was digested with BamHI-HF (New England Biolabs, R3136S) and SnaBI (New England Biolabs, R0130L) according to manufacturer's instructions.

After nicking/digestion the DNA was precipitated, washed and solubilised as above with the addition of 300 mM Sodium Acetate pH 5.2 at the first ethanol addition.

2.4.10. 2D Gel Electrophoresis of replication intermediates or catenated plasmid replication products

Purified DNA was separated in the first dimension by electrophoresis in 0.4% MegaSieve/MegaBase Agarose (Scientific Laboratory Supplies, H15608), 1X TBE (90 mM Tris, 90 mM Boric Acid, 10 mM EDTA).

For DNA catenation analysis of plasmid pRS316 from *top2-4* cells, 1st dimension gels were run at room temperature for 16-18 hrs at 30V. A portion of the gel containing small amount of each DNA sample was excised and stained in 0.5 μ g/ml Ethidium Bromide 1XTBE to reveal the extent of genomic DNA mobility. The remaining non-stained gel slices containing the plasmid were excised and embedded in 1.2%

MegaSieve/MegaBase Agarose 1X TBE and run in the 2nd dimension in 1XTBE at 4°C for 16-17 hrs, 120V.

For analysis of paused replication intermediates from plasmid pRS426-RFB, for 1st dimension gels were run at room temperature for 15.5 hrs at 30V. 1st dimension gels were stained in 0.5 µg/ml Ethidium Bromide in 1XTBE and gel slices containing the replication intermediates were excised and embedded in 1% MegaSieve/MegaBase Agarose 1X TBE 0.3 µg/ml Ethidium bromide gels. 2nd dimension gels were run in 1XTBE at 4°C for 8 hrs, 120V with re-circulation of the running buffer.

2.4.11. Southern Blotting

Following 2D electrophoresis, gels were washed sequentially in Depurination buffer (0.125M HCl), Denaturation buffer (0.5 M NaOH, 1.5 M NaCl) and Neutralisation buffer (0.5 M Tris-HCl, 1.5 M NaCl pH 7.5) with washes in ddH₂O in-between each buffer. DNA was transferred onto Hybond-N+ membrane (GE Healthcare) by capillary action in 20X SSC (3M NaCl, 350 mM NaOC Trisodium Citrate pH 7.0). Membranes were cross-linked using a UV Stratalinker 1800 (Stratagene) at 1200J/m and subsequently blocked in hybridisation buffer (5X SSC, 5% Dextran sulphate (Sigma-Aldrich, D8906) 0.2% Tropix I-Block (Applied Biosystems, T2015), 0.1% SDS) for at least 1 hr at 60°C.

Catenated pRS316 plasmids or replication intermediates from pRS416-RFB were probed with DNA amplified from pRS316 (probing specifically for the *URA3* gene). Labelling and detection used random prime labelling incorporating fluorescein tagged dUTP (Roche). Following probing, hybridized fluorescein tagged dUTP was detected with alkaline phosphatase tagged anti fluorescein Fab fragments (Roche), revealed with CDP-Star (GE Healthcare) and non-saturating exposures acquired on an ImageQuant LAS4000 system (GE Healthcare). Densitometry analysis was carried out using ImageQuant TL software.

2.4.12. SDS-PAGE and western blotting

Protein extracts prepared by TCA extraction were run on either 8%, 10% or 12% SDS-PAGE gels before being wet-transferred to nitrocellulose membranes at 50V for 90 min,

4°C. Proteins were visualised by staining in Ponceau for 30 seconds before membranes were blocked in 5% milk (Marvel) PBS 0.2% Tween-20 (PBS-T). All primary antibodies were diluted in 5% milk PBS-T and incubated overnight at 4°C. In between primary and secondary antibody incubations membranes were washed 3 times with PBS-T for 15 min. Secondary antibodies were diluted in 5% milk PBS-T and incubated with membranes for 1 hr at room temperature. See table 2.6 for all antibodies used. Proteins were detected using Western Lightning Plus-ECL (Perkin-Elmer, NEL104001EA) and images were acquired on an ImageQuant LAS4000 system (GE Healthcare). Densitometry analysis was carried out using ImageQuant TL software.

Antibody & Species reactivity	Raised in	Supplier/Product code	Dilution used
α -Oct-A (FLAG)	Mouse	Santa Cruz Biotechnologies sc-166355	1:1000
α -Csm3 (<i>S. cerevisiae</i>)	Sheep	Labib lab (Gambus et al., 2006)	1:2000
Peroxidase anti-peroxidase (PAP)	Mouse	Sigma-Aldrich P1291	1:1000
α -HA	Mouse	Roche 12CA5	1:1000
α -Rad53 (<i>S. cerevisiae</i>)	Rabbit	Abcam ab104232	1:2000
α -Timeless (<i>Homo sapiens</i>)	Mouse	Abcam ab50943	1:1000
α -Timeless (<i>H. sapiens</i>)	Mouse	Santa-Cruz Biotechnologies sc-393122	1:1000
α -GAPDH (<i>H. sapiens</i>)	Mouse	Abcam ab8245	1:1000
α -mouse-HRP	Rabbit	Dako P0260	1:1000
α -rabbit-HRP	Goat	Dako P0448	1:1000
α -sheep-HRP	Donkey	Sigma-Aldrich A3415	1:10000

Table 2.6: Primary and secondary antibodies used in this thesis

3. Generation and Characterisation of C-terminal Tof1 mutants in *S. cerevisiae*

3.1. Introduction

As discussed in chapter 1.3, Timeless family proteins are evolutionarily conserved, with orthologues found in all eukaryotes. In the budding yeast *S. cerevisiae*, this orthologue is “topoisomerase interacting factor 1”, or Tof1, a replisome-associated protein with numerous roles in replication fork stability and genome maintenance. Despite advances in our understanding of the numerous roles that Tof1 performs, our knowledge of these functions comes almost entirely from work carried out in *tof1Δ* mutants. This not only limits our understanding of how these proteins carry out their roles in detail, but gives no information as to how Tof1 structure relates to its multiple functions.

Consequently, a more detailed understanding of Tof1 requires a more comprehensive structure-function analysis. This chapter aims to address this problem by generating a series of specific Tof1 mutants in *S. cerevisiae* cells. These mutants, once characterised, can later be used to study the contribution of different Tof1 domains to its various functions in DNA replication and genome stability.

3.2. Results

3.2.1. Generation of C-terminal Tof1 mutants in *S. cerevisiae*

To carry out a structure-function analysis of the Tof1 protein in budding yeast, I started by using site-directed mutagenesis to create a series of C-terminal truncation mutations in the Tof1 protein. Generation of Tof1 mutations at the endogenous yeast Tof1 locus involved several steps: first, a base vector was constructed by Dr Andrea Keszthelyi, by insertion of a *TOF1* gene sequence codon-optimised for yeast expression (Yeeles et al., 2017) (gift from John Diffley) into the NotI site upstream of the natNT2 gene of vector pFA-natNT2 (Janke et al., 2004). Codon-optimisation of the *TOF1* gene is necessary for cloning steps due to the toxicity of this sequence to *E. coli* cells (Foss, 2001). This generated vector pBAK004 which was then utilised for all subsequent mutagenesis (Figure 3.1A). The codon-optimised *TOF1* of pBAK004 was then subjected to site-

directed mutagenesis using commercial PCR-based kits and primers designed to introduce premature stop codons into the *TOF1* ORF (Figure 3.1B). Successful mutagenesis of the vectors was confirmed by sequencing. Primers were subsequently designed to amplify the mutagenised *TOF1* sequences from these plasmids with 5' and 3' arms homologous to the upstream and downstream regions of the endogenous *TOF1* locus on *S. cerevisiae* chromosome XIV. This allowed for transformation of the codon-optimised, mutagenised sequence into the *TOF1* locus to ensure that the mutant alleles were expressed from the endogenous *TOF1* promoter (Figure 3.1C).

Initially, this thesis aimed to focus on the C-terminus of the Tof1 protein due to the proposed interaction of this region with the type IIB topoisomerase, Top1 in budding yeast (Park and Sternglanz, 1999), and the potential for this finding to overlap significantly with the interests of the Baxter lab in DNA topology. I reasoned that disruption of the Tof1 C-terminus and, as a consequence, its interaction with Top1, would enable me to investigate the contribution of this interaction to the known roles of Tof1 in budding yeast cells. Therefore, using the approach described above, C-terminal Tof1 truncation mutants were generated with premature stop codons at positions aa 627, aa 762, aa 830, aa 997 and aa 1182 of the 1238 amino acid protein (Figure 3.1D).

An important consideration is the possibility that these Tof1 C-terminal truncation mutants could be especially disruptive to protein structure and function if the cut-off point of a particular truncation resides within a feature of protein secondary structure. These secondary structure features, such as α -helices and β -sheets, are closely related to both the overall fold of the protein and its inherent functions. At the time of generation of the Tof1 C-terminal truncation mutants, no structural data was available for the Tof1 protein, making predictions into the effect of truncations on the secondary structure of the protein more difficult. Instead, the Jpred secondary structure prediction tool was used to see whether the truncations generated could potentially disrupt a predicted feature of secondary structure (Drozdetskiy et al., 2015). The results of this prediction are summarised in Figure 3.2. Of the five mutants described in Figure 3.1, *tof1-627* and *tof1-1182* are predicted to truncate the protein in unstructured regions (Figure 3.2). The other three truncations, *tof1-762*, *tof1-830* and *tof1-997*, are predicted to truncate the protein close to a secondary structure boundary, but not directly inside it (Figure 3.2). Whilst only a prediction, this suggests that the truncations generated do not disrupt any major features of secondary structure within the Tof1 protein.

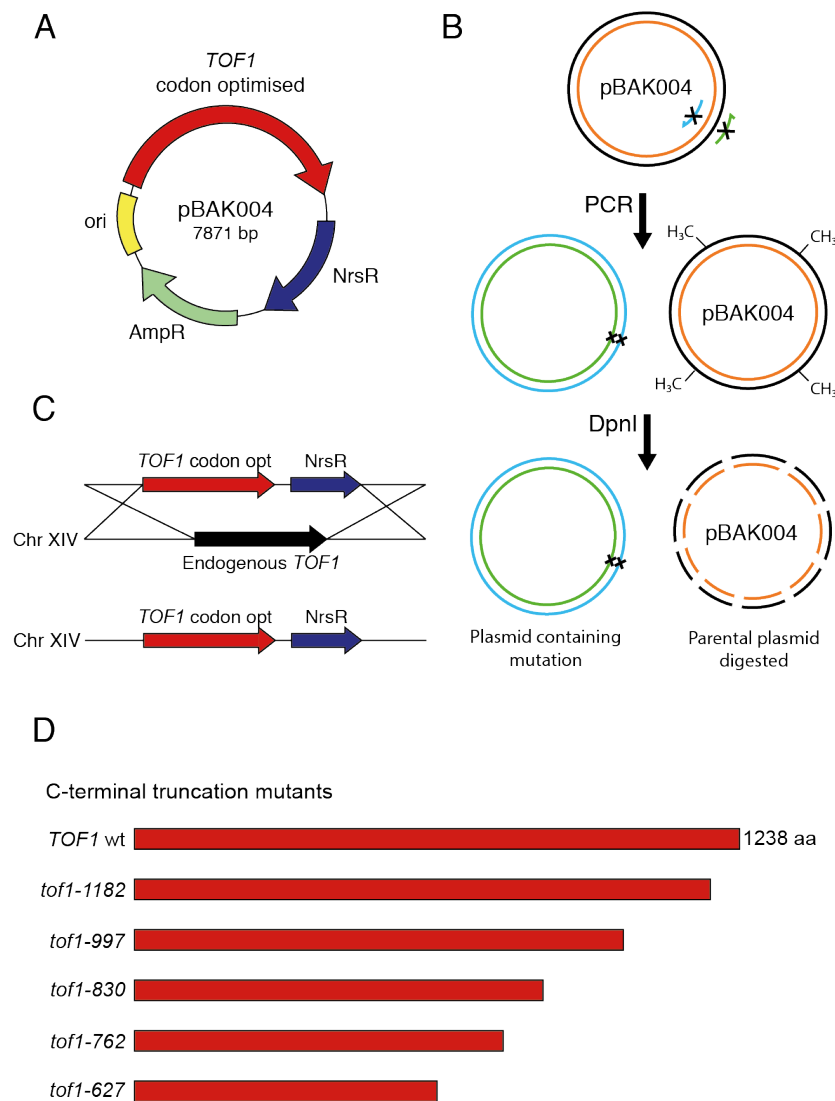


Figure 3.1: Schematic of workflow to generate Tof1 mutants in *S. cerevisiae* cells

A) Schematic map of plasmid pBAK004 used for all site-directed Tof1 mutagenesis. pBAK004 was generated by insertion of a *TOF1* ORF, codon-optimised for yeast expression (Yeeles et al., 2017) (gift from John Diffley) into pFA6a-natNT2 (Janke et al., 2004). pBAK004 was generated by Dr. Andrea Keszthelyi.

B) Plasmid pBAK004 (black/orange) was mutagenised by PCR using primers designed to incorporate premature stop codons into the *TOF1* codon optimised ORF. Mutagenic primers denoted with 'x'. After several rounds of amplification with the mutagenic primers this results in a new vector containing the desired mutations (light blue/green). Incubation of the PCR reaction with the restriction enzyme DpnI results in digestion of the template (non-mutagenised) DNA only, due to template DNA extracted from *E. coli* cells being methylated compared to the newly synthesised DNA.

C) A gene targeting cassette amplified from mutagenised pBAK004 plasmids, consisting of the entire *TOF1* ORF and Nourseothricin resistance gene, was inserted at the endogenous *TOF1* locus on chromosome XIV in wild-type *S. cerevisiae* cells (in the w303 background). Cells were selected for integration by growth on plates containing NAT and PCR was used to determine correct integration at the *TOF1* locus.

D) Schematic of C-terminal Tof1 truncation mutants generated in this study.

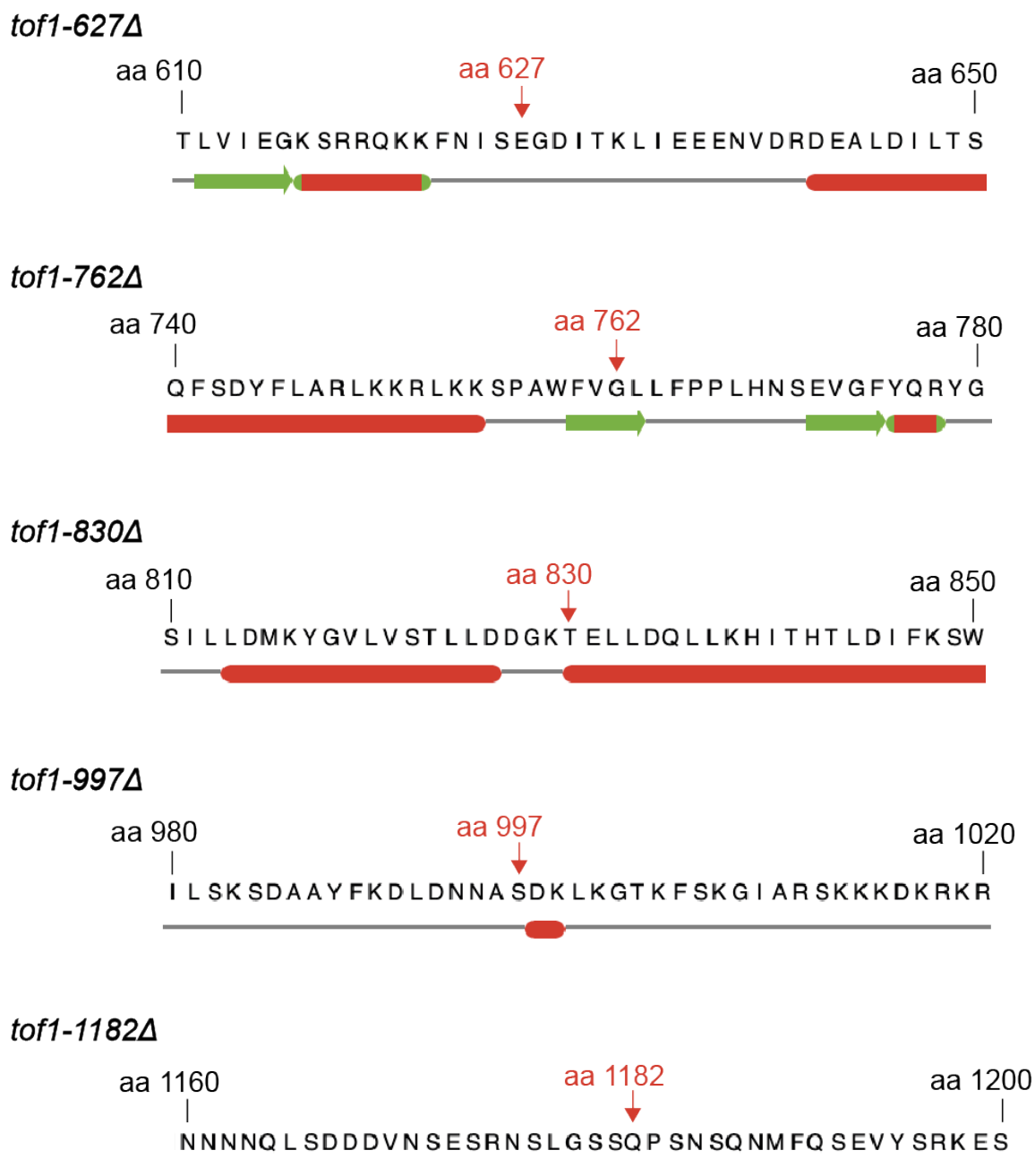


Figure 3.2: Tof1 truncation mutant cut-off sites compared with Tof1 predicted secondary structure

Schematic representations of the position of each Tof1 truncation cut-off point compared to secondary structure prediction of the Tof1 C-terminus. Secondary structure prediction was obtained from the Jpred Protein Secondary Structure Prediction Server (Drozdetskiy et al., 2015). Red tubes = predicted α -helices. Green arrows = predicted β -sheets.

3.2.2. Codon-optimised Tof1 truncations at residues 627, 762, 830, 997 and 1182 are stably expressed in cells at wild-type levels

Having generated Tof1 mutants lacking significant portions of the protein it was essential to determine whether these Tof1 mutants were stably expressed in cells before carrying out functional analyses. To assess the expression levels of the Tof1 truncation mutants in cells, C-terminal epitope tagging and western blot analysis was performed. Briefly, Tandem Affinity Purification (TAP) tags were introduced into the C-terminus of the truncated proteins by insertion of the TAP-tag coding sequence directly into the desired point of truncation (Figure 3.3A). Protein extracts from mid-log phase cultures were subsequently prepared and subjected to western blotting using peroxidase anti-peroxidase (PAP), which immunoreacts with the protein-A portion of TAP tags. This approach was used to assess the stability and relative expression levels of *TOF1* truncation mutants *tof1-627*, *tof1-762*, *tof1-830*, *tof1-997* and *tof1-1182*. Using this method, bands corresponding to the expected sizes of the truncated Tof1 proteins were observed, confirming that these *TOF1* truncations were expressed stably in cells (Figure 3.3B). In addition, endogenous, non-codon-optimised *TOF1* was C-terminally TAP-tagged and checked for expression levels by western blotting. Importantly, this analysis showed that codon-optimisation of the Tof1 protein does not influence its expression in cells, as codon-optimised Tof1 and endogenous Tof1 were expressed in cells at comparable levels (Figure 3.3B).

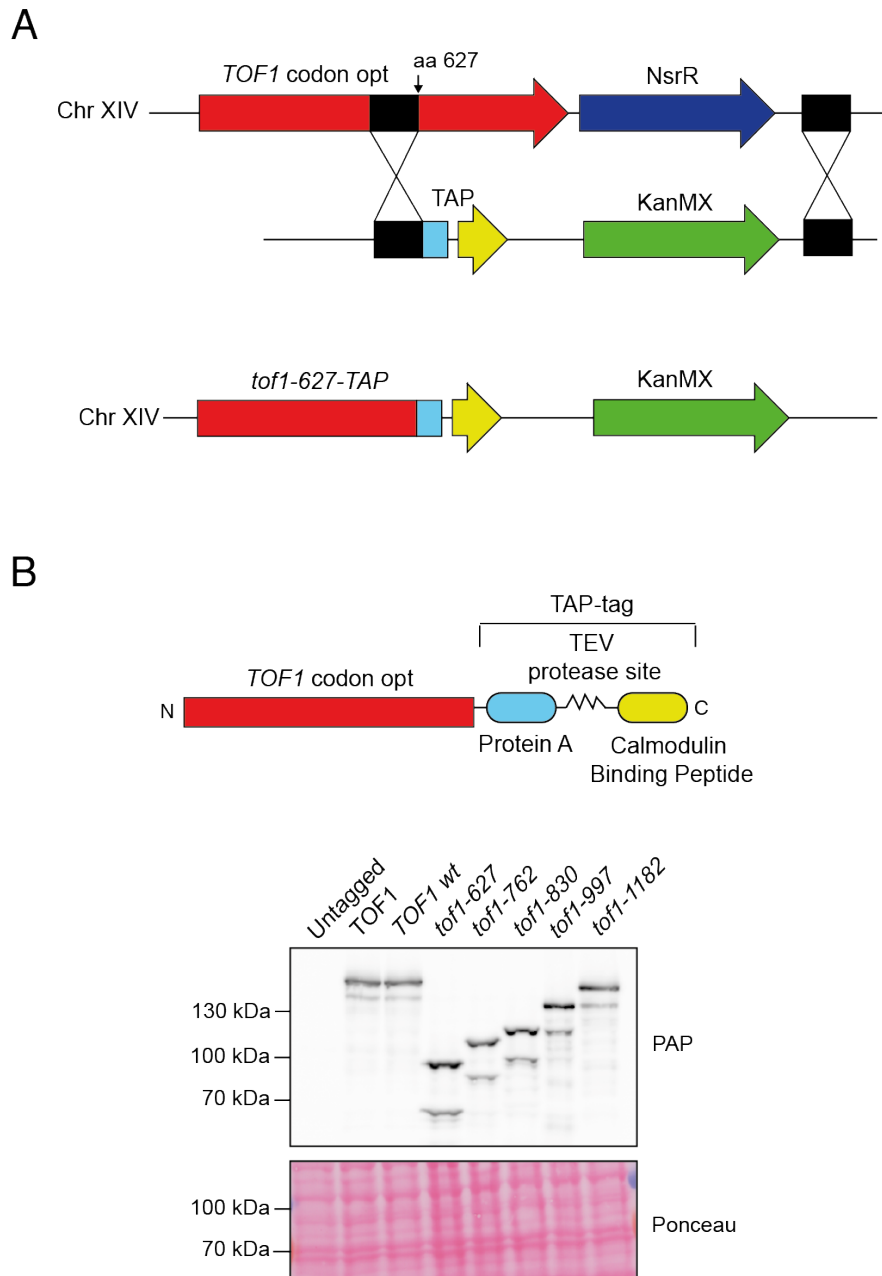


Figure 3.3: Construction and expression of C-terminal Tof1 TAP-tagged mutants

A) Schematic of strategy used to construct strains expressing TAP-tagged Tof1 proteins used for protein analysis in panel B. A construct containing C-terminal TAP tag sequence and Kanamycin resistance gene (KanMX) flanked by regions of homology to the desired insertion point (amino acid 627 shown as example) was transformed to cells containing wild-type *TOF1* codon-optimised gene at the endogenous *TOF1* locus on chromosome XIV. An additional strain containing TAP at the C-terminus of wild-type (not codon optimised) Tof1 was also constructed.

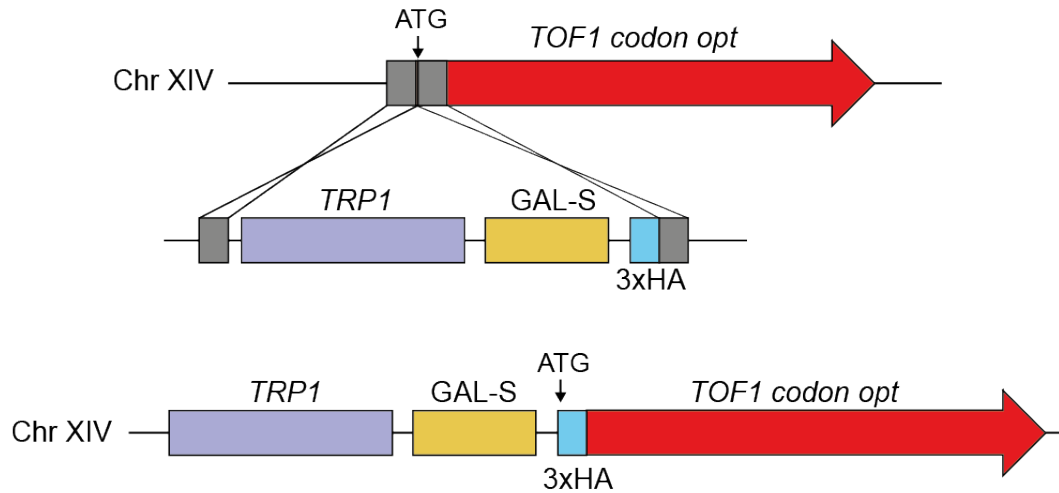
B) Schematic representation of TAP-tagged Tof1 proteins (top) and anti-PAP western blot (bottom) showing relative levels of Tof1 protein in C-terminally TAP tagged: wild type Tof1 (not codon optimised), full length codon optimised Tof1 (*TOF1* wt), and Tof1 truncation mutants 627, 762, 830, 997 and 1182. An untagged strain is included as a control for non-specific antibody binding and the ponceau-stained membrane is shown as a loading control. A representative western of 2 independent experiments is shown.

Interestingly, a second, smaller band was observed in all 5 Tof1 truncation mutants running consistently around 30 kDa below the full-length proteins (Figure 3.3B). This second band was found to be present and consistently smaller than the full-length protein in all truncations including the smallest *tof1-627* truncation (Figure 3.3B). This suggests that the smaller band is produced from cleavage of a ~30 kDa fragment N-terminal to aa 627 of the protein. Such cleavage would result in two products: a non-detectable (due to losing the TAP tag) N-terminal fragment, and the TAP-tag containing fragment which varies in length between the Tof1 truncation mutants. This is consistent with the band pattern seen in Figure 3.3B.

To investigate the possibility that the N-terminus of Tof1 is modified to generate different isoforms, I used western blotting to probe for N-terminally tagged Tof1 protein. Specifically, I generated additional strains in which haemagglutinin (HA) tags were inserted into the N-terminus of Tof1 and performed western blotting using antibodies against the HA epitope (Figure 3.4). Insertion of epitope tags at the Tof1 N-terminus was achieved using a PCR-based tagging strategy. First, the *NsrR* gene in pYM-N32 was replaced by the *Kluyveromyces lactis TRP1* gene, amplified from pYM22. This gave rise to a new vector, pYM-N32-TRP which was used to amplify a gene targeting cassette containing a 3XHA epitope tag downstream of the *GAL-S* promoter and *TRP1* gene. This linear construct was designed with arms homologous to the 80bp upstream and downstream of the *TOF1* ATG start codon, allowing for insertion of the cassette directly upstream of the *TOF1* coding sequence. This generated strains with N-terminally tagged *HA-TOF1* constructs under the control of the *GAL-S* promoter (Janke et al., 2004) (Figure 3.4A). The resulting cells were grown to mid-log phase in galactose to induce expression of the tagged proteins, before collection of samples for TCA protein extraction. The protein lysates were subsequently subjected to western blotting using antibodies against the HA epitope tag.

Using this approach, the N-terminus of Tof1 was visualised in cells containing full length codon-optimised Tof1 protein as well as in Tof1 mutants truncated at aa 627, 997 and 1182. As before, bands corresponding to the predicted protein sizes for each mutant were observed in all cases (Figure 3.4B). Surprisingly however, a smaller band was not observed in any case (Figure 3.4B), suggesting that alternative isoforms of Tof1 observed by C-terminal tagging are generated either by an alternative translation initiation site in the ORF, are unstable in cells or are not able to be visualised using the method used.

A



B

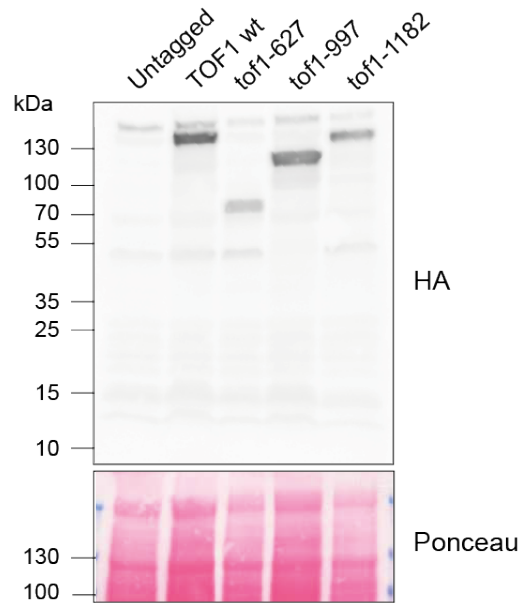


Figure 3.4: Construction and expression of N-terminally HA-tagged Tof1 mutants

A) Schematic of strategy used to construct strains expressing HA-tagged Tof1 proteins used for protein analysis in panel B. A construct containing the *K. lactis TRP1* gene, *GAL-S* promoter (Janke et al., 2004) and an N-terminal HA tag sequence, flanked by regions of homology upstream and downstream to the Tof1 ATG, was transformed to the endogenous *TOF1* locus in Tof1 truncation mutant cells.

B) Western blot using anti-HA antibodies showing relative levels of N-terminally HA-tagged Tof1 proteins in full length codon optimised Tof1 (*TOF1 wt*) and Tof1 truncation mutants 627, 997 and 1182. An untagged strain is included as a control for non-specific antibody binding and the ponceau-stained membrane is shown as a loading control. A representative western of 2 independent experiments is shown.

3.2.3. Residues 762-830 of Tof1 are crucial for Csm3 binding

In mammalian cells it is well established that Tim/Tipin form a constitutive heterodimer, with depletion of one of these proteins resulting in destabilisation and loss of the other (Chou and Elledge, 2006, Ünsal-Kaçmaz et al., 2007). The same does not appear to be strictly true for budding yeast, as deletion of Csm3 in *S. cerevisiae* cells has been reported to lead to no visible decrease in cellular Tof1 levels (Bando et al., 2009). However, deletion of Tof1 and thus disruption of the Tof1/Csm3 complex does lead to a partial reduction of detectable Csm3 in whole cell extracts, suggesting that Tof1/Csm3 complex formation is required for Csm3 stabilisation (Bando et al., 2009). In human cells, aa 1-813 of Timeless is the minimal region required for its stable interaction with Tipin (Holzer et al., 2017). In budding yeast, a recent cryo-EM structure of Tof1, Csm3 and Mrc1 bound to CMG has revealed key regions of contact between Tof1/Csm3, most notably an alpha-helix between aa 750-770 of Tof1 termed the 'Csm3 binding element', or CBE (Baretić et al., 2020). However, whether other regions are required for Tof1/Csm3 heterodimer stability is not known.

To address this, I carried out co-immunoprecipitation (co-IP) experiments to investigate whether the truncated Tof1 proteins were stably associated with Csm3 in cells. Specifically, protein extracts obtained from TAP-tagged Tof1 truncation mutants were subject to pulldown analysis using IgG-coupled sepharose beads, which bind to the protein A portion of the TAP tag used. The resulting eluates were then separated by SDS-PAGE and subjected to western blotting using antibodies against Csm3. Even in the input sample, Csm3 levels were reduced in *tof1-627* and *tof1-762* mutants compared to cells expressing full-length Tof1, suggesting Tof1/Csm3 destabilisation in these mutants (Figure 3.5, top). Indeed, when the eluates from co-IP experiments were probed using the same antibodies, no detectable Csm3 was seen in *tof1-627* and *tof1-762* pulldowns, confirming that these Tof1 truncation mutants are unable to stably bind Csm3 (Figure 3.5, bottom). Importantly, Csm3 was found to co-IP with wild-type Tof1, *tof1-830*, *tof1-997* and *tof1-1182* (Figure 3.5, bottom). Therefore, these data show that residues 762-830 of Tof1 are required for its stable association with Csm3.

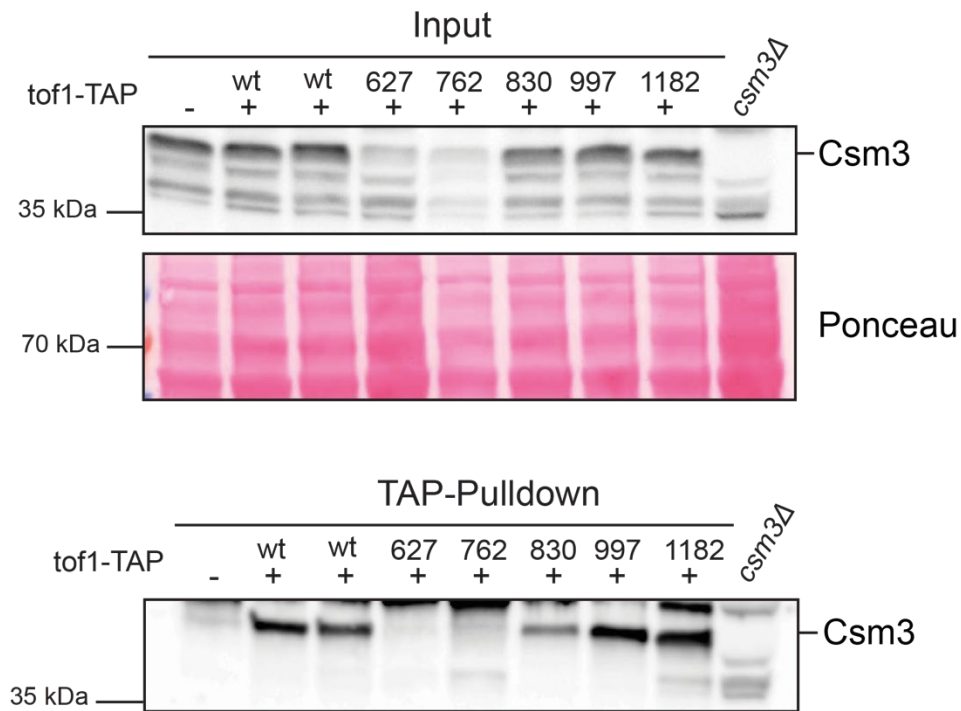


Figure 3.5: Residues 762-830 of Tof1 are required for its stable association with Csm3

Western blots showing input and eluate samples from the same co-immunoprecipitation experiment. Truncated Tof1 proteins containing C-terminal TAP tags were immunoprecipitated from cell lysates using IgG sepharose beads, and the resulting eluates were separated by SDS-PAGE gel electrophoresis before western blotting using antibodies against Csm3. Ponceau of the input samples included as loading control. Representative westerns of 2 independent experiments are shown.

3.2.3. Mutations within aa 810-950 can regulate Tof1 stability and/or function

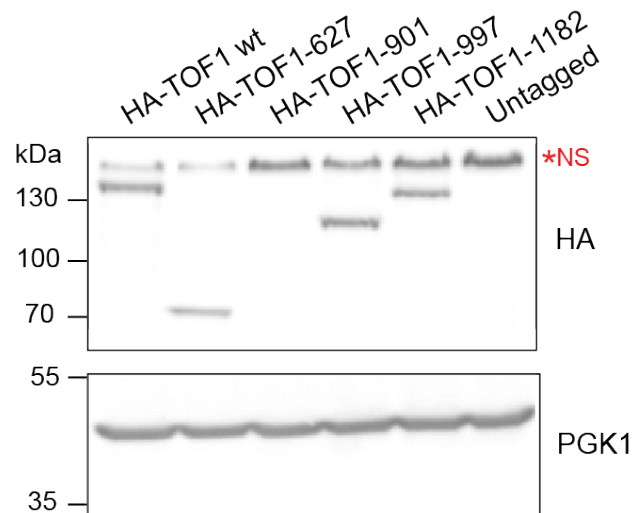
In addition to the five initial Tof1 truncations constructed for this study (*tof1-627*, *tof1-762*, *tof1-830*, *tof1-997* & *tof1-1182*), a sixth Tof1 truncation mutant was originally generated at residue 901 of the 1238 aa protein using the same method outlined in 3.2.1 (Figure 3.6A). As with the other truncations, this *tof1-901* strain was epitope tagged to characterise its stable expression in cells. Specifically, *tof1-901* was targeted with the N-terminal HA tag described in 3.2.2 and protein extracts from these cells subjected to western blotting using antibodies against the HA. Interestingly, western blot analysis of this truncation mutant revealed that *tof1-901* was completely undetectable by western blotting (Figure 3.6B). This suggested that truncation of Tof1 at residue 901 produces a protein product that is unstable in cells, which was enigmatic considering the smaller Tof1 truncations at aa 627, aa 762 and aa 830 were all detected in cells using the same methods (Figure 3.3B).

To further investigate the hypothesis that truncation of Tof1 at residue 901 produces an unstable protein product, I investigated whether *tof1-901* was able to confer resistance to CPT. Cells lacking Tof1 are hypersensitive to CPT (Redon et al., 2006) and so this was used as a quick and direct readout of Tof1 function in cells. Compared to wild-type cells, hypersensitivity to CPT was observed in *tof1-627* cells (Figure 3.6C), which is further investigated and discussed in chapter 4 of this thesis. Importantly, truncation of Tof1 at aa 901 resulted in extreme sensitivity to CPT, comparable with *tof1Δ* cells (Figure 3.6C), supporting the biochemical evidence for its instability in cells.

A



B



C

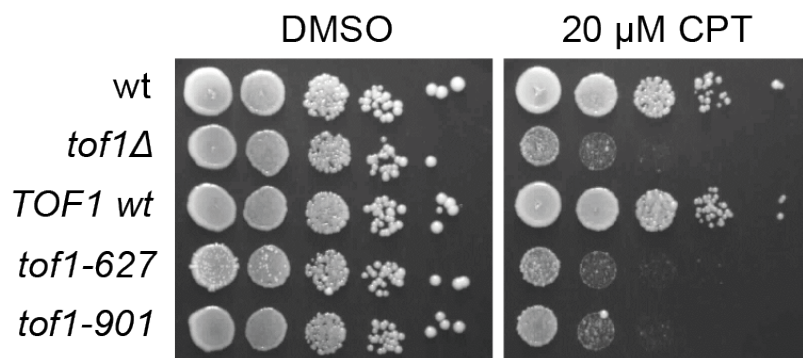


Figure 3.6: Truncation of Tof1 at amino acid 901 leads to its destabilisation

A) Schematic of the *tof1-901* truncation in relation to full-length Tof1.

B) Western blot showing expression of N-terminally HA tagged *tof1* proteins truncated at residues 627, 901, 997 and 1182, as well as full length Tof1. '*NS' indicates a non-specific band recognised by the anti-HA antibody (present in the untagged control). Western blot carried out by MSc student David Jones under supervision. A representative western of 2 independent experiments is shown.

C) Spot tests comparing growth of wild type, *tof1Δ*, TOF1 wt, *tof1-627* and *tof1-901* cells on YPD plates supplemented with DMSO (control) or 20 μM CPT. Photos were taken of plates 48 hours after incubation at 30°C. A representative image of 3 independent experiments is shown.

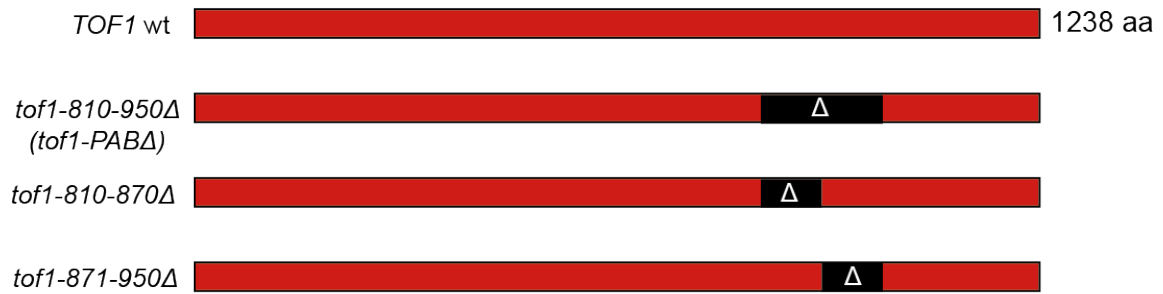
The observation that disruption of Tof1 at aa 901 leads to loss of this protein was particularly intriguing considering that smaller truncations at residues 627, 762 or 830 are apparently stably expressed in cells. Potentially, a specific region of the Tof1 C-terminus, close to residue 901, is important for regulation of its stability. This led to the question of which region may be responsible for these results. Presently, no information on the domain structure of budding yeast Tof1 has been published regarding this region of the protein. The only structure-function insight into this region of Timeless protein orthologues comes from two published analyses of human Timeless as a PARP-1 binding protein. Specifically, two back-to-back studies have characterised a region between amino acids 1000-1098 of hTim as a PARP-1 binding domain (termed the 'PAB' domain) (Xie et al., 2015, Young et al., 2015). Interestingly, around the same time that I had generated and analysed the *tof1-901* mutant, a collaborator of the Baxter lab, Luca Pellegrini, threaded the Tof1 predicted structure to the published Tim structures of the N-terminus (Holzer et al., 2017) and the C-terminal PAB domain (Xie et al., 2015) and informed us that residues 810-950 of Tof1 are predicted to be highly similar in structure to the hTim PAB domain (unpublished). Intriguingly, there is no known PARP-1 orthologue in yeast, and therefore the function of the Tof1 PAB domain is enigmatic.

Given that truncation of Tof1 within the PAB domain (at aa 901) can result in its destabilisation, I reasoned that disruption of the PAB domain might lead to Tof1 destabilisation and therefore loss of Tof1 function in cells. To test this hypothesis, I generated additional Tof1 mutants containing internal deletions of the entire Tof1 PAB domain (*tof1 810-950Δ/tof1 PABΔ*) or each half of the PAB domain (*tof1-810-870Δ* and *tof1-871-950Δ*) (Figure 3.7A). To determine whether these Tof1 mutants were functional within cells I assessed their growth on plates containing 20 μM CPT as before (Figure 3.7B). Surprisingly, deletion of the entire PAB domain did not disrupt Tof1 function, as these cells were no more sensitive to CPT than cells containing full length, wild-type *TOF1* (Figure 3.7B, top panel). However, disruption of the PAB domain by removing smaller regions (specifically the first or second half) resulted in a moderate sensitivity to CPT, suggesting that whilst removal of the entire PAB domain does not impair Tof1 stability and/or function, disruption of the residues inside this domain can (Figure 3.7B, bottom).

I next decided to ask which region specifically of the 140 amino acid PAB domain was required for Tof1 function. Therefore, I used an approach to narrow this region down to 10 amino acids. A further set of Tof1 truncation mutants were generated every 10 aa

between residues 840-890 of the 1238 residue protein (Figure 3.8A). The resulting mutants were then assessed for Tof1 function using CPT sensitivity assays as before. Using this analysis, it was observed that truncation of Tof1 at residues 840, 850 and 860 appeared to suppress CPT sensitivity to wild-type levels similarly to the *tof1-830* truncation, indicative of normal Tof1 function in these cells (Figure 3.8B). However, further truncation at Tof1 residues 870, 880 and 890 resulted in hypersensitivity to CPT, indicating that disruption of the Tof1 protein specifically between residues 860-870 results in loss of Tof1 function (Figure 3.8B).

A



B

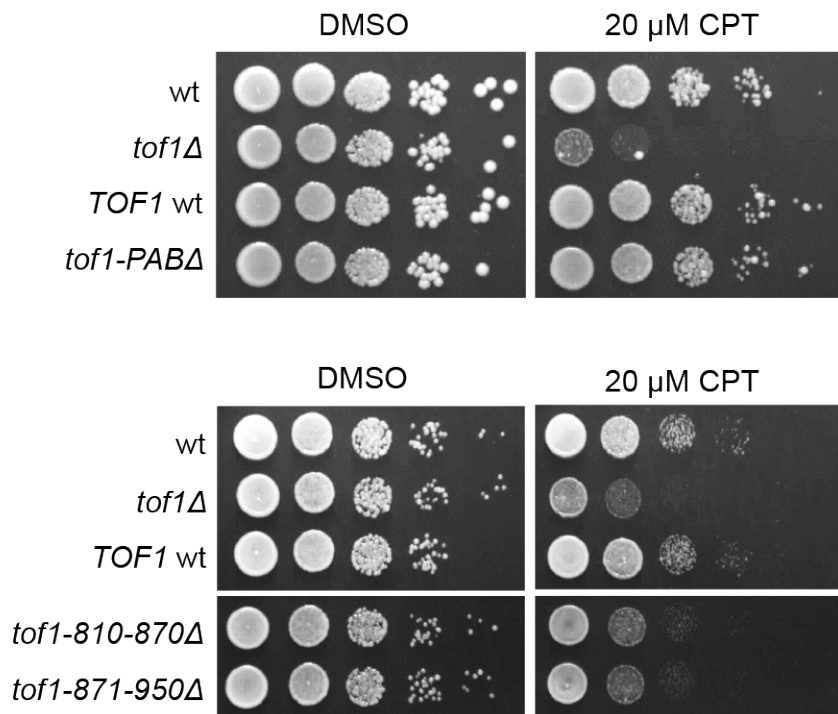


Figure 3.7: Disruption of the Tof1 C-terminal PAB domain impairs Tof1 function in cells

A) Schematic of additional Tof1 mutants containing internal deletions of amino acids 810-950 (*tof1 PABΔ*), 810-870 or 871-950.

B) Spot tests comparing growth of wild type, *tof1Δ*, TOF1 wt, *tof1 PABΔ*, *tof1-810-870Δ* and *tof1-871-950Δ* cells on YPD plates supplemented with DMSO (control) or 20 μ M CPT. Photos were taken of plates 48 hours after incubation at 30°C. Representative images of 2 independent experiments are shown.

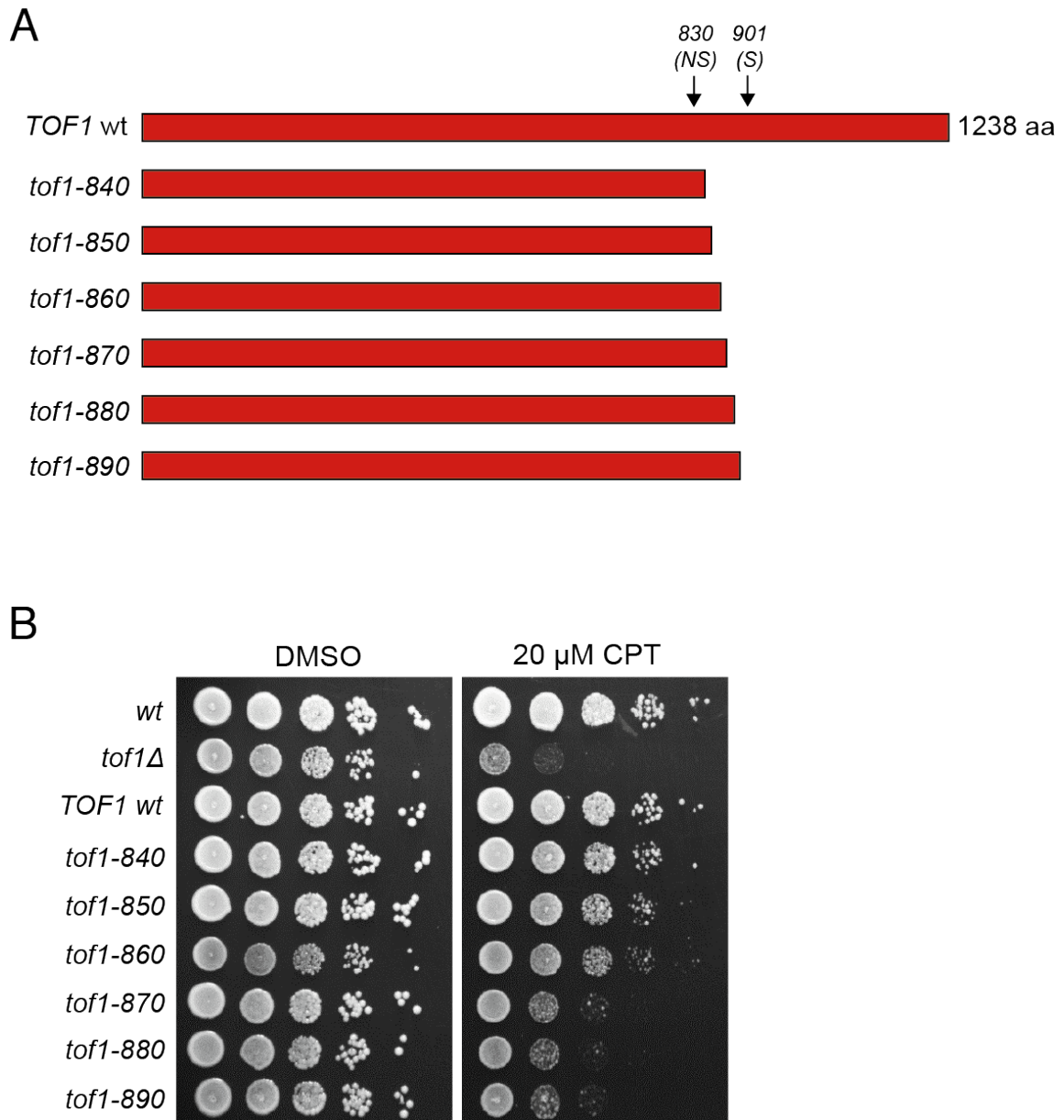


Figure 3.8: Disrupting Tof1 between residues 860-870 leads to loss of Tof1 function

A) Schematic of Tof1 truncations generated between aa 840 – aa 890. On wild-type Tof1 the relative positions of the *tof1-830* truncation which is not sensitive to CPT (NS) and the *tof1-901* truncation which is sensitive to CPT (S), are shown.

B) Spot tests comparing growth of wild type, *tof1 Δ* , *TOF1* wt and Tof1 truncations shown in (A) on YPD plates supplemented with DMSO (control) or 20 μ M CPT. Photos were taken of plates 48 hours after incubation at 30°C. A representative image of 3 independent experiments is shown.

3.3. Discussion

Tof1, the *S. cerevisiae* orthologue of mammalian Timeless, is a conserved replisome factor with roles in replication fork pausing and stability, intra-S checkpoint activation and sister chromatid cohesion, amongst others. To date there are no published structure-function analyses of Tof1 in budding yeast, and the domains responsible for the many reported Tof1 functions are not defined. This chapter has addressed the root of this problem by generating and characterising C-terminal Tof1 mutants, with the intention of using the resulting mutants for such a structure-function analysis. Specifically, I have generated a series of Tof1 truncation mutants, five of which, at residues 627, 762, 830, 997 and 1182 of the 1208 amino acid protein, are used throughout this PhD thesis. A summary of the phenotypes of these mutants described in this chapter can be found in table 3.1 (page 105).

Importantly, I have shown using C-terminal epitope tagging that all five of these mutants are stably expressed in cells, allowing me to be confident that any further phenotypes observed cannot be attributed to complete loss of Tof1 protein. Interestingly this analysis also shows that, due to the presence of two bands observed in the TAP-tagged Tof1 strains, two distinct Tof1 isoforms exist in budding yeast cells. How this second isoform is generated and its function within cells is unclear. Potentially, Tof1 is subject to proteolytic degradation, producing smaller protein products which are visible by western blotting. If this were non-specific, it would be expected that several small bands would be seen, producing a 'ladder' of degradation products. However, the consistency in size difference between the two Tof1 bands across all mutants tested suggests instead that the two bands are produced from a regulated process, for example in which a specific region of the protein is targeted for degradation.

However, the data presented in this chapter suggests that any resulting N-terminal Tof1 cleavage product, which was predicted from the size difference between the two bands to be ~30 kDa in size, is not detectable in cells. Potentially this smaller Tof1 fragment is degraded and is not stable enough to visualise by western blotting. Alternatively, the different Tof1 protein isoforms may arise from differential ATG usage during translation. It would be interesting to investigate the smaller Tof1 band that was visualised by C-terminal tagging, to determine which residues of the protein correspond to this smaller

isoform. This could be achieved by excising the smaller Tof1 band from SDS-PAGE gels and performing mass spectrometry analysis on the samples.

Next, I have shown that residues 762-830 of Tof1 are crucial for its ability to bind Csm3. The recently published cryo-EM structure of the yeast replisome has defined a hydrophobic alpha helix close to this region as a key Csm3 binding element. Specifically, this domain forms an interface with a tetra-helical 'helix turn helix' (HTH) domain at the C terminus of Csm3 allowing the two proteins to interact (Baretić et al., 2020). However, it is not clear from the pulldown analysis whether additional regions and/or specific residues of Tof1 are required for this interaction, particularly those in the N-terminus. In human cells, cross-linking mass spectrometry analysis has revealed that interactions between Timeless and Tipin are extensive, involving residues 1-814 of Tim (Holzer et al., 2017). To fully define the minimal region of Tof1 required for Csm3 interaction it would be necessary to truncate regions of the N-terminus and perform additional pulldowns, to determine exactly which residues are required for stabilisation of the Tof1/Csm3 heterodimer in budding yeast cells. As discussed in the introduction to this thesis, the Tof1/Csm3 heterodimer interacts physically and functionally with a third protein, Mrc1. Unfortunately, I have not been able to determine whether the C-terminal Tof1 truncation mutants generated in this chapter are capable of interacting with Mrc1, despite several attempts to co-IP Mrc1 using TAP-tagged Tof1 strains. Therefore, it remains that there is currently no insight into which regions of Tof1 are required to sustain this interaction. Therefore, further optimisation of this experiment would have been prioritised, had time allowed.

This chapter has given novel insights into the role of the conserved Tof1 PARP-binding (PAB) domain in budding yeast cells. Given that there is no known PARP-1 orthologue in *S. cerevisiae*, the structural conservation between the hTim and ScTof1 PAB domains is enigmatic. Interestingly, in this chapter I have shown that in budding yeast, mutations within this region can regulate Tof1 stability (in the case of *tof1-901*) and/or function in cells. Specifically, I have demonstrated that specific mutations designed to disrupt the PAB domain can result in loss of Tof1 stability, and consequently, function. Paradoxically, truncation of Tof1 at residue 830 produces a stable protein product, whereas truncation at a more C-terminal residue, 901, results in destabilisation of the protein and loss of Tof1 function. Similarly, deletion of the entire PAB domain does not result in cellular sensitivity to CPT, whereas removing just half of the domain does. These findings support a model

in which disruption within the PAB domain could alter the Tof1 tertiary structure and result in Tof1 destabilisation. For example, truncation of Tof1 at residue 901 may result in exposure of a destabilising region within the protein that is normally buried in the physiological tertiary structure. In contrast, removal of the residues that result in protein destabilisation in the first place (e.g. in the case of the *tof1-830* mutant) would prevent any protein destabilisation, resulting in stable Tof1 expression. Importantly however, western blotting analysis would be required to say with confidence that all mutations generated within the PAB domain physically destabilise the protein, as it is possible that these mutations disrupt Tof1 function without affecting protein abundance within the cell. For example, disruption of important interactions or 3D folding of the protein could impair Tof1 function without destabilising the protein. Furthermore, to add weight to the argument that a specific region of Tof1 can impair protein stability and/or function, additional mutants in which internal deletions within the *tof1-901* truncation mutant could be generated, to investigate whether removal of specific residues within the PAB domain lead to re-stabilisation of the *tof1-901* mutant.

In summary, this chapter has defined a series of novel Tof1 mutants and characterised their expression in budding yeast cells. Truncation of Tof1 at residues 627, 762, 830, 997 or 1182 does not alter Tof1 expression in cells, whilst truncation at residue 901 of the protein results in its destabilisation and consequently a hypersensitivity to CPT comparable with *tof1Δ* cells. In addition, expression analysis has shown that two Tof1 isoforms exist in cells as visualised by C-terminal epitope tagging, although the exact nature of the smaller isoform remains unknown. Importantly, a region within the Tof1 C-terminus (specifically aa 762-830) is required for Csm3 binding, most likely through a recently identified alpha helix in this region positioned to dock Csm3 to the replisome (Baretić et al., 2020). Lastly, I have defined a region within the Tof1 protein, specifically between residues 810-950, that appears to play a role in regulating the stability of Tof1 in cells, through an undetermined mechanism.

Mutant	Produces stable protein product	Supports Tof1-Csm3 Binding	Suppresses CPT sensitivity
<i>tof1</i> Δ	N/A	N/A	X
<i>csm3</i> Δ	N/A	N/A	X
<i>mrc1</i> Δ	N/A	NT	✓
Tof1 wt	✓	✓	✓
<i>tof1-627</i>	✓	X	X
<i>tof1-762</i>	✓	X	X
<i>tof1-830</i>	✓	✓	✓
<i>tof1-901</i>	X	NT	X
<i>tof1-997</i>	✓	✓	✓
<i>tof1-1182</i>	✓	✓	✓
<i>tof1-810-950</i> Δ	NT	NT	✓
<i>tof1-810-870</i> Δ	NT	NT	X
<i>tof1-871-950</i> Δ	NT	NT	X
<i>tof1-840</i>	NT	NT	✓
<i>tof1-850</i>	NT	NT	✓
<i>tof1-860</i>	NT	NT	✓
<i>tof1-870</i>	NT	NT	X
<i>tof1-880</i>	NT	NT	X
<i>tof1-890</i>	NT	NT	X

Table 3.1: Summary of phenotypes of Tof1 mutants described in chapter 3 compared with the phenotypes of *tof1* Δ , *csm3* Δ and *mrc1* Δ mutants.

NT = Not tested.

4. Investigating the structure-function relationship of the Tof1 protein in topological stress, protein-DNA roadblocks and in helicase/polymerase coupling in *S. cerevisiae*

4.1. Introduction

Timeless proteins have been well-established as important factors in the response to impediments to replication fork progression. *S. cerevisiae* Tof1 has been shown to play a key role in the stabilisation of replisomes. For example, *tof1* Δ cells have higher levels of replication fork rotation in response to topological stress, are unable to pause the replisome at well-defined proteinaceous roadblocks and are unable to couple helicase and polymerase activities in response to HU (Dalgaard and Klar, 2000, Katou et al., 2003, Krings and Bastia, 2004, Calzada et al., 2005, Tourrière et al., 2005, Schalbetter et al., 2015).

However, the mechanisms behind Tof1's functions in these processes is relatively poorly understood. In addition, it is not clear whether the roles of Tof1 in responding to these various replisome impediments are functionally linked or can be separated from one another. This chapter aims to address these questions. To investigate the roles of Tof1 in these processes I have used the C-terminal truncations described in Chapter 3 to functionally complement *tof1* Δ cells. By investigating the ability of these C-terminal Tof1 mutants to restrict replisome rotation, support fork pausing and to allow helicase/polymerase coupling, this chapter aims to dissect the structure-function relationship of Tof1 in the context of these different physical impediments to replisome progression.

4.2. Results

4.2.1. The far C-terminus of Tof1 restricts replication fork rotation

I was initially interested in the role of Tof1 in suppressing replication fork rotation. During replisome progression, the CMG helicase separates the parental DNA strands without removing the intertwining between them, leading to a local increase in helical stress ahead of the replication fork. Such increases in torsional stress can impede replisome progression if not resolved (Keszthelyi et al., 2016). It has been established previously that Tof1 plays a key role in the response of the replisome to topological stress. In the absence of Tof1, replisomes experience increased levels of fork rotation upon encountering increased topological stress which leads to increased intertwining, or catenation, between the newly replicated sister chromatids (Schalbetter et al., 2015). However, the mechanism for Tof1's role in restricting replisome rotation remains unclear. One possibility is that the absence of Tof1 results in an unstable replisome structure that is more susceptible to passive replisome rotation events. Alternatively, Tof1 could play a direct role in preventing rotation of the fork. Interestingly, Tof1 was initially identified in a yeast two-hybrid screen designed to find Topoisomerase 1-interacting proteins. Residues 981-1238 of Tof1 were shown to interact with Top1 by this method (Park and Sternglanz, 1999). Potentially, Tof1's role in restricting replisome rotation could be through direct recruitment of Top1 to the replication fork, allowing for direct resolution of topological stress ahead of the unwinding CMG. If this were the case, C-terminal truncation alleles of Tof1 unable to mediate the Tof1-Top1 interaction would be expected to produce a hypercatenation phenotype similar to that seen in *tof1* Δ cells.

To assess the role of the Tof1 C-terminus and this proposed interaction in restricting excessive replication fork rotation, a previously established assay to visualise catenanes on plasmids extracted from post-replicative cells was used (Schalbetter et al., 2015). This experimental approach is outlined in Figure 4.1.

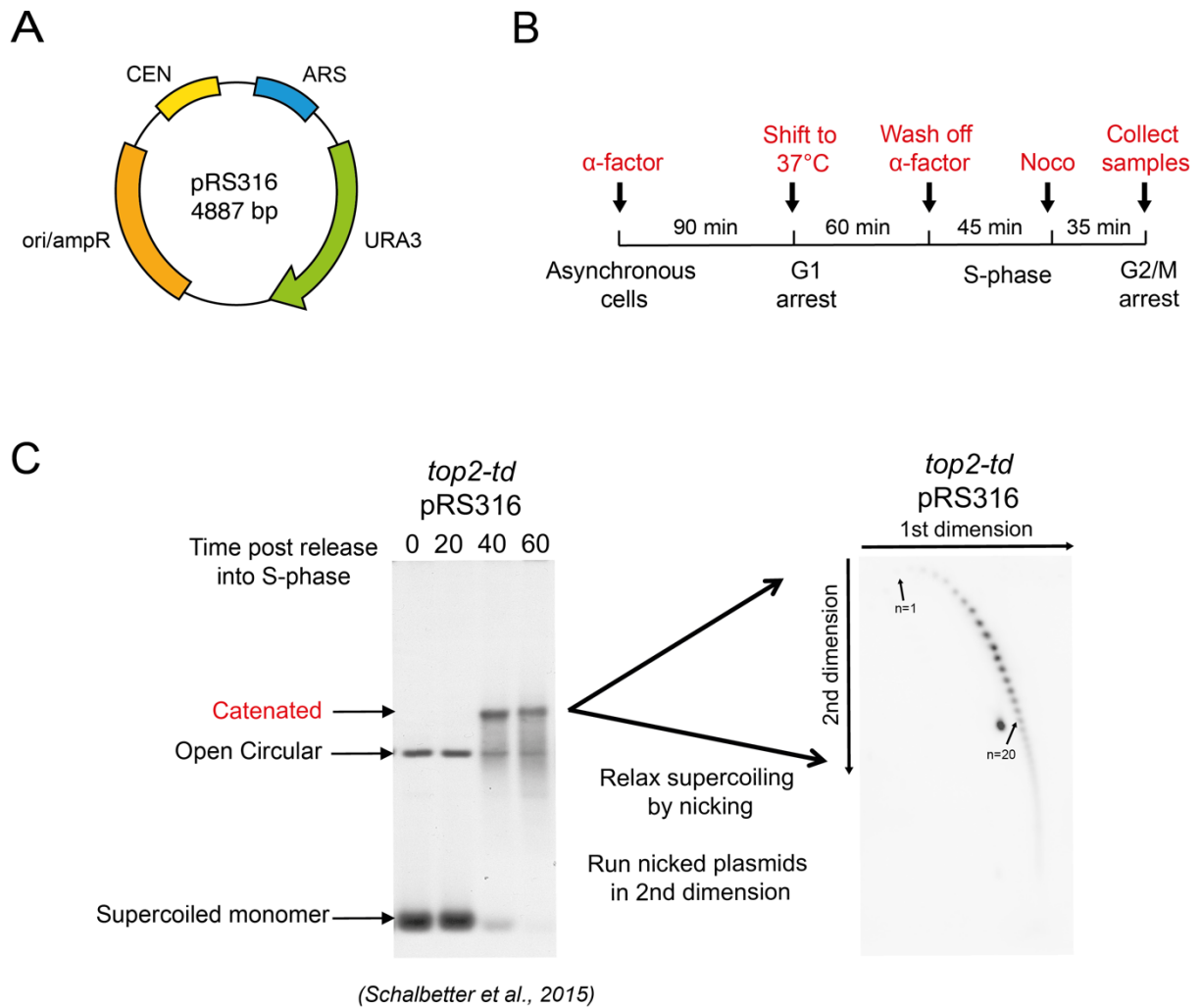


Figure 4.1: Visualisation of replication fork rotation events *in vivo*

(A) Schematic diagram of yeast episomal plasmid pRS316. The plasmid contains the *URA3* gene for yeast selection along with a single yeast DNA replication origin and centromere.

(B) Schematic of the time course carried out to follow replication fork rotation *in vivo*. Budding yeast cells were arrested prior to replication in G1 phase by addition of alpha factor before release into S-phase at the restrictive temperature to eliminate Top2 activity. Nocodazole was added to arrest cells at the G2/M transition.

(C) Adapted from Schalbetter et al., 2015. Catenated plasmids extracted from post-replicative cells run in 1st dimension agarose gels as a smear (left). These can be resolved according to number of catenanes by running the gel slice containing catenated plasmids in the 2nd dimension, where they can be visualised by Southern blotting (right). Each spot corresponds to replicated plasmids with a specific number of catenanes, from $n=1$ to $n=20$ and above.

Cells containing the yeast episomal plasmid pRS316, C-terminal Tof1 truncations and the temperature-sensitive *top2-4* allele (Holm et al., 1985) were arrested in G1 using alpha-factor peptide before being released into S-phase at the restrictive temperature (37°C) to eliminate Top2 activity specifically during DNA replication. Cells were then arrested prior to cell division using the microtubule depolymerising drug nocodazole. Replicated plasmids extracted from these post-replicative cells were subject to two-dimensional gel electrophoresis and Southern blotting as previously described (Schalbetter et al., 2015) (for details see methods, chapter 2, sections 2.4.9 - 2.4.12). In the first dimension after being nicked, catenated plasmids run as a smear which are then cut from the gel and resolved by running in the second dimension (Figure 4.1C). Using a probe against the *URA3* gene to visualise pRS316, Southern blotting allows visualisation of individual spots which correspond to plasmids with increasing numbers of catenanes (Figure 4.1C).

Previously work from our group was able to establish that *tof1Δ top2-4* cells experience extremely high levels of replication fork rotation as visualised by this technique. Plasmids extracted from these cells are highly catenated and therefore the proportion of these plasmids with over 20 intertwines is dramatically increased (Schalbetter et al., 2015) (schematic representation in Figure 4.2A). To investigate the role of the Tof1 C-terminus in suppression of fork rotation events, this approach was used and the plasmid population containing 20 or more catenanes was visualised as described. As the truncation mutants were generated using codon-optimised *TOF1* (see chapter 3), *tof1Δ* cells were first complemented with wild-type codon-optimised *TOF1* to ensure that any changes in fork rotation could not be attributed to codon-optimisation of the gene. As expected, the hypercatenation phenotype was rescued by codon optimised wild-type Tof1 and these cells experience only background levels of fork rotation events (Figure 4.2B). However, complementation with truncations *tof1-627*, *tof1-762*, *tof1-830* or *tof1-997* resulted in a hypercatenation phenotype (Figures 4.2C-F). Interestingly, in all four mutants this phenotype was as strong as was previously observed for *tof1Δ* cells. However, a truncation at *tof1-1182* suppressed fork rotation events to wild type levels (Figure 4.2G). Therefore, these data suggest that a far C-terminal region of the Tof1 protein, specifically between amino acids 997 and 1182, is required to suppress DNA replication fork rotation in response to topological stress.

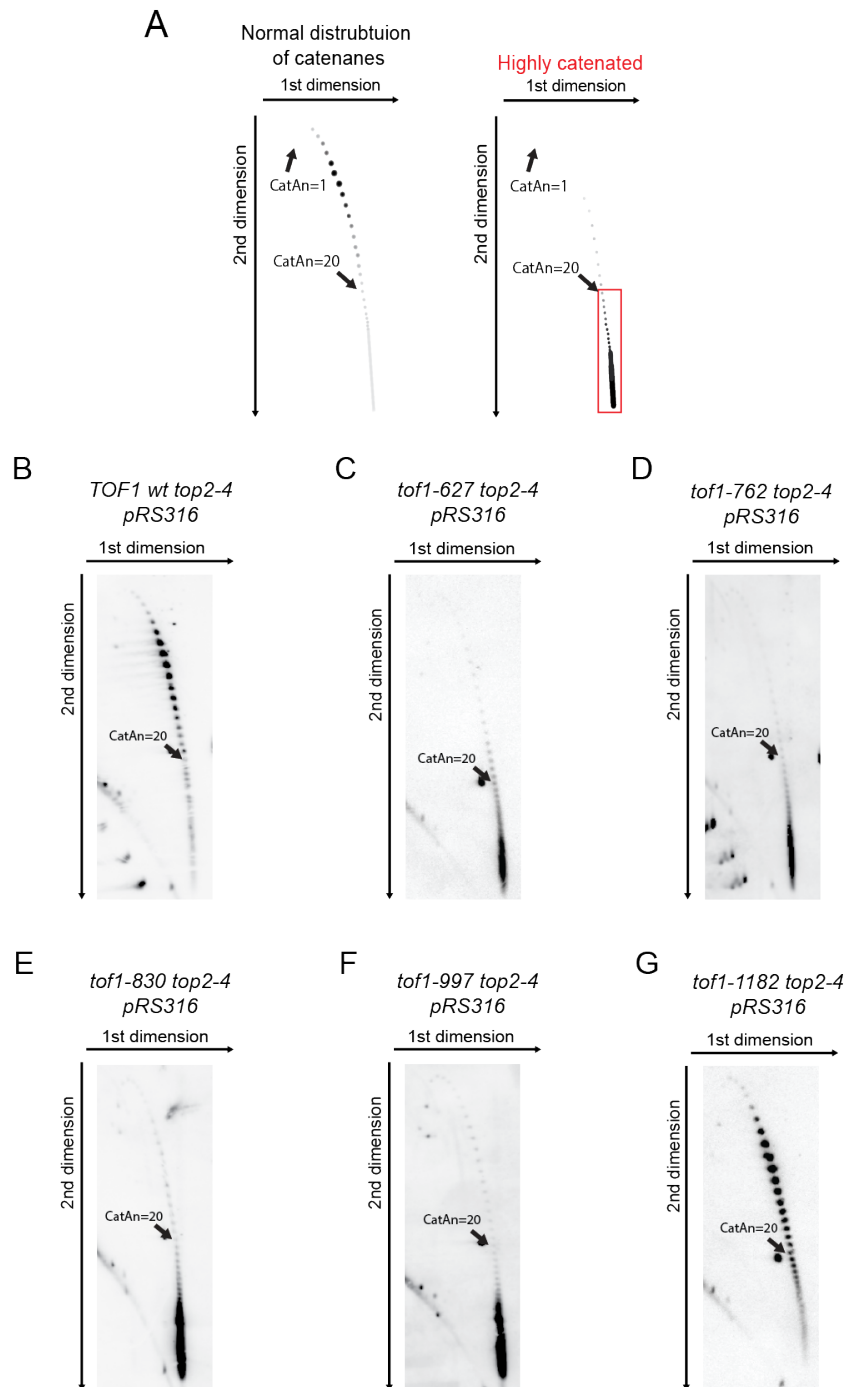


Figure 4.2: Residues 997-1182 of ToF1 are required to suppress replication fork rotation in response to topological stress

(A) Schematic representation of normal (left) and hypercatenated (right) distributions of intertwined pRS316 plasmids extracted from post-replicative cells and resolved by 2D gel electrophoresis. Hypercatenation is expected in cells with loss of ToF1 function as described in Schalbetter et al., 2015.

(B-G) Analysis of pRS316 catenation in cells containing the temperature sensitive *top2-4* allele and (B) wild-type ToF1 (C) *tof1-627* (D) *tof1-762* (E) *tof1-830* (F) *tof1-997* (G) *tof1-1182*. Representative images of 2 independent experiments are shown for each mutant.

4.2.2. The replisome pausing function of Tof1 at the rDNA RFB requires residues 762-830 of the protein

I next sought to investigate the role of Tof1 in replisome pausing at stable protein-DNA barriers. Specifically, I utilised the well-characterised 'replisome fork blocking' (RFB) pause site that serves a physiological role at the rDNA repeats on budding yeast chromosome XII. The RFB sequence is located within the non-transcribed NTS1 region of the rDNA repeats and is stably bound by the Fork Blocking 1 (Fob1) which blocks replication forks in a unidirectional manner to prevent head-on collisions between replication forks and transcription complexes on the highly transcribed rDNA genes (Brewer and Fangman, 1988, Kobayashi and Horiuchi, 1996, Kobayashi, 2003, Ward et al., 2000).

It has been established that Tof1 is required for this pausing, although it is unknown whether its role in replisome pausing may be linked to its other functions. Thus, I set out to investigate the requirement of the Tof1 C-terminus in this function and whether the roles of Tof1 in restriction of replication fork rotation and replisome pausing at stable protein-DNA barriers may be linked. To this end, I generated a yeast episomal plasmid containing a single rDNA RFB sequence. Briefly, the sequence corresponding to *S. cerevisiae* chromosome XII (sequence 459799 – 460920) was PCR amplified from a 4xRFB plasmid (Gift from Luis Aragon) and cloned into pRS426 using Gibson assembly to generate plasmid pRS426-RFB (Figure 4.3A). This plasmid was transformed to yeast haploid cells carrying the Tof1 truncation mutants and then extracted from exponentially growing cells. In a log phase culture, a proportion of cells are in mid-S phase and actively replicating plasmid pRS426-RFB. Digestion of these plasmids with endonucleases SnaBI and BamHI results in a range of replication intermediates, ranging from those which are linear and unreplicated to those which are almost entirely replicated, with a range of Y-shaped molecules in-between. Resolution of these various intermediates using two-dimensional gel electrophoresis and visualisation using Southern blotting results in the characteristic 'Y-arc' shape originally described by Brewer and Fangman in 1987 (schematic representation shown in figure 4.3C). However, a number of rightward-moving replication forks from the replication origin will be paused in a unidirectional manner at the RFB/Fob1 site on pRS426-RFB, which can be clearly observed as a distinct spot corresponding to a build-up of these paused fork molecules (Figure 4.3C). As expected,

these paused replication intermediates could clearly be detected using this technique in cells containing wild-type codon-optimised Tof1 (Figure 4.4B) (spot marked by arrow) but were completely lost in *tof1* Δ cells (Figure 4.4A). Using this method I was able to ask whether the C-terminal Tof1 truncation mutants that were unable to restrict replisome rotation were able to support replisome pausing at the fork barrier on pRS426-RFB. Notably, the *tof1-627* and *tof1-762* truncation mutants were unable to pause replication forks at the RFB (Figures 4.4C & D) whilst truncations at aa 830, aa 997 and aa 1182 all restored the fork pausing function of Tof1 in this context (Figures 4.4E-G). Therefore, a distinct region of the Tof1 protein between aa 762 and aa 830 is required for its role in replisome pausing at the RFB. Interestingly therefore, this suggests that the role of Tof1 in restricting replisome rotation is separable from its role in pausing replication forks at stable protein-DNA roadblocks.

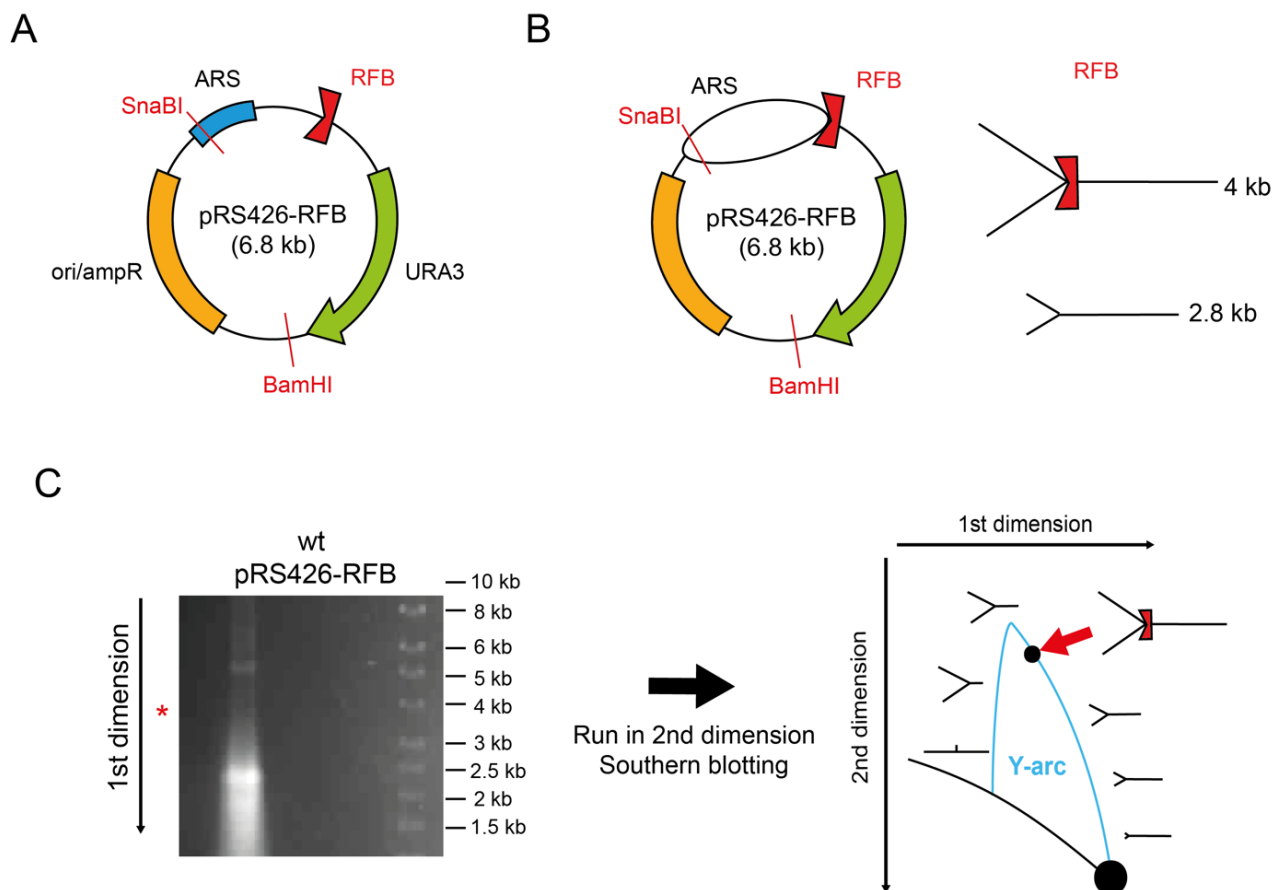


Figure 4.3: Analysis of paused replication intermediates *in vivo*

(A) Plasmid pRS426-RFB containing the *S. cerevisiae* RFB sequence downstream of the yeast replicative origin (ARS), *URA3* gene for yeast selection, bacterial origin (*ori*) and Ampicillin antibiotic resistance gene for bacterial selection (*ampR*).

(B) Schematic representation of two potential Y-shaped replication intermediates generated from digestion of pRS426-RFB extracted from replicating yeast cells. An accumulation of Y-shaped molecules with forks blocked at the RFB are expected in wild-type cells.

(C) Left: example of a 1st dimension gel containing digested replication intermediates after ethidium bromide staining. Red asterisk marks position of expected RFB replication intermediate size (4kb) after digestion with *Sna*BI and *Bam*HI, Right: schematic of expected Y-arc shape following resolution of 1st dimension intermediates in the 2nd dimension and visualisation by Southern blotting.

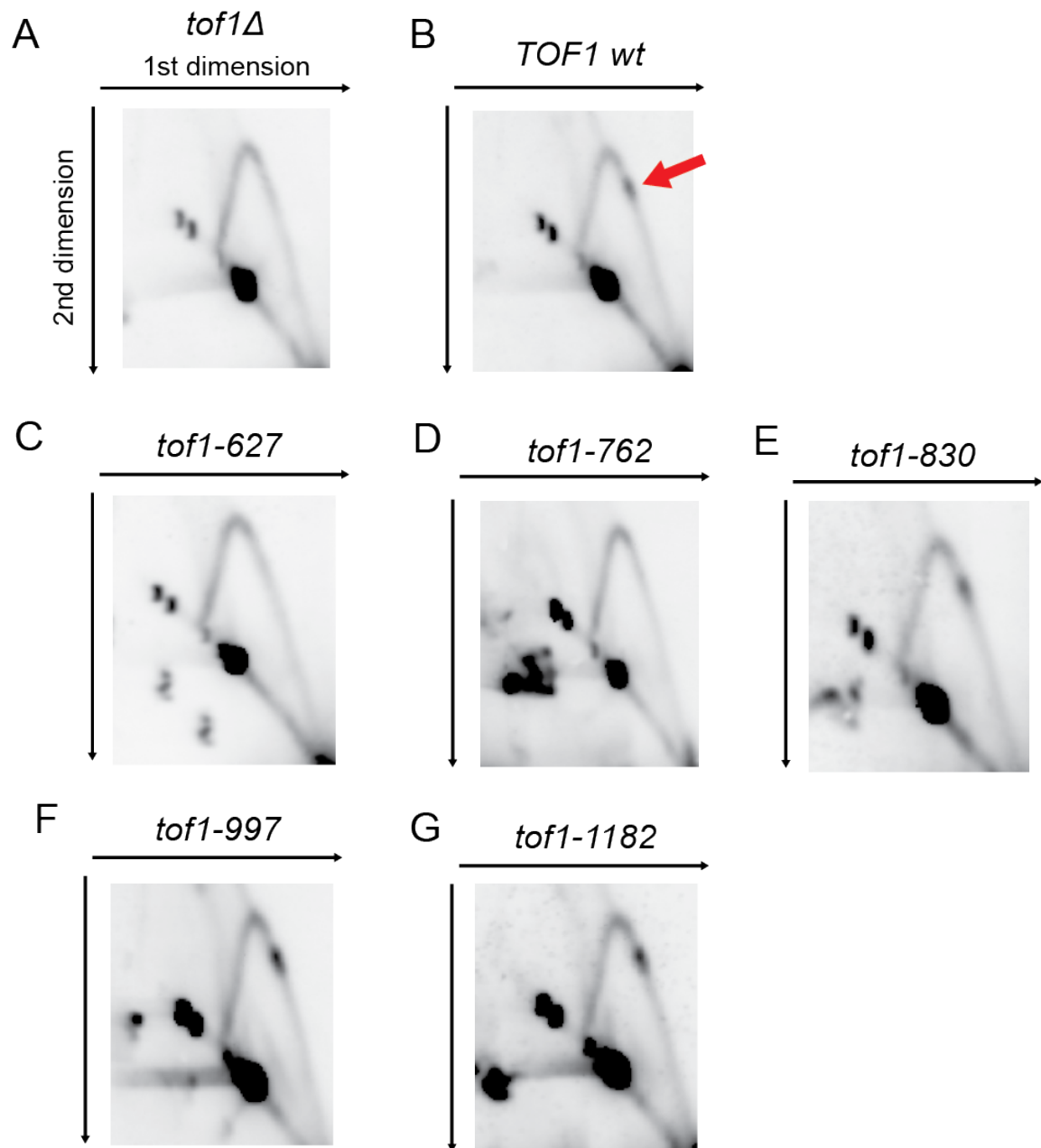


Figure 4.4: Tof1 residues 762-830 are required for replisome pausing at the RFB

(A-G) 2D-gels of *Sna*BI and *Bam*HI double-digested pRS426-RFB plasmids extracted from log phase yeast cells containing (A) *tof1*Δ (B) wild-type Tof1 (red arrow indicates build-up of paused fork intermediates) (C) *tof1*-627 (D) *tof1*-762 (E) *tof1*-830 (F) *tof1*-997 (G) *tof1*-1182. Representative images of 2 independent experiments are shown for each mutant.

4.2.3. The role of Tof1 in responding to CPT-induced DNA-protein crosslinks is linked to its role in replisome pausing

Cells lacking Tof1 are hypersensitive to the Top1 poison camptothecin (CPT) (Redon et al., 2006). CPT binds in the catalytic pocket of Top1, stabilising the Top1-DNA covalent complex (known as Top1-ccs) and preventing the re-ligation step of the Top1 catalytic cycle (Liu et al., 2006). This is thought to induce replication stress in numerous ways. Top1-ccs are DNA-protein crosslinks which may prevent CMG progression along the DNA duplex. In addition, these lesions are associated with a single-stranded break and increased topological stress which may result in replication fork run-off or limited CMG progression, respectively. It is not clear what causes the hypersensitivity of *tof1* Δ cells to CPT. Potentially the inability to pause the replisome at a Top1-cc would result in replication fork run-off and the generation of a toxic single-ended double strand break (DSB). Alternatively, CPT could induce much higher than wild-type levels of topological stress through its Top1-trapping action, which may be more problematic for *tof1* Δ cells that experience increased sister chromatid intertwining in response to topological stress (Schalbetter et al., 2015).

To test these two hypotheses, I assessed the viability of the C-terminal Tof1 truncations in response to chronic exposure to CPT. As expected, adding back wild-type codon-optimised *TOF1* to *tof1* Δ cells rescued their extreme sensitivity to the drug (Figure 4.5A). In contrast, *tof1-627* and *tof1-762* cells showed the same hypersensitivity to CPT as *tof1* Δ cells (Figure 4.5A). Finally, truncations at aa 830, aa 997 and aa 1182 all rescued this hypersensitivity (Figure 4.5A). Interestingly, these data together show that the C-terminal Tof1 truncation *tof1-997* which is unable to suppress replication fork rotation, is not sensitive to CPT, suggesting that CPT sensitivity in *tof1* Δ mutants is not linked to its role in responding to increased levels of topological stress. Instead, the same region of Tof1 required for replisome pausing at the RFB (aa 762-830) is crucial for suppression of CPT hypersensitivity, suggesting that Tof1 senses and responds to Top1-cc's in the same manner as other physical proteinaceous blocks to replication.

A recent study of Tof1 reported partial loss of pausing function at the rDNA RFB in C-terminal Tof1 mutants that were unable to bind Top1 (Shyian et al., 2019). This led to the hypothesis that topoisomerase action is required for replisome pausing at protein-DNA

barriers. However, the data presented here seem to contradict this finding as the C-terminal *tof1-997* truncation mutant, which is unable to suppress replication fork rotation, appears proficient in its fork pausing function. One of the primary differences between the two studies is the method used to generate the C-terminal truncations. As outlined in chapter 3 of this thesis, the truncation mutants used throughout this study were generated by inserting premature stop codons into a codon-optimised *TOF1* ORF at the endogenous *TOF1* locus. However, Shyian and colleagues generated their truncation mutants by inserting C-terminal FLAG epitope tags into the desired truncation point in the *TOF1* ORF (Shyian et al., 2019). As described in chapter 3, the region of Tof1 between aa 810-950 appears to be highly sensitive to disruption and can result in destabilisation of the protein. I therefore hypothesised that adding C-terminal epitope tags close to this region of Tof1 could lead to partial loss of Tof1 stability and function, which may contribute to the differences between the two studies. To test this hypothesis, I used CPT sensitivity as a readout for Tof1 function and performed spot tests to compare growth of my Tof1 C-terminal truncations with the equivalent epitope-tagged truncation. Specifically, I utilised the C-terminally TAP-tagged Tof1 truncations described in chapter 3 to assess the effects of inserting epitope tags into this region of the Tof1 protein. After 48 hrs growth on CPT, both Tof1 wild-type-TAP and *tof1-627-TAP* strains were no more sensitive to the drug than their untagged counterparts (figure 4.5B). Interestingly however, TAP-tagged *tof1-997* cells had a clear growth defect in response to CPT when compared with the untagged *tof1-997* strain (figure 4.5B), suggesting that insertion of epitope tags into this region of the protein leads to a subtle but clear loss of Tof1 function, potentially accounting for the loss of pausing function seen in C-terminally tagged Tof1 mutants.

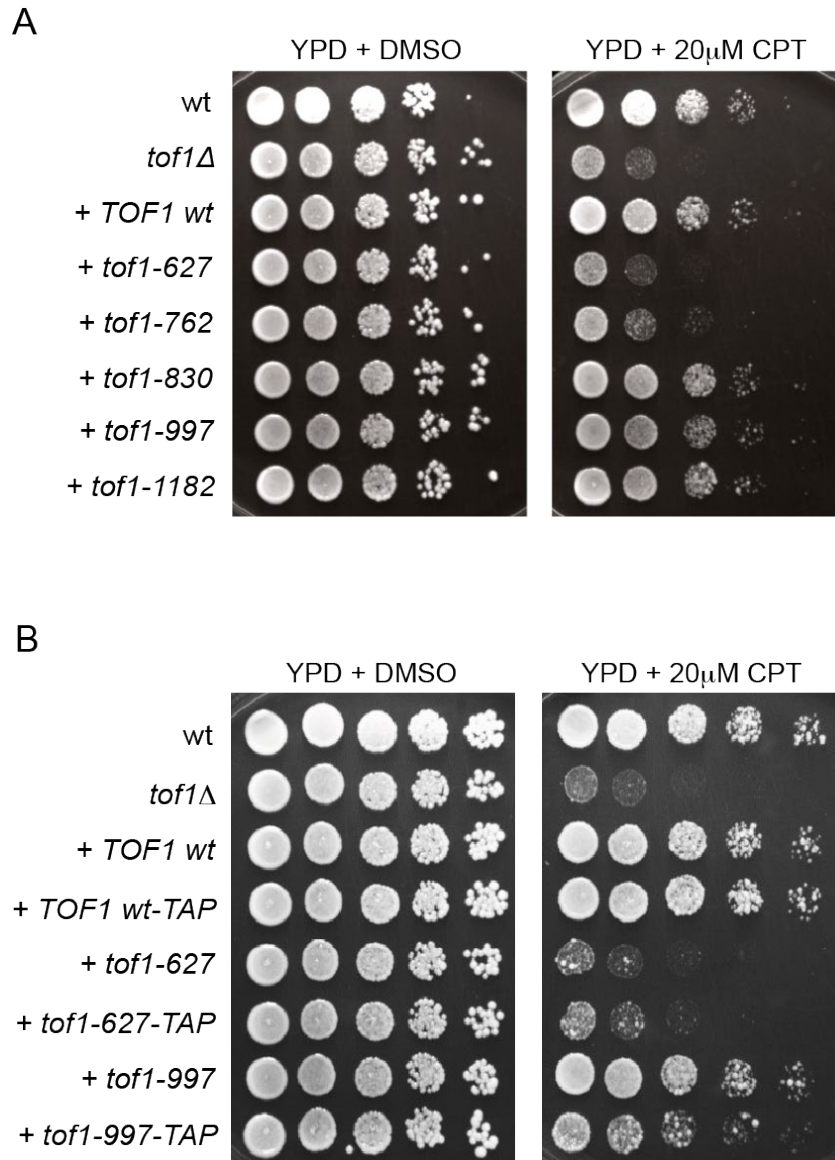


Figure 4.5: C-terminal *Tof1* mutants are sensitive to CPT

(A) Spot viability assays of C-terminal *Tof1* truncations grown on YPD and DMSO (control) or 20 μ g/ml CPT. Photos were taken 48 hrs after plating and cells were grown at 30°C. Images shown are one of three independent experiments.

(B) Spot viability assays of C-terminal *Tof1* truncations and their TAP-tagged counterparts grown on YPD + DMSO (control) or YPD + 20 μ g/ml CPT. Photos were taken 48 hrs after plating and cells were grown at 30°C. Images shown are one of two independent experiments.

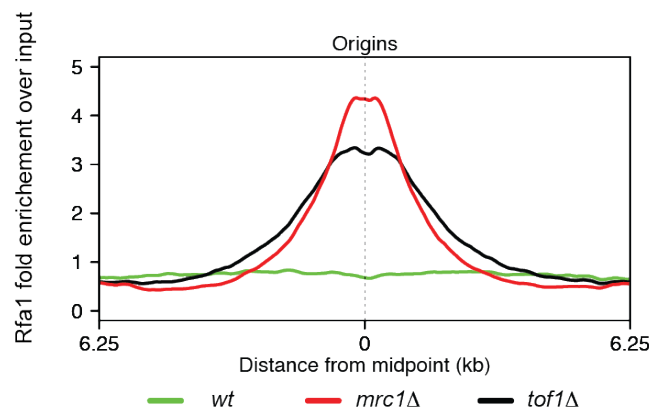
4.2.4. The helicase/polymerase coupling function of Tof1 is closely associated to its replisome pausing function

I next decided to investigate the role of Tof1 in responding to nucleotide depletion during DNA synthesis. Cells lacking Tof1 are mildly sensitive to the drug HU, a potent inhibitor of ribonucleotide reductase (RNR) (Redon et al., 2006). This suggests that Tof1 plays a role in stabilisation of replisomes under these conditions. In addition, deletion of either *TOF1* or *MRC1* has been linked to an inability to couple CMG helicase unwinding with nascent DNA synthesis by the replicative polymerases (Katou et al., 2003). This is thought to lead to generation of single-stranded DNA (ssDNA) as the parental strands become exposed. To confirm this prediction, a ChIP-Seq approach was used by Dr. Andrea Keszthelyi and Dr. Nicola Minchell to detect RPA accumulation around replication origins. This experimental approach is outlined in figure 4.6A. Briefly, *tof1Δ* or *mrc1Δ* cells were arrested in G1 using alpha factor before being released into 200 mM HU for 1 hour. Cells were then collected and used for ChIP-Seq analysis using Rfa1 antibodies. In these conditions, wild-type cells did not appear to have increased RPA binding around replication origins (Figure 4.6B). However, as predicted in *tof1Δ* cells RPA accumulation was dramatically increased and to an even greater extent in *mrc1Δ* cells, suggesting high levels of helicase/polymerase uncoupling in these cells (Figure 4.6B). To assess the role of the Tof1 C-terminus in replisome uncoupling, *tof1Δ* cells were complemented with the C-terminal truncations and ChIP-Seq was carried out as before. Expression of *TOF1 wt* and *tof1-830* in these cells completely suppressed the accumulation of RPA around replication origins, whilst expression of either *tof1-627* or *tof1-762* only partially suppressed this phenotype (Figure 4.6C). Thus, these data argue that the same region of Tof1 required for replisome pausing at the RFB and for suppressing CPT-induced lethality (residues 762-830) is required to couple helicase progression to nascent strand synthesis in nucleotide-limiting conditions. Interestingly, the *tof1-627* and *tof1-762* truncation mutants consistently appeared to accumulate less RPA around replication origins than *tof1Δ* cells, suggesting that they retained some activity in helicase/polymerase coupling (Figure 4.6C).

A



B



C

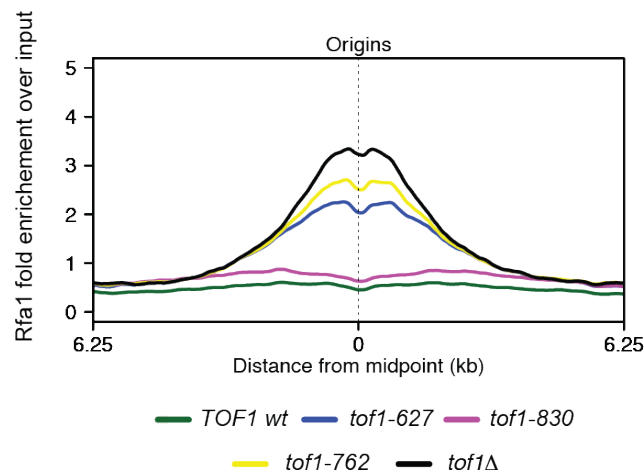


Figure 4.6: The Tof1 C-terminus is required to suppress replication fork uncoupling and the accumulation of ssDNA around replication origins (ChIP-seq performed by Dr. Andrea Keszthelyi and Dr. Nicola Minchell)

(A) Schematic representation of the time course performed for ChIP-seq experiments. Asynchronous cells were arrested in G1 using alpha factor before being released into S-phase in the presence of 200 mM HU to deplete nucleotide pools. Cells were collected for ChIP-seq analysis 60 minutes after the release into S-phase. Antibodies against Rfa1 were used for ChIP-seq to enrich for RPA-coated ssDNA.

(B) Metadata analysis of Rfa1 (RPA) enrichment around all replication origins 60 minutes after release into 200 mM HU in wild-type, *tof1Δ* and *mrc1Δ* cells.

(C) Metadata analysis of Rfa1 (RPA) around all replication origins 60 minutes from release into 200 mM HU in *tof1Δ* cells and *tof1Δ* cells complemented with either codon-optimised wild-type Tof1, *tof1-627*, *tof1-762* or *tof1-830*. Data shown is from merging two independently conducted Rfa1 ChIP-SEQ experiments in each background.

4.3. Discussion

In this chapter I have utilised the Tof1 truncation mutants described in chapter 3 to functionally characterise the C-terminal half of the protein in *S. cerevisiae*. Specifically, I have carried out assays to investigate the contribution of the Tof1 C-terminus to its roles in suppressing replication fork rotation, in replisome pausing at stable DNA-protein blocks and in coupling helicase unwinding to nascent strand synthesis. A summary of these findings can be found in table 4.1 (page 123).

Firstly, I have been able to show that Tof1's role in suppressing replication fork rotation in response to topological stress is dependent on residues 997-1182 of the protein, in the far C-terminus. Amino acids 981-1238 of Tof1 have been previously described as the minimal region required for Top1 interaction and more recently a Tof1 truncation mutant at residue 981 has been shown to be unable to bind to Top1 (Park and Sternglanz, 1999, Shyian et al., 2019). Therefore, although I have not yet been able to confirm this myself using pulldown analysis, it is likely that the *tof1-997* truncation mutant is unable to bind Top1. Presumably, recruitment of Top1 directly to elongating forks would allow for rapid and continuous resolution of topological stress along the DNA duplex, reducing the requirement for replication fork rotation events as a back-up pathway to resolve this stress. Interestingly, *csn3Δ* cells are also unable to restrict replication fork rotation (Schalbetter et al., 2015), suggesting that proper positioning of Top1 by Tof1 ahead of the fork requires Csm3 association with the replisome.

The data presented in this chapter have led to the identification of a Tof1 separation-of-function mutant, *tof1-997*, as Tof1's role in responding to topological stress can be separated from both its role in replisome stabilisation at the rDNA RFB and at CPT-induced Top1-ccs. Rather than the far C terminus, the region of Tof1 required for replisome stability at these protein-DNA blocks instead appears to reside between residues 762-830 of the protein. Interestingly, it is this region that I have found to be crucial for the stable interaction of Tof1 with Csm3 in pulldown analyses (see chapter 3), suggesting that the stable binding of Csm3 by Tof1 is crucial for stable replisome pausing at protein-DNA barriers.

Whilst the mechanism for Top1 trapping on the DNA by CPT has been elucidated in detail, the way in which these lesions are sensed and responded to by the replisome has thus far remained unclear. CPT treatment is known to cause DNA DSBs during S-phase and local increases in topological stress (Ryan et al., 1994, Koster et al., 2007). However it is not clear from these observations how much each of these consequences contributes to the extreme cellular toxicity of *tof1Δ* cells to this drug. The identification of the *tof1-997* separation of function mutant which cannot respond normally to topological stress but is able to support fork pausing indicates that the hypersensitivity of *tof1Δ* cells is not linked to its inability to respond normally to topological stress but instead its ability to stabilise the replisome at protein-DNA barriers. In the case of Top1-ccs this pausing action would be expected to be particularly important to prevent replication fork run-off of the single-stranded break generated by Top1, predicted to generate toxic one-ended DSBs (Strumberg et al., 2000). This then provides a ready explanation of why Tof1 mutants unable to stably pause the replisome at these blocks are hypersensitive to Top1 poisons.

A recent study by Shiyan et al. (2020) has reported a loss of fork pausing function in a C-terminal Tof1 truncation unable to bind Top1. This truncation, at residue 981 of Tof1, appeared to have a small but significant loss of function in fork pausing activity at the rDNA RFB as visualised by 2D gel electrophoresis. However, the data presented in this chapter has found conflicting results. These differences may be explained by the methods used to generate the two mutants. Shiyan and colleagues used C-terminally epitope tagged truncations to analyse replisome pausing, whereas in this thesis all mutants were generated by inserting premature stop codons into the *TOF1* open reading frame. By performing spot tests using C-terminally tagged Tof1 mutants I have been able to show in this chapter that the C-terminus of Tof1 is sensitive to epitope tagging and can result in a partial loss of Tof1 function. Therefore, the loss in pausing observed in Shiyan et al. (2020) may be explained by a general loss of stability and/or Tof1 function rather than a mechanistic role for this region of the protein in replisome pausing at the RFB. Alternatively the differences between the two studies could be explained by the context of the RFB site used. Shiyan and colleagues (2020) assessed replication fork pausing at the endogenous RFB located within the rDNA repeats on chromosome XII in *S. cerevisiae*, whereas this study has used a yeast episomal plasmid containing the RFB sequence to study pausing. The rDNA is one of the most actively transcribed regions of the genome which leads to high levels of topological stress at this region (Brill et al., 1987). It is possible that topoisomerase action is required to assist stable replisome

pausing as well as progression in this unique chromosomal environment, although whether this reflects a general mechanism for replication fork pausing at all protein-DNA barriers genome-wide is unclear.

The ChIP-seq data presented in this chapter has linked the helicase-polymerase coupling function of Tof1 to the mid-C-terminal region of Tof1, between residues 762-830. Similar to its function in replisome pausing, this suggests that the ability of Tof1 to stably associate with Csm3 and recruit it to the replisome is important for fork stability and for coordinating CMG progression with polymerase activity, an observation which is consistent with other similar studies (Katou et al., 2003, Errico et al., 2007). Given that Tof1/Csm3 are positioned at the front of the replisome (Baretić et al., 2019), it is an interesting question to consider how they can mediate strand synthesis occurring at the back of the progressing fork. Mrc1 is also required for helicase/polymerase coupling in response to HU (Katou et al., 2003). The recent cryo-EM structure of the fork protection complex bound to CMG suggests that Mrc1 spans the replisome, which would make it an ideal candidate for a factor able to coordinate the functions of the front and back of elongating replisomes (Baretić et al., 2019). Potentially, the ability of Tof1/Csm3 to stably associate with the replisome promotes Mrc1 stability and/or function, enabling it to carry out its role in coupling.

Taken together, the results presented here demonstrate that the C-terminus of Tof1 has distinct roles in restricting fork rotation, in replisome pausing and in helicase/polymerase coupling. The region of Tof1 between residues 997-1182 appears to be essential for its role in restricting replication fork rotation, potentially by recruiting Top1 directly to the replisome. This function can be separated from its other roles in stable replisome pausing which require the same region of Tof1 needed for stable Csm3 binding, between aa 762-830. This mid C-terminal region of the protein is required to pause forks at the Fob1/RFB site and to suppress CPT sensitivity, likely by preventing replication fork run-off at these lesions. The same region is also required to couple CMG progression with nascent strand synthesis in nucleotide limiting conditions, potentially through positioning Csm3 and Mrc1 correctly within the replisome to connect the front and back of this complex molecular machine.

Mutant	Suppresses CPT sensitivity	Suppresses fork rotation	Pauses forks at RFB	Helicase/ polymerase coupling in HU
<i>tof1Δ</i>	X	X	X	X
<i>csm3Δ</i>	X	X	X	X
<i>mrc1Δ</i>	✓	✓	✓	X
Tof1 wt	✓	✓	✓	✓
<i>tof1-627</i>	X	X	X	Partial
<i>tof1-762</i>	X	X	X	Partial
<i>tof1-830</i>	✓	X	✓	✓
<i>tof1-997</i>	✓	X	✓	✓
<i>tof1-1182</i>	✓	✓	✓	✓
<i>tof1-wt-TAP</i>	✓	NT	NT	NT
<i>tof1-627-TAP</i>	✓	NT	NT	NT
<i>tof1-997-TAP</i>	X	NT	NT	NT

Table 4.1: Summary of phenotypes of Tof1 mutants described in chapter 4 compared with the phenotypes of *tof1Δ*, *csm3Δ* and *mrc1Δ* mutants.

NT = Not tested.

5. Investigating the structure-function relationship of Tof1 in the intra-S checkpoint and fork restart in *S. cerevisiae*

5.1. Introduction

As discussed in chapter 1, in response to replication stress and genotoxic agents cells activate the ATR pathway to elicit a checkpoint response (Iyer and Rhind, 2017). In *S. cerevisiae* the ATR homolog, Mec1, is activated following recruitment to RPA coated ssDNA by Ddc2, where it phosphorylates and activates a number of substrates within the immediate vicinity of the DNA damage or replication stress signal (Paciotti et al., 2000). In turn, this results in phosphorylation and activation of the effector checkpoint kinase Rad53, the functional homolog of Chk1 in higher eukaryotes. Rad53 is able to freely diffuse throughout the cell and is thought to phosphorylate hundreds of substrates to enact multiple checkpoint responses, including repression of further origin firing, upregulation of specific genes and prevention of mitotic entry (Pardo et al., 2016).

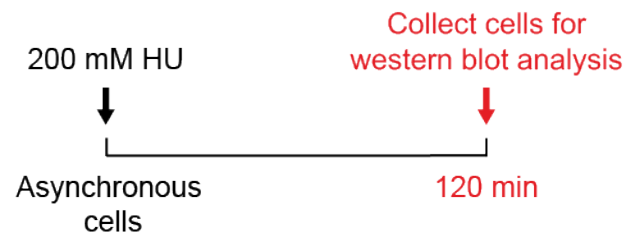
In *S. cerevisiae*, Rad53 can be activated via two distinct branches of the checkpoint response. In all stages of the cell cycle, Rad9 is able to mediate Rad53 phosphorylation and activation as part of the DNA damage checkpoint (DDC) (Weinert and Hartwell, 1988, Weinert and Hartwell, 1993). However, Rad53 can be activated in a Rad9-independent manner specifically during S-phase by Mrc1 and Tof1, which forms the DNA replication checkpoint (DRC) (Foss, 2001, Alcasabas et al., 2001). As a replisome component, Tof1 is ideally placed to mediate checkpoint activation and is thought to do so by assisting the key role of Mrc1 in DRC activation. However, it is unclear how Tof1 carries out this mediator function and whether it is related to its other reported roles, as the structure-function relationship of Tof1 in checkpoint activation is yet to be explored. In this chapter, I have utilised the truncation mutants previously described in chapters 3 and 4 to investigate the role of the Tof1 C-terminus in its role as a mediator of the DRC. Specifically, this chapter focuses on the role of Tof1 in mediating Rad53 phosphorylation in response to HU, along with the implications of this checkpoint response for replication fork restart and cellular viability following replication stress.

5.2. Results

5.2.1. The C-terminus of Tof1 is dispensable for intra-S-checkpoint activation

I first set out to ask whether the C-terminus of Tof1 is required for its mediator function in the intra-S checkpoint. As discussed above, checkpoint pathways in *S. cerevisiae* can be divided into two distinct branches, the Rad9-dependent DDC and the Mrc1/Tof1-dependent DRC. Due to the overlapping nature of these responses, only cells lacking both Tof1 and Rad9 are unable to elicit Rad53 activation in response to replication stressing agents (Foss, 2001). Therefore, to investigate the importance of the Tof1 C-terminus specifically in DRC activation, the Tof1 truncation mutants previously generated were combined with a *RAD9* deletion so that cells were lacking the DDC. These cells were then tested for their ability to activate Rad53 in response to HU. HU is a potent inhibitor of dNTP synthesis and so treatment with HU results in prolonged fork arrest and activation of the checkpoint (Bianchi et al., 1986, Iyer and Rhind, 2017). Briefly, exponentially growing cells were treated with 200 mM HU for 2 hours to arrest replication forks, before collecting samples for TCA extraction and analysis by western blot (Figure 5.1A). Upon checkpoint activation, Rad53 becomes hyper-phosphorylated, which can be visualised as a phospho-shift (resulting from phosphorylated protein species running slower through SDS-PAGE gels) on western blots. Treatment of wild-type cells with HU resulted in a detectable phospho-shift of the Rad53 protein, indicating that checkpoint activation is robustly activated under these experimental conditions (Figure 5.1B). As expected, cells lacking either Tof1 or Rad9 were still proficient in activating the checkpoint, whilst *tof1Δ rad9Δ* cells showed a dramatic decrease in Rad53 hyperphosphorylation (Figure 5.1B). Interestingly, complementation of these cells with either *tof1-627*, *tof1-762*, *tof1-830*, *tof1-997* or *tof1-1182* restored checkpoint activation following HU treatment (Figure 5.1B). This observation indicates that the N-terminus of Tof1 alone is sufficient to support its mediator role in activation of the intra-S checkpoint. This argues that the checkpoint function of Tof1 can be separated from its roles in replication fork pausing and coupling, suppression of CPT sensitivity and in restricting replication fork hyper rotation, all of which require the region between 762-830 of Tof1 for normal function (see chapter 4).

A



B

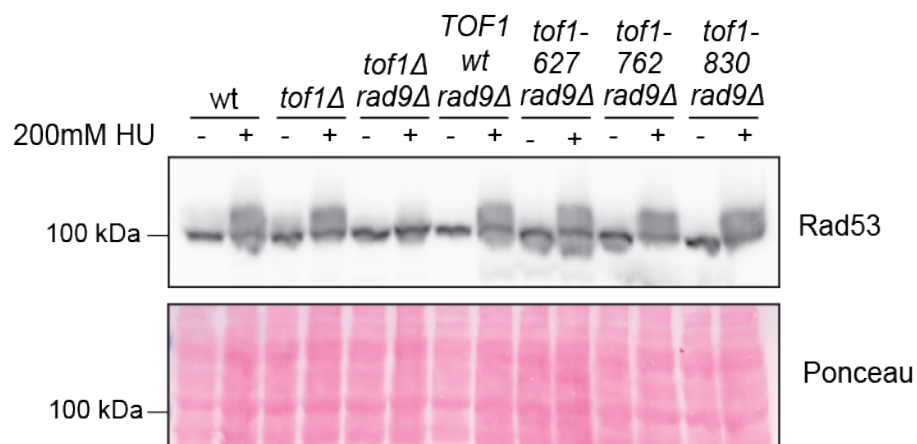


Figure 5.1: The C-terminus of Tof1 is not required for Rad53 phosphorylation in response to HU treatment

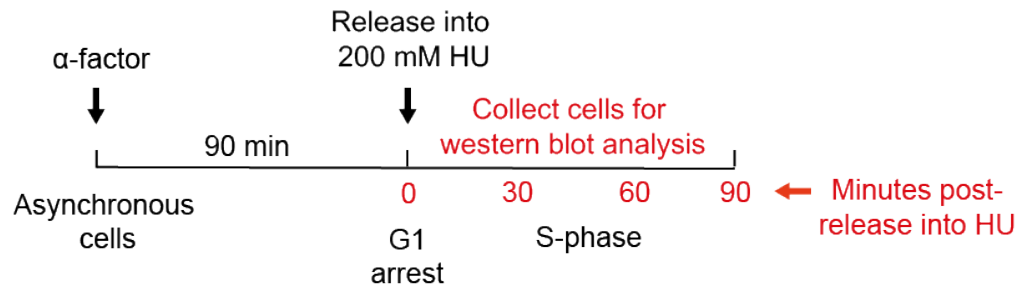
(A) Schematic of experiment performed in B. Asynchronous yeast cells growing in YPD were re-suspended in YPD medium containing 200 mM HU and collected for TCA extraction and western blot analysis after 120 min in HU.

(B) Western blot using anti-Rad53 antibodies to visualise Rad53 phospho-shift indicative of checkpoint activation in wt, *tof1Δ*, *tof1Δ rad9Δ*, *TOF1 wt rad9Δ*, *tof1-627 rad9Δ*, *tof1-762 rad9Δ* and *tof1-830 rad9Δ* cells. Ponceau-stained blot included as loading control. Image shown is from one of two equivalent independently conducted experiments.

I next set out to establish whether the C-terminal Tof1 truncations were able to activate the intra-S checkpoint with wild-type kinetics. Previous studies have indicated that DRC-mediated Rad53 hyperphosphorylation can be visualised by as early as 30 minutes in response to HU (Alcasabas et al., 2001). The timely activation of Rad53 in response to replication stress is crucial for the earliest of checkpoint responses, such as inhibition of late origin firing. Potentially, C-terminal mutants able to mediate Rad53 activation may do so with slower kinetics, which could have implications on downstream checkpoint responses. Such a delay to activation would not be visible in the previous assay used, where cells were collected one hour after exposure to HU. Therefore, I decided to use the same technique to follow checkpoint activation in the Tof1 mutants at earlier time points.

The experimental outline is shown in Figure 5.2A. Briefly, cells were arrested in G1 phase using alpha-factor peptide before being released into S-phase in the presence of 200 mM HU. Samples were collected at 0, 30, 60 and 90 minutes after release into HU and subjected to TCA extraction and western blotting using Rad53 antibodies as before. Consistently, *tof1Δ rad9Δ* mutants failed to activate the checkpoint after 1 hour in HU (Figure 5.2B). Even at the latest time point observed, 90 minutes, no detectable Rad53 phosphorylation was observed (Figure 5.2B). Using this assay, I could detect no visible Rad53 phosphorylation in either *TOF1 wt rad9Δ* or *tof1-627 rad9Δ* cells at 30 minutes. However, after 60 minutes in HU robust Rad53 phosphorylation was detected in both cells complemented with *TOF1 wt* or *tof1-627*, indicating that the DRC activation observed in C-terminal Tof1 truncations occurs with the same kinetics as in wild-type cells (Figure 5.2B).

A



B

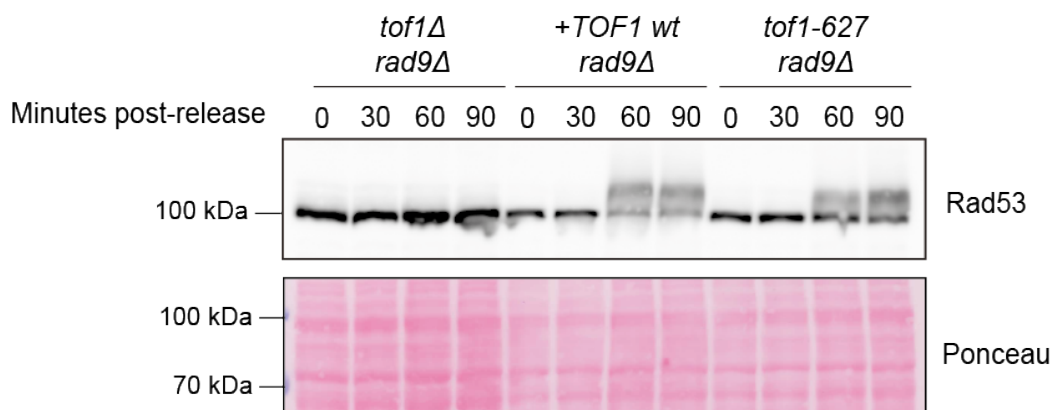


Figure 5.2: The N-terminus of Tof1 activates the intra-S checkpoint with wild-type kinetics

(A) Schematic of experiment performed in B. Asynchronous yeast cells growing in YPD were arrested in G1 phase using alpha factor before being released into S-phase in the presence of 200 mM HU. Samples for western blot analysis were collected at 0, 30, 60 and 90 min post-release into HU.

(B) Western blot using anti-Rad53 antibodies to visualise Rad53 phospho-shift indicative of checkpoint activation in *tof1Δ rad9Δ*, *TOF1 wt rad9Δ*, and *tof1-627 rad9Δ* cells. Ponceau-stained blot included as loading control. Image shown is from one of two equivalent independently conducted experiments.

5.2.2. The intra-S-checkpoint function alone of Tof1 is not sufficient to maintain cellular viability in response to replication stress

The data presented above show that the N-terminus of Tof1, specifically residues 1-627, are sufficient for activation of the DRC in response to HU. I next wanted to investigate the contribution of this DRC function of Tof1 in maintaining genome stability following nucleotide limitation. Activation and maintenance of the DRC is crucial for fork stabilisation, to limit further origin firing, and to halt cell cycle progression (Iyer and Rhind, 2017, Pardo et al., 2016). However, the links between DRC activation by mediators such as Tof1 and Mrc1 and how this function relates to their other functions in fork stabilisation at physical blocks to replication are not clear. Potentially, checkpoint activation mediated by Tof1 at the replisome is sufficient to protect stalled forks and ensure subsequent faithful completion of DNA replication. Alternatively, the C-terminal roles of Tof1 in replisome pausing and coupling (discussed in chapter 4) may also be required to maintain genome stability and cell viability following replication stress.

To distinguish between these possibilities, I started by investigating whether Tof1-dependent mediation of the DRC is sufficient to allow cells to survive chronic HU treatment. To do this, I performed spot tests using *tof1Δ rad9Δ* mutants, to ask whether chronic HU treatment would affect the viability of these cells. As expected, deletion of both *TOF1* and *RAD9* led to extreme sensitivity to the drug, consistent with a loss of checkpoint function leaving cells unable to recover from HU-induced replication fork arrest (Figure 5.3). Interestingly however, when these cells were complemented with checkpoint-proficient *tof1-627* or *tof1-762* truncations, this was unable to rescue the viability of cells grown on HU (Figure 5.3), suggesting that checkpoint activation alone is not sufficient to allow cells to recover from chronic HU treatment. Addition of *tof1-830* rescued viability to wild-type levels (Figure 5.3) indicating that the same region of Tof1 required for its pausing and coupling functions (aa 762-830) is required for recovery after chronic exposure to HU.

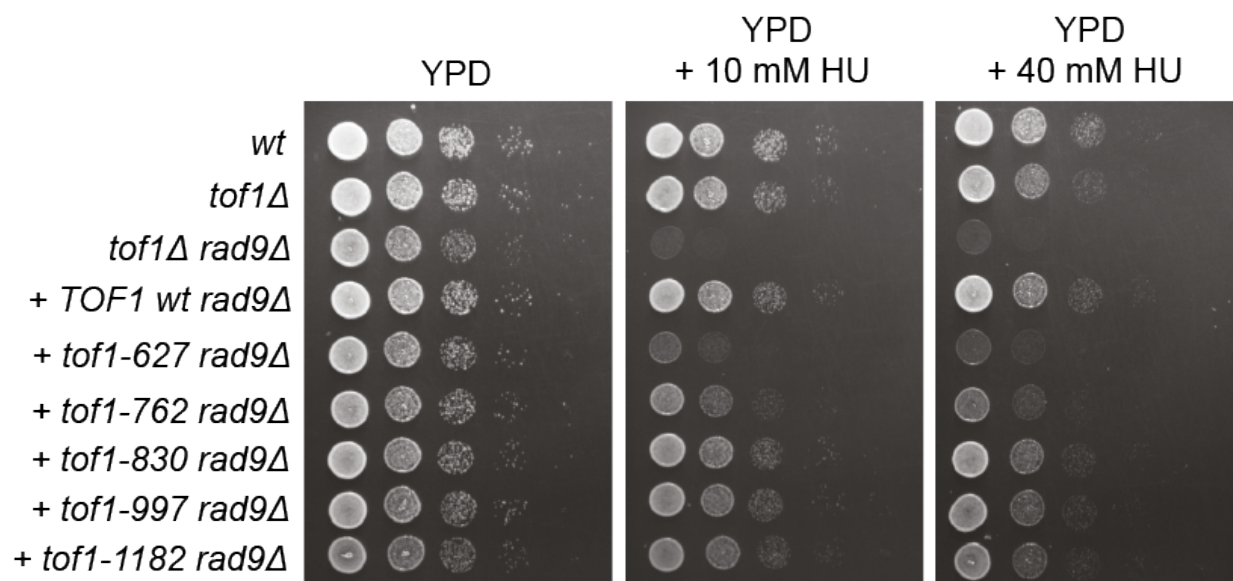


Figure 5.3: The C-terminus of Tof1 is required for cell viability in response to chronic HU treatment in DRC-proficient cells

Spot viability assays of C-terminal Tof1 truncations grown on YPD, 10 mM HU or 40 mM HU. Photos were taken 48 hrs after plating and cells were grown at 25°C. Images shown are from one of two equivalent independently conducted experiments.

5.2.3. Intra-S checkpoint activation by the Tof1 N-terminus allows cells to resume DNA replication following acute HU treatment

I next wanted to investigate the cause of toxicity in DRC-proficient Tof1 truncation mutants treated with HU. As well as suppressing origin firing and halting progression through the cell cycle, it is speculated that the essential function of the DRC is to stabilise stalled replication forks to ensure they can be restarted (Paciotti et al., 2001, Iyer and Rhind, 2017, Tercero and Diffley, 2001). Potentially, Tof1 mutants with an active checkpoint may be unable to resume DNA synthesis following HU treatment, which would lead to terminal fork arrest and failure to complete chromosome duplication and segregation. Based on the spot test assays shown in Figure 5.3, it would be presumed that this function of Tof1 in resuming DNA synthesis following HU treatment depends on its mid-C-terminal region, between residues 762-830. To test this hypothesis, I used FACS analysis to ask whether the C-terminal Tof1 mutants were able to resume DNA synthesis following HU-mediated arrest and subsequent release. The experimental outline is shown in Figure 5.4A. Briefly, cells were arrested in G1 phase using alpha factor before being released into 200 mM HU for 1 hour. Cells were then released from the HU arrest into YPD medium to allow recovery. FACS samples were taken at regular time points to monitor DNA content of the cells throughout the time course. In all cases, cells showed a 1C DNA content after HU arrest consistent with the replication arrest induced by inhibition of dNTP synthesis (Figure 5.4B). As expected, *tof1Δ rad9Δ* cells showed a severe defect in resuming DNA replication after release from HU into YPD. This defect was seen as late as 2 hours post-release from the HU block, where DNA synthesis had not been completed in *tof1Δ rad9Δ* cells (Figure 5.4B). Complementation of these cells with either *TOF1* wild-type or *tof1-830* led to apparent completion of DNA synthesis by 100 min post-release from the HU block (Figure 5.4B). Surprisingly, *tof1-627 rad9Δ* cells were also able to resume DNA synthesis after acute HU treatment, with the same kinetics as seen in *TOF1* wt *rad9Δ* cells (Figure 5.4B).

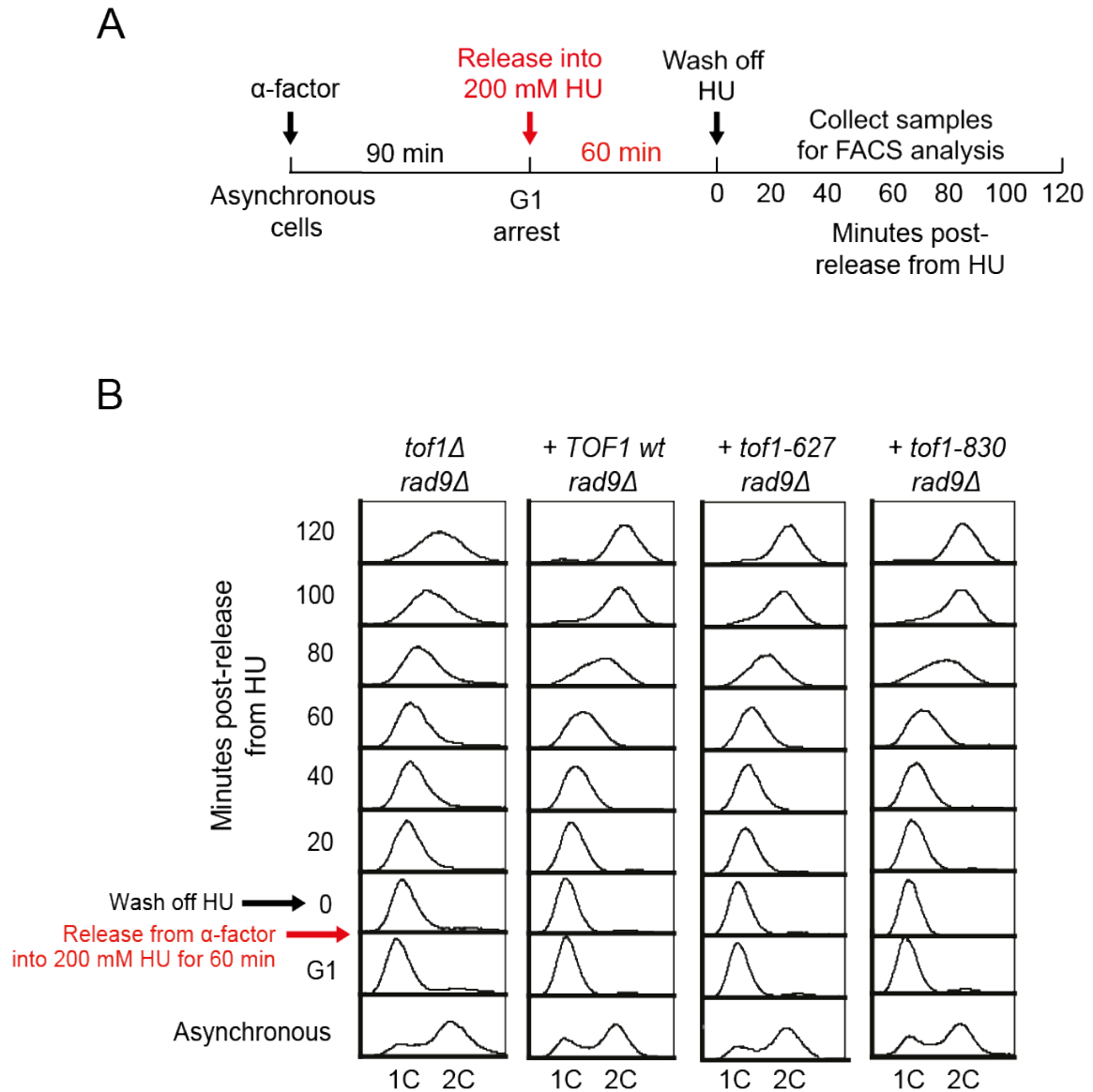


Figure 5.4: The N-terminal DRC function of Tof1 is sufficient for resumption of DNA synthesis after HU-induced fork stalling and release

(A) Schematic of experiment performed in B. Asynchronous yeast cultures were arrested in G1 with alpha factor before releasing cells into 200 mM HU for 1 hour to stall replication forks (red arrow). Cells were then released from the HU arrest into fresh YPD medium and samples were collected for FACS analysis at 0, 20, 40, 60, 80, 100 and 120 min after release from the HU arrest. (B) FACS profiles showing DNA content of *tof1Δ rad9Δ*, *TOF1 wt rad9Δ*, *tof1-627 rad9Δ* and *tof1-830 rad9Δ* cells at indicated time points after HU-induced fork arrest and release. Images of DNA content FACS shown are from one of two equivalent independently conducted experiments.

This suggests that intra-S checkpoint activation in the absence of other Tof1 mediated activities is sufficient to allow cells to resume DNA synthesis after replication forks have been stalled in HU and subsequently released. Interestingly, these data therefore suggest that the sensitivity to chronic HU treatment observed in *tof1-627 rad9Δ* and *tof1-762 rad9Δ* cells is not due to an inability to resume and complete bulk DNA replication.

5.2.4. Completion of bulk DNA replication in C-terminal Tof1 truncation mutants is not sufficient to rescue cellular viability after acute HU treatment

As shown in Figure 5.3, addition of *tof1-627* or *tof1-762* into *tof1Δ rad9Δ* cells is not sufficient to rescue the poor viability of these cells when grown on YPD plates containing 10 mM or 40 mM HU. However, it is not clear whether cells only exposed briefly to HU and allowed to resume replication may be more viable than cells continuously exposed to the drug, as chronic exposure to HU may only lead to toxicity after several cell divisions. Therefore, I next investigated whether the apparent resumption and completion of DNA replication following release from an acute HU-induced arrest would be sufficient for viability. To do so, G1-arrested cells were released into 200 mM HU as before, but following the 60-minute HU arrest cells were diluted in YPD, counted and plated to YPD agar plates (Figure 5.5A and 5.5B). In order to plate between 200-400 cells per YPD plate, cells require multiple serial dilution steps, effectively washing out any HU from the growth medium. After a 48-hour recovery period, viability was calculated as the percentage of cells originally plated that were able to form visible colonies. As expected, checkpoint deficient *tof1Δ rad9Δ* cells showed extremely poor recovery from the acute HU treatment with on average only 6.4% of cells plated able to form visible colonies, presumably as a result of an inability to stabilise stalled replication forks and prevent subsequent fork collapse (Figure 5.5B and 5.5C). When complemented with either wild-type *TOF1* or *tof1-830*, average viability increased to a base level of 51.4% and 56.4%, respectively. However, when complemented with *tof1-627*, recovery from HU was only partially recovered to 22.2% viability, despite the previously observed DNA resumption seen in these cells (Figure 5.5C). Taken together, these data suggest that intra-S checkpoint activation and the ability to complete bulk DNA replication following HU arrest and release is only partly sufficient for cells to recover from HU-induced lesions. This is consistent with the region of Tof1 between aa 762-830 having an additional, separate function in responding to HU-induced stress to ensure viability in dNTP-limiting conditions.

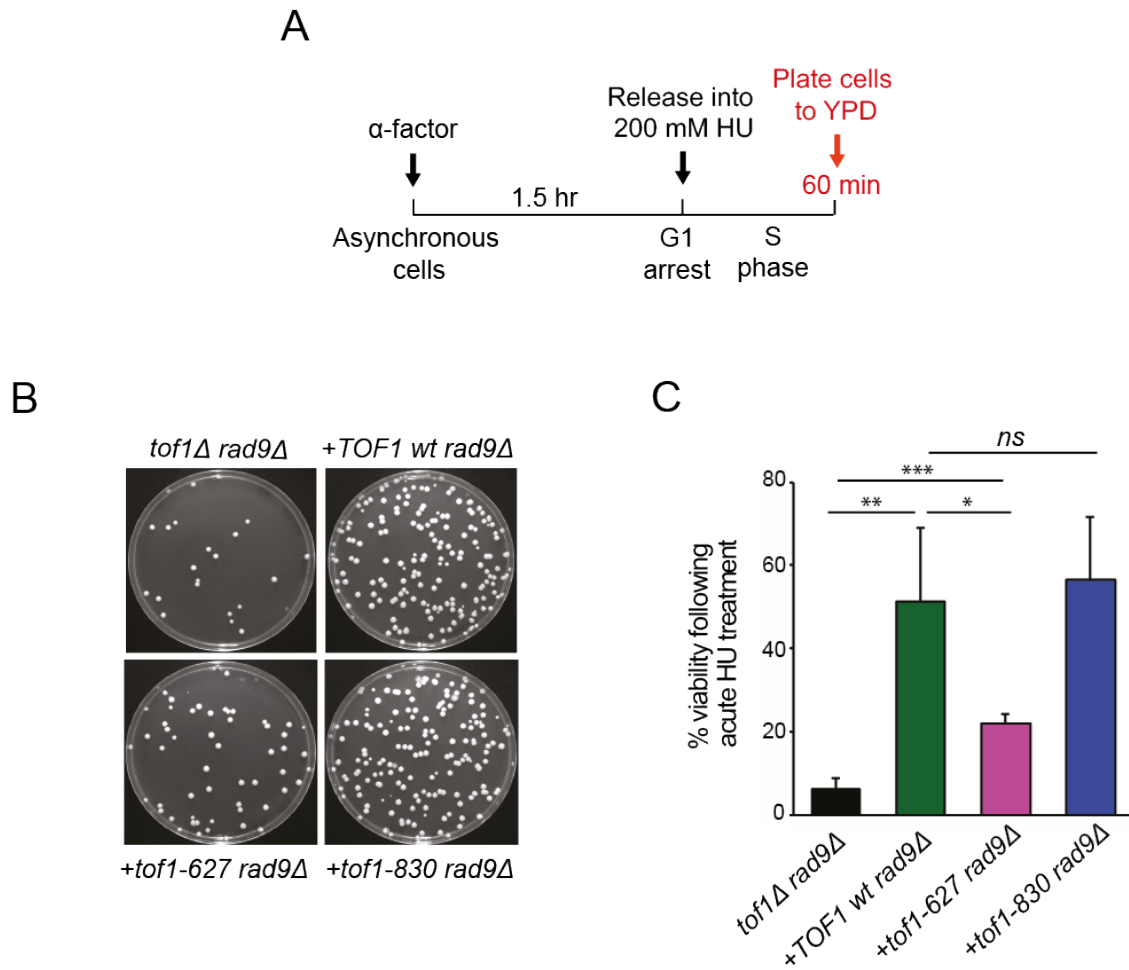


Figure 5.5: Amino acids 762-830 of *Tof1* are required to rescue cellular viability in DRC-proficient mutants treated acutely with HU

(A) Schematic of experiment performed in B and C. Asynchronous cells were arrested in G1 phase with alpha factor before being released into 200 mM for 1 hour. HU was then washed out by dilution of the cells in YPD before plating 200, 300 or 400 cells to one YPD plate. Plates were incubated for 48 hours to allow cells to form colonies before imaging and colony counting.

(B) Representative images of YPD plates 48 hrs after plating 200 *tof1Δ rad9Δ*, *TOF1 wt rad9Δ*, *tof1-627 rad9Δ* and *tof1-830 rad9Δ* cells acutely treated with HU. Representative images from 4 independent experiments are shown.

(C) Quantitation of (B). Percent viability was calculated as the percentage of cells able to form colonies as a fraction of the total number of cells plated. Statistical significance was calculated using a two-tailed unpaired student's *t-test*, with *n*=4. Significance values: *= <0.05 , **= <0.01 , ***= <0.001 .

5.2.5. The N-terminal checkpoint function of Tof1 is sufficient to restart HU-stalled replication forks but fork progression is compromised

Thus far, the data presented in this chapter suggest that DRC activation mediated by the N-terminus of Tof1 is sufficient to ensure that the bulk of DNA synthesis can be resumed and completed following release from HU-induced arrest. However, in Tof1 truncation mutants lacking residues 762-830 of the protein, DRC activation and apparent completion of DNA synthesis is not sufficient to fully rescue the sensitivity of these cells to even acute HU treatment. This led to the question of what might be responsible for cell death in these mutants.

Potentially, the bulk DNA quantification generated by FACS analysis could be misleading. The FACS data presented in Figure 5.4 does not allow for distinction between two possibilities: either replication forks stalled in HU are restarted following release from the block, or stalled forks are rescued by firing of dormant origins. If bulk DNA synthesis is completed by firing of dormant origins rather than restart of forks stabilised in HU, this could suggest that the DRC activation mediated by the N-terminus of Tof1 is not sufficient to stabilise forks arrested in HU, in a manner compatible with restart. The failure to stabilise stalled forks is associated with fork collapse, a phenomenon thought to lead to double-strand breaks and unusual, potentially toxic fork structures (Lopes et al., 2001). In this scenario collapsed forks would be undetectable by FACS but would likely have a significant impact on cellular viability.

To see whether stalled replication forks in the Tof1 mutant backgrounds were effectively restarted, Dr Andrea Keszthelyi performed Sync-Seq to analyse copy number increases across the genome in cells treated with HU and subsequently released from the HU block, allowing for visualisation of restart from individual forks after the HU arrest. Sync-Seq data uses copy-number analysis to follow progression of replication during S-phase (Batrakou et al., 2020). A schematic representation of the data profile obtained from Sync-seq experiments is shown in Figure 5.6 (for simplicity, the schematic shows an example of the copy number profile that could be generated in one cell rather than in a population of cells where differences in origin firing and fork speed will influence the profile). In G1 phase before cells have begun to replicate their DNA, all regions of the genome should be present in equal copy number, resulting in equal reads for all genomic loci being

returned from NGS analysis (Figure 5.6A). When cells have initiated DNA replication and begin to synthesise nascent strands, this results in increases in copy number and as a result, increased reads around replication origins which are visualised as sharp peaks (Figure 5.6B). These peaks broaden as replication forks move along the DNA duplex and copy number increases along the chromosome (Figure 5.6C). The valleys between peaks represent regions that are unreplicated at the point of sample collection (Figure 5.6C).

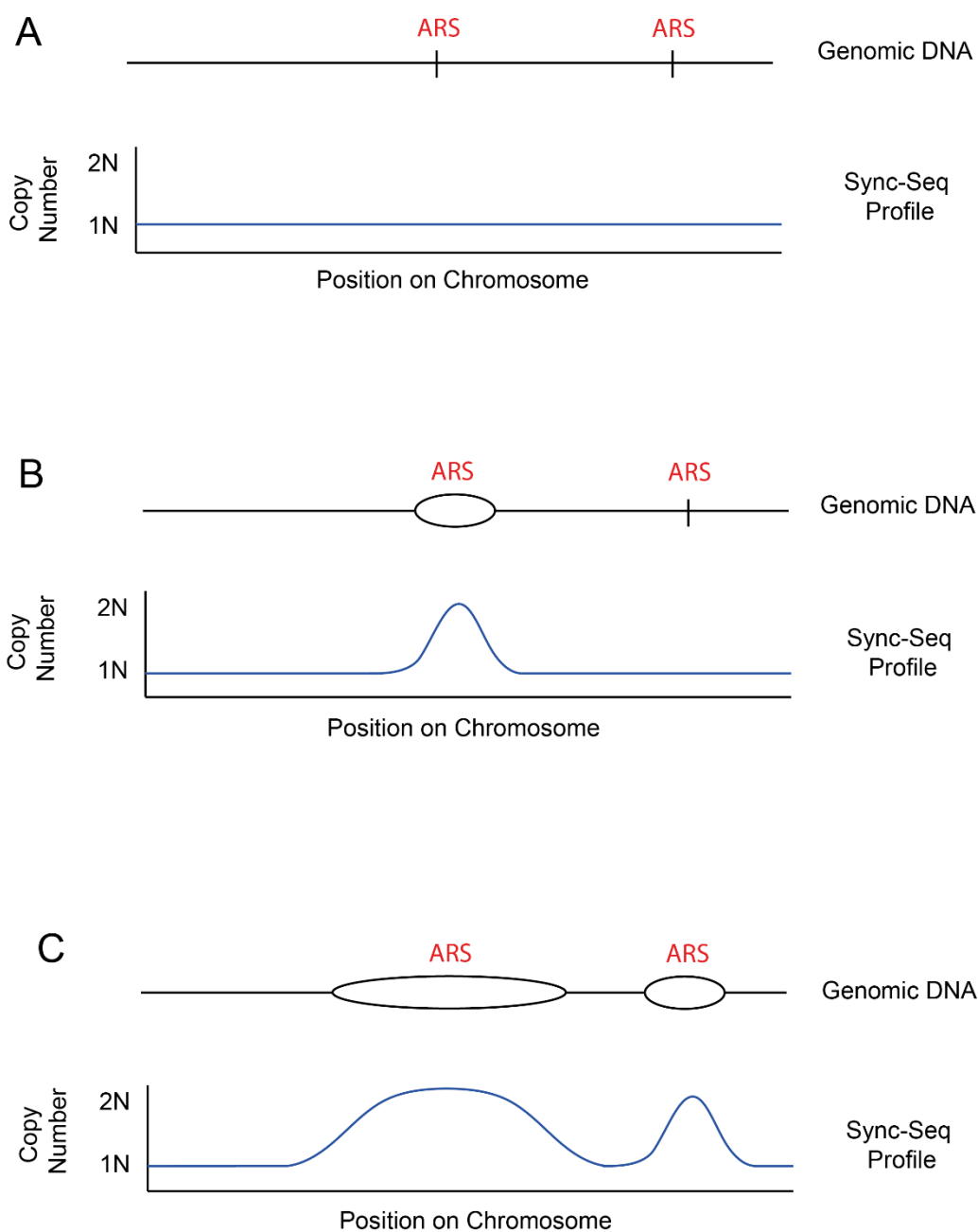


Figure 5.6: Schematic representation of Sync-Seq data from replicating cells (for simplicity, figure shows a representation of copy number analysis in one cell)

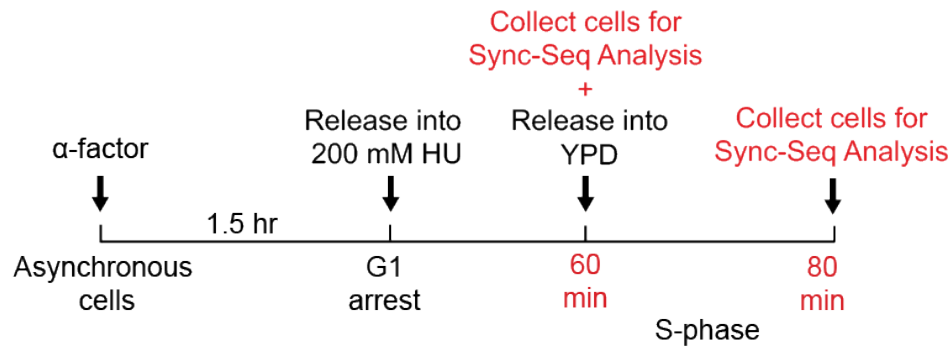
(A) Before replication initiation, all genomic loci are present in equal amounts resulting in a flat profile after sequencing analysis.

(B) After initiation, increases in copy number are observed around origins that fire resulting in sharp peaks after sequencing analysis

(C) As replication forks progress along the chromosome the sharp peaks generated by initiation events broaden, with valleys between peaks representing unreplicated regions.

To follow individual replication fork stalling and restart, cells deleted for *RAD9* and containing either *tof1Δ*, *TOF1* wt, *tof1-627* or *tof1-830* were synchronised in G1 phase using alpha factor before being released into S-phase in the presence of 200 mM HU for 1 hour to stall forks. Cells were then released from the HU arrest into YPD medium and allowed to recover and resume DNA replication (Figure 5.7A). Samples for Sync-Seq analysis were taken at 1 hour in HU to visualise where forks originating from defined origins were stalled, and at 80 minutes after release from the HU block to monitor their progression through S-phase. 80 minutes was chosen as previous FACS analysis had shown that at this time point, replication would be expected to be taking place after the HU arrest but would not be complete (Figure 5.4). After 1 hour in 200 mM HU, similar copy number increases very close to replication origins were seen in all backgrounds, indicating that replication forks fired normally in every strain but then stalled in the absence of dNTP synthesis (Figure 5.7, left panels). As expected, 80 minutes after release from the HU block, checkpoint deficient *tof1Δ rad9Δ* cells showed a severe defect in restarting the same replication forks stalled by HU treatment, as compared with *TOF1* wt *rad9Δ* cells and *tof1-830 rad9Δ* cells (Figure 5.7, right panels). Increases in copy number close to the previously stalled forks were seen to spread further along the chromosome in *TOF1* wt *rad9Δ* and *tof1-830 rad9Δ* backgrounds, indicating that nascent strand synthesis occurred from the same locus and therefore most likely from the same forks stalled in HU (Figure 5.7, upper right panel). Interestingly, *tof1Δ rad9Δ* cells complemented with *tof1-627* showed an increase in copy number further away from replication origins, suggesting that the DRC function of *tof1-627* cells is sufficient to restart HU-stalled replication forks (Figure 5.7, bottom right panel). However, these copy number increases were not seen as far away from origins as in *TOF1* wt and *tof1-830* cells, indicating that although replication forks can restart in this background, these restarted forks cannot progress as far as in wild-type cells.

A



B

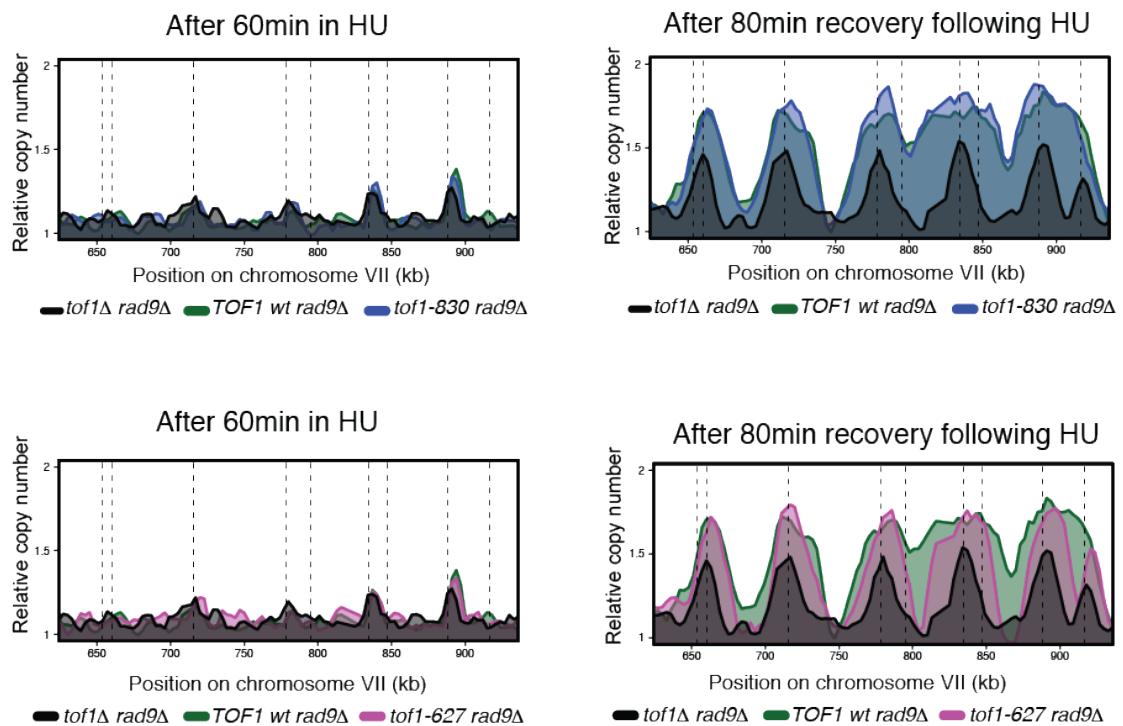


Figure 5.7: The DRC function mediated by the Tof1 N-terminus is sufficient for fork restart, but not for normal elongation following restart (Sync-Seq performed by Dr Andrea Keszthelyi)

(A) Schematic of experiment performed in B. Asynchronous cells were arrested in G1 using alpha factor before being released into 200 mM HU for 1 hour to stall replication forks. Cells were subsequently released into YPD medium to allow cells to recover from the HU arrest. Samples for copy number analysis (Sync-Seq) were collected at 60 min in HU and 80 min post-release from the HU arrest.

(B) Copy number analysis of *tof1Δ rad9Δ*, *TOF1 wt rad9Δ*, *tof1-627 rad9Δ* and *tof1-830 rad9Δ* cells after 60 min in HU and at 80 min following release from HU arrest. Data from one of two independent sync-seq experiments is shown as representative of both replicates.

5.3. Discussion

As a replisome component, Tof1 is placed in the ideal position to assist with activation of the DRC specifically at replication forks encountering DNA damage or replication stress. Deletion of *TOF1* in budding yeast cells lacking the Rad9-dependent DDC sensitises cells to replication stressing agents MMS, UV and HU, highlighting its importance in this pathway (Foss, 2001). However, previous analysis of the role of Tof1 in the DRC has focused on using gene deletion studies in yeast or siRNA knockdown in mammalian cells, which has limited the understanding of the mechanisms by which Tof1 contributes to DRC activation, as well as whether this role can be linked to its other known functions. In this chapter I have addressed this problem by utilising C-terminal Tof1 truncation mutants to investigate the role of the Tof1 C-terminus in intra-S checkpoint activation. Specifically, I have investigated whether the C-terminus of the protein is required for Rad53 phosphorylation in response to HU-induced fork arrest, whether replication forks stalled in HU can restart in these mutants, and whether checkpoint-mediated fork restart is sufficient for cell viability under conditions of replication stress. A summary of these findings can be found in table 5.1 (page 146).

First, I have shown that the N-terminus of Tof1, specifically residues 1-627, is sufficient for Rad53 activation in response to HU treatment. Importantly, Rad53 phosphorylation was observed with wild-type kinetics. This was surprising given the failure of the *tof1-627* mutant to perform several other Tof1-mediated functions as discussed in chapter 4. Interestingly, this identifies *tof1-627* as a new separation-of-function mutant, which is capable of mediating DRC activation in response to HU but cannot support the fork stabilisation functions discussed in chapter 4. This suggests that the roles of Tof1 in fork pausing, in responding to topological stress, and in replisome coupling are not related to its ability to activate the intra-S checkpoint.

Potentially, the role of Tof1 in checkpoint activation is through its ability to stabilise Mrc1 in the replisome, allowing it to carry out its well-known checkpoint mediator function efficiently. Tof1 interacts with Mrc1, and both proteins travel with replication forks (Bando et al., 2009, Katou et al., 2003, Lewis et al., 2017, Baretić et al., 2019, Gambus et al., 2006). In budding yeast, *mrc1Δ rad9Δ* cells are inviable (Alcasabas et al., 2001). In

contrast, *tof1Δ rad9Δ* cells, although slow growing, are viable unless treated with replication stressing agents. This suggests that the role of Mrc1 in mediating the DRC is required even under unchallenged growth conditions, whereas Tof1 may act to promote or assist this function under conditions of high replication stress. The data presented in this chapter argues that the N-terminus of Tof1 alone is capable of doing so. Potentially, this is mediated by a physical interaction between the Tof1 N-terminus and Mrc1. The recently published cryo-EM structure of Tof1-Csm3 and Mrc1 bound to the CMG supports this; cross-linking mass spectrometry experiments have found interactions between the N-terminus of Tof1, specifically at aa 203, aa 499 and aa 616, with Mrc1 (Baretić et al., 2020). To see whether the *tof1-627* truncation is able to interact with Mrc1, pulldown analysis was started with the Tof1 truncation mutants, but was unable to be completed due to time restrictions. This analysis will be important to confirm that the ability to associate with Mrc1 allows *tof1-627* to carry out its DRC mediator function.

A surprising observation from the data presented in this chapter is that the ability of *tof1-627* to mediate the DRC is not sufficient for cellular viability in response to HU treatment in cells lacking the DDC, as I have shown that *tof1-627 rad9Δ* cells which can mediate the DRC are as sensitive to chronic HU treatment as DRC deficient *tof1Δ rad9Δ* cells and partially sensitive to acute HU treatment. This gives rise to an important question: why can DRC proficient cells not recover from HU-induced replication fork arrest? In attempt to answer this question, in this chapter I have also investigated the contribution of Tof1-mediated DRC activation to replication fork restart and cell viability following HU-induced fork stalling.

Interestingly, I have shown that the decreased viability seen in *tof1-627 rad9Δ* cells following HU treatment is not linked to an inability to resume DNA replication after an acute HU treatment. Using FACS analysis to monitor DNA content over time, I have been able to show that *tof1-627 rad9Δ* cells are capable of completing bulk DNA synthesis following release from HU. This is likely attributed to the essential fork stabilisation role of the DRC, as checkpoint defective *tof1Δ rad9Δ* cells cannot complete DNA replication under the same conditions. Mec1 and Rad53-dependent checkpoint activation protects stalled replication forks from collapse, an event that is poorly understood at the molecular level but generally refers to a stalled replication fork being destabilised into a structure that cannot be restarted (Tercero and Diffley, 2001, Lopes et al., 2001).

If *tof1-627 rad9Δ* cells can resume DNA replication after acute HU treatment, what then, is the reason for the poor viability of these cells under these conditions? In this chapter, Sync-seq has been used to attempt to address this question, by following DNA replication at specific genomic loci under conditions of HU arrest and subsequent release. This was used to give a clearer view of the restart and elongation dynamics of individual replication forks stalled in and released from HU. This was necessary as the initial increases in DNA content as visualised from the FACS data could have been accounted for by increased dormant origin firing in these cells. The resulting Sync-seq data presented in this chapter suggests that whilst *tof1-627 rad9Δ* cells can restart HU-stalled replication forks, there are large regions of unreplicated DNA in these cells compared to *TOF1 wt rad9Δ* and *tof1-830 rad9Δ* cells at the time points observed. This observation could be explained by several scenarios.

First, it is important to note that whilst the Sync-seq data strongly suggests replication forks can restart after release from a HU-induced arrest in *tof1-627 rad9Δ* cells, it cannot absolutely rule out rescue of stalled forks by dormant origins lying very close to the stalled fork, as the resolution of the experiment does not allow for this distinction. A single-molecule approach such as DNA combing and staining with thymidine analogues would be necessary to definitively show that arrested forks can restart (Bensimon et al., 1994, Tourrière et al., 2017). However, several observations suggest that dormant origin firing is not responsible for the increases in DNA content close to forks stalled in and released from HU. First, elongation from forks stalled in HU appears to occur at genomic loci where there is no known ARS or previously identified dormant origin, suggesting that the elongation is most likely to continue from forks already fired and stalled before the release. Second, *tof1Δ rad9Δ* cells have significantly less DNA content than *tof1-627 rad9Δ* cells, despite being expected to be unable to perform DRC-mediated suppression of origin firing. Lastly, to produce a peak with the symmetry observed in our Sync-seq profiles, dormant origins within a close vicinity to both sides of the initial origin would have to fire, otherwise the peaks from these dormant origins would be visible as separate peaks on the profile. This cannot be completely ruled out, but is unlikely. Therefore, the Sync-seq data strongly suggests that replication forks stalled in and released from HU can be restarted in a checkpoint-dependent manner in *tof1-627 rad9Δ* cells.

Potentially, the restarted forks in *tof1-627 rad9Δ* cells could have an elongation defect, resulting in slower moving forks. Recent work using reconstituted replication *in vitro* has

shown that both Tof1 and Csm3 are required for maximum rates of DNA replication (Yeeles et al., 2017). The region between aa 762-830 of Tof1, and the stable interaction of Tof1 with Csm3, may therefore be required to ensure wild type rates of replisome progression. Alternatively, replication fork restart kinetics may be slower in certain Tof1 mutants, a question that has not yet been investigated. To determine whether either or both of these problems contributes to the difference between *tof1-627* and *tof1-830* cells, Sync-seq could be repeated, with earlier time points being studied. If the difference between *tof1-627 rad9Δ* and *tof1-830 rad9Δ* cells is due to an inability to maintain a stable fork structure after restart alone, this would be visible if earlier time points were studied, with the difference between *tof1-627* and *tof1-830* only becoming visible as replication forks elongate after restart. If, however, *tof1-627 rad9Δ* cells are simply slower at restarting stalled forks, Sync-seq data would show this delay at the very earliest of time points following release from the HU arrest.

Slower moving forks or slower rates of fork restart alone are unlikely to explain the significant loss of cell viability in *tof1-627 rad9Δ* cells as compared to *tof1-830 rad9Δ* cells following acute HU treatment. Instead, the data suggest that the C-terminal region of Tof1 between residues 762-830 is required following restart to ensure the replisome is stable enough to avoid toxic lesions. However, to be more confident that the cause of lethality in *tof1-627 rad9Δ* cells following HU arrest and release is indeed the accumulation of unrecoverable lesions, it would first be necessary to show that these cells experience DNA damage under these conditions, as the data presented in this chapter do not strictly show this. This could be achieved by visualisation of increased global and/or local levels of DNA-damage marker γ H2AX by western blotting or ChIP-Seq respectively, in cells acutely treated with HU. Interestingly, the same region of Tof1 required for suppression of lethality after acute HU treatment (aa 762-830) is required for the association of Tof1 with Csm3 (see chapter 3), suggesting that this interaction is crucial to maintain a stable replisome structure following replication fork restart. Alternatively, Mec1-dependent phosphorylation of the Tof1 C-terminus may be required to ensure the stability of restarted replication forks. Serine 654 of Tof1 is phosphorylated in response to checkpoint activation (Bastos de Oliveira et al., 2015). Currently the function of this modification and other candidate phosphorylation sites in the Tof1 C-terminus are not known but may play a role in regulating the stability of replication forks undergoing stress. To experimentally test whether this is the case, extra mutations in which these phosphorylation sites are mutated to alanine residues could be generated. This would then enable me to investigate

the contribution of these phosphorylation sites to DRC-mediated replication fork restart and stability.

In summary, in this chapter I have been able to demonstrate that the role of Tof1 in mediating intra-S checkpoint activation is achieved through the N-terminus of the protein, separating this function from its other roles in topological stress, replisome pausing and helicase/polymerase coupling. However, DRC activation alone is not sufficient to rescue viability of HU-treated cells lacking the Tof1 C-terminus, specifically between residues 762-830. This is the same region of Tof1 required for the stable association with Csm3, suggesting that this interaction is crucial for maintaining the stability of replication forks restarted after experiencing replication stress and stalling of the replisome. In cells lacking the Rad9-mediated DDC, this fork instability then results in unrecoverable replication defects that result in cell inviability. The interesting question of exactly what lesion or structure results in this inviability remains to be answered and may lead to new insights into how Tof1-Csm3 act at the fork in a checkpoint-independent manner to prevent replication fork collapse and the accumulation of toxic recombination intermediates.

Mutant	Rad53 phosphorylation (in <i>rad9Δ</i> background)	Suppresses sensitivity to chronic HU exposure (in <i>rad9Δ</i> background)	Restarts replication after acute HU exposure (in <i>rad9Δ</i> background)	Suppresses lethality after acute HU exposure (in <i>rad9Δ</i> background)
<i>tof1Δ</i>	X	X	X	X
<i>csm3Δ</i>	NT	NT	NT	NT
<i>mrc1Δ</i>	Inviabile	Inviabile	Inviabile	Inviabile
Tof1 wt	✓	✓	✓	✓
<i>tof1-627</i>	✓	X	Partial	Partial
<i>tof1-762</i>	✓	X	NT	NT
<i>tof1-830</i>	✓	✓	✓	✓
<i>tof1-997</i>	✓	✓	NT	NT
<i>tof1-1182</i>	✓	✓	NT	NT

Table 5.1: Summary of the phenotypes observed in Tof1 truncation mutants described in chapter 5 compared with the phenotypes of *tof1Δ*, *csm3Δ* and *mrc1Δ* mutants.

NT = Not tested.

6. Developing cell lines to investigate the S-phase functions of Timeless in human cells

6.1. Introduction

The role of Timeless proteins in replication fork protection and maintaining genome stability are conserved across eukaryotes. Human cells depleted for Timeless using siRNA show increased spontaneous γ H2AX and Rad51 foci formation, increased chromosomal breakage and increased sensitivity to genotoxic agents such as UV, MMS and CPT (Chou and Elledge, 2006, Leman et al., 2010). However, similarly to *S. cerevisiae* Tof1, previous analyses of Tim function in higher eukaryotes has been complicated by its multiple reported roles, and in-depth functional analysis of the protein in mammalian systems is lacking. Overexpression of *TIM* is observed in a number of different tumour types to protect cancer cells from increased levels of replication stress, highlighting the importance of deepening our understanding of Tim function in humans (Mao et al., 2013, Bianco et al., 2019).

As discussed in chapters 3-5 of this thesis, I have performed a structure-function analysis of the Tof1 protein in *S. cerevisiae*. However, a similar analysis of the functions of human Tim has been primarily hindered by the lack of genetic tools available to study the protein in higher eukaryotes. Such a study would first require a cell line deleted for the endogenous *TIM* gene in which various Tim mutants could be ectopically expressed. However, whilst it is possible to make *tof1* Δ mutants in *S. cerevisiae*, deletion of *TIM* leads to early embryonic lethality in mice (Gotter et al., 2000) and has been described as a core fitness gene in genome-wide CRISPR knockout screens using human cell lines (Hart et al., 2015), suggesting that Timeless has an essential role in mammals. In recent years, a powerful tool, Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas9) technology, has been heavily used to generate a vast range of human knockout cell lines (Cong et al., 2013, Mali et al., 2013). Despite this progress, a *TIM* knockout cell line is yet to be published and will not be possible to generate if *TIM* is indeed cell essential in metazoa. As a consequence, the functions of mammalian Tim have thus far been elucidated exclusively by studies utilising siRNA depletion. Whilst these studies have been fundamental to demonstrate the various

conserved roles of the protein in genome stability, siRNA depletion has several caveats. The level of siRNA-mediated protein depletion can vary dramatically, is known to have off-target effects, and cannot easily be combined with cell-synchronisation studies due to the length of time often required for complete depletion. Consequently, this has made it difficult to study the cell-cycle specific effects of loss of Timeless function.

Therefore, there is a need to develop mammalian cell lines in which Tim can be conditionally depleted, which would allow regulated and rapid depletion of the protein with minimal off-target effects. To try and address this, I set out to create human cell lines in which the Timeless protein could be conditionally depleted at the post-translational level. Specifically, I aimed to fuse a double degron tag to the Timeless protein that would allow for its rapid and reversible degradation in h-TERT immortalised human retinal epithelial pigment (RPE-1) cells. If successful, this would enable me to observe the phenotypes of human cells depleted of Timeless in a highly specific manner, and would allow for depletion of Timeless specifically in G1 phase (when combined with synchronisation techniques), to build a better understanding of both the general and S-phase specific roles of this protein in humans. To generate a human cell line in which Tim could be conditionally depleted, I decided to utilise the SMASh-mAID double degron approach outlined below.

6.1.1. The SMASh-mAID double degron system

In recent years the SMASh-mAID double degron approach has been described to induce rapid and efficient depletion of target proteins in human cells (Lemmens et al., 2018, Hégarat et al., 2020). This system consists of two distinct degron tags used together: a Small-Molecule-Assisted-Shutoff (SMASh) tag (Chung et al., 2015) and a mini-Auxin-Inducible-Degron (mAID) (Nishimura et al., 2009, Natsume et al., 2016) (Figure 6.1A).

The SMASh tag makes use of the activity of the hepatitis C virus (HCV) non-structural protein 3 (NS3) protease. A protein of interest is separated from a degron sequence by this protease and an intervening protease cleavage site. Under normal conditions, the NS3 protease cleaves the SMASh tag from the protein leaving protein levels unaffected (Figure 6.1B). This can be prevented by the addition of the non-toxic NS3 inhibitor asunaprevir (ASV), which prevents proteolytic cleavage of the degron tag, leading to the protein being targeted for proteasomal destruction (Figure 6.1C).

The mAID tag results in protein degradation upon the addition of the auxin family plant hormone indole-3-acetic acid (IAA). A prerequisite of this system is the integration and expression of the *Oryza sativa* F-box protein Transport Inhibitor Response 1 (OsTIR1). In the RPE-1 OsTIR1 cells I utilised for construction of Timeless degron cell lines (gift from Hocheegger lab), OsTIR1 is stably integrated under a tetracycline inducible promoter at the *Rosa26* locus, a genomic locus found on chromosome 3 frequently used for gene targeting (Irion et al., 2007). In these cells, OsTIR1 is inducibly expressed upon addition of tetracycline or its analogue doxycycline (DOX). Upon its ectopic expression, the F-box protein OsTIR1 can then subsequently bind to mammalian SKP1 forming an E3 ubiquitin ligase SCF complex. Upon addition of IAA to the cells, this E3 ligase is targeted via OsTIR1 to the mAID tag, resulting in polyubiquitination and targeting of the protein for proteasomal degradation (Nishimura et al., 2009) (Figure 6.1C).

The combination of both the SMASh and mAID degron tags has a distinct advantage: this double degron approach ensures highly effective depletion of both newly synthesised and old protein molecules. Newly synthesised proteins that have not self-cleaved the SMASh portion of the tag are targeted for degradation by both degrons, whilst old molecules that have just the mAID portion of the tag remaining can still be targeted for degradation by IAA. Under normal conditions, self-cleavage of the SMASh portion of the tag ensures that by leaving just the mAID tag, consisting of only 68 amino acids (corresponding to ~7.8 kDa), this minimises the risk of disruption of the target protein (Natsume et al., 2016).

As *TIM* is an essential gene in human cells (Hart et al., 2015), it was reasoned that by fusing Tim protein to this conditional degron tag, protein function would be unaffected under normal conditions, with Tim only being depleted upon the addition of DOX, IAA and Asv (hereafter collectively referred to as DIA). RPE-1 OsTIR1 cells were used for gene targeting as these cells have a stable diploid karyotype and have previously been used to successfully target Cdc6, Cyclin A2 and B1 with SMASh-mAID tags (Lemmens et al., 2018, Hégarat et al., 2020).

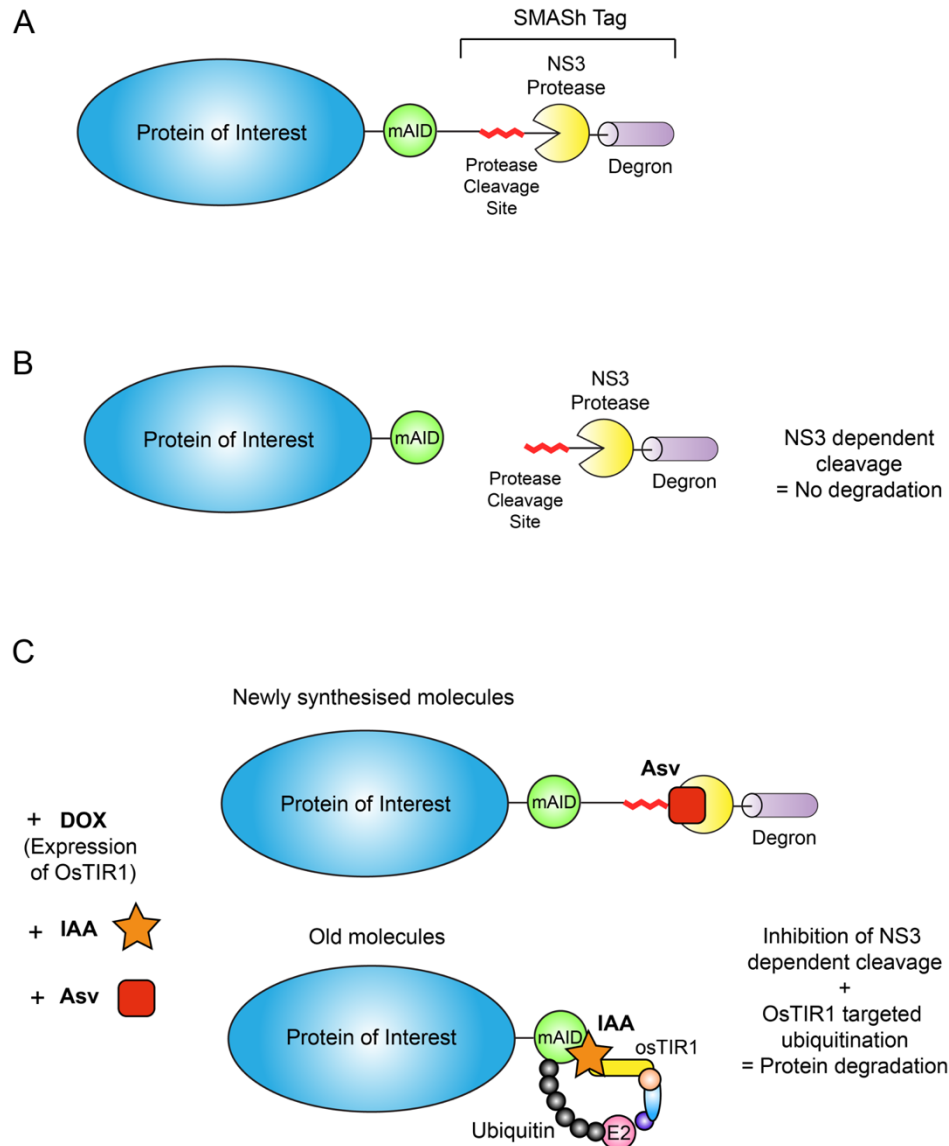


Figure 6.1: Schematic of the SMASH-mAID double degron system

(A) The protein of interest is fused to a double-degron tag consisting of a mini-Auxin-Inducible-Degrone (mAID) (Natsume et al., 2016) and a Small-Molecule-Assisted- Shutoff (SMASH) tag (Chung et al., 2015). The SMASH tag consists of a degron peptide sequence, the viral NS3 protease and a NS3 targeting protease peptide sequence.

(B) Under physiological conditions, the NS3 protease portion of the SMASH tag will self-cleave, removing the degron sequence from the protein of interest, leaving the protein unaffected in cells. The small mAID tag remains fused to the protein of interest but under these conditions is unable to target the protein for degradation.

(C) Upon addition of the NS3 protease inhibitor asunaprevir (Asv), the SMASH tag self-cleavage system is prevented and the degron portion of the tag remains fused to the protein of interest. This targets newly synthesised molecules for degradation by the proteasome. The mAID tag, upon the addition of DOX and IAA, targets any old molecules that have lost the SMASH degron tag for destruction. DOX induces expression of OsTIR1, which is targeted to the mAID tag by the addition of IAA. OsTIR1 forms an E3 ubiquitin ligase complex with host accessory factors and polyubiquitinates the protein of interest targeting it for proteasomal degradation.

6.2. Results

6.2.1. Generation of Timeless-SMASH-mAID RPE-1 OsTIR1 cell lines

I decided to attempt both N- and C-terminal tagging of the protein as it was unknown which tag would work more efficiently or be less disruptive to the protein under normal conditions. Previous studies have primarily used N-terminal tagging of human Tim (Ünsal-Kaçmaz et al., 2005, Young et al., 2015) although my work in budding yeast shows that the structurally conserved *S. cerevisiae* Tof1 can be epitope tagged both at the N- and C- terminus without loss of protein levels or loss of function (see chapter 3).

6.2.1.1. Construction and introduction of vectors to SMASH-mAID tag the Tim N- and C-termini

To target Tim with either an N- or C- terminal SMASH-mAID tag I decided to use a CRISPR-Cas9 mediated approach to ‘knock-in’ the double degron tag into the endogenous *TIM* locus on chromosome 12. This first required the design and construction of four vectors to use for CRISPR-Cas9 mediated genome editing. Here, a brief overview of the gene targeting strategy is described. For details of vector construction and specific sequences, see materials and methods (chapter 2.4.8).

First, I manipulated a previously generated vector, eSpCas9. This vector co-expresses the *Streptococcus pyogenes* Cas9 nuclease (SpCas9) along with a single guide RNA (sgRNA) designed to target the SpCas9 nuclease to specific genomic loci (Slaymaker et al., 2015). Specifically, I designed and inserted 20 nucleotide sgRNA sequences containing homology to regions either proximal to the *TIM* initiation (for N-terminal tagging) or termination (for C-terminal tagging) codons. These guide sequences were cloned directly downstream of the ‘scaffold’ RNA portion of the vector, which is required for recruitment of the sgRNA to the SpCas9 nuclease and therefore for targeting of the endonuclease to specific genomic loci (Figure 6.2A) (Ran et al., 2013). The Benchling online CRISPR tool was used to select optimum candidate sgRNA sequences (Benchling, 2020).

Next, two vectors were generated for co-transfection with the SpCas9-sgRNA plasmids to provide the donor template for HR-mediated repair of the Cas9-induced break. These

vectors contain the SMASh-mAID degron tag sequence and Neomycin resistance gene surrounded by homologous arms to the regions immediately upstream and downstream of the initiation (for N-terminal targeting) or termination (for C-terminal targeting) codons of the *TIM* gene (Figure 6.2B). When transfected together, the SpCas9-induced break close to the initiation or termination codon of the *TIM* gene will undergo repair by homologous recombination. Depending on the transfection efficiency, in a subset of cells this repair may use the exogenous template vector transfected as a template, due to the inclusion of homologous arms to the genomic regions targeted. After successful repair this results in incorporation of the degron sequence into the endogenous *TIM* locus (Figure 6.2B).

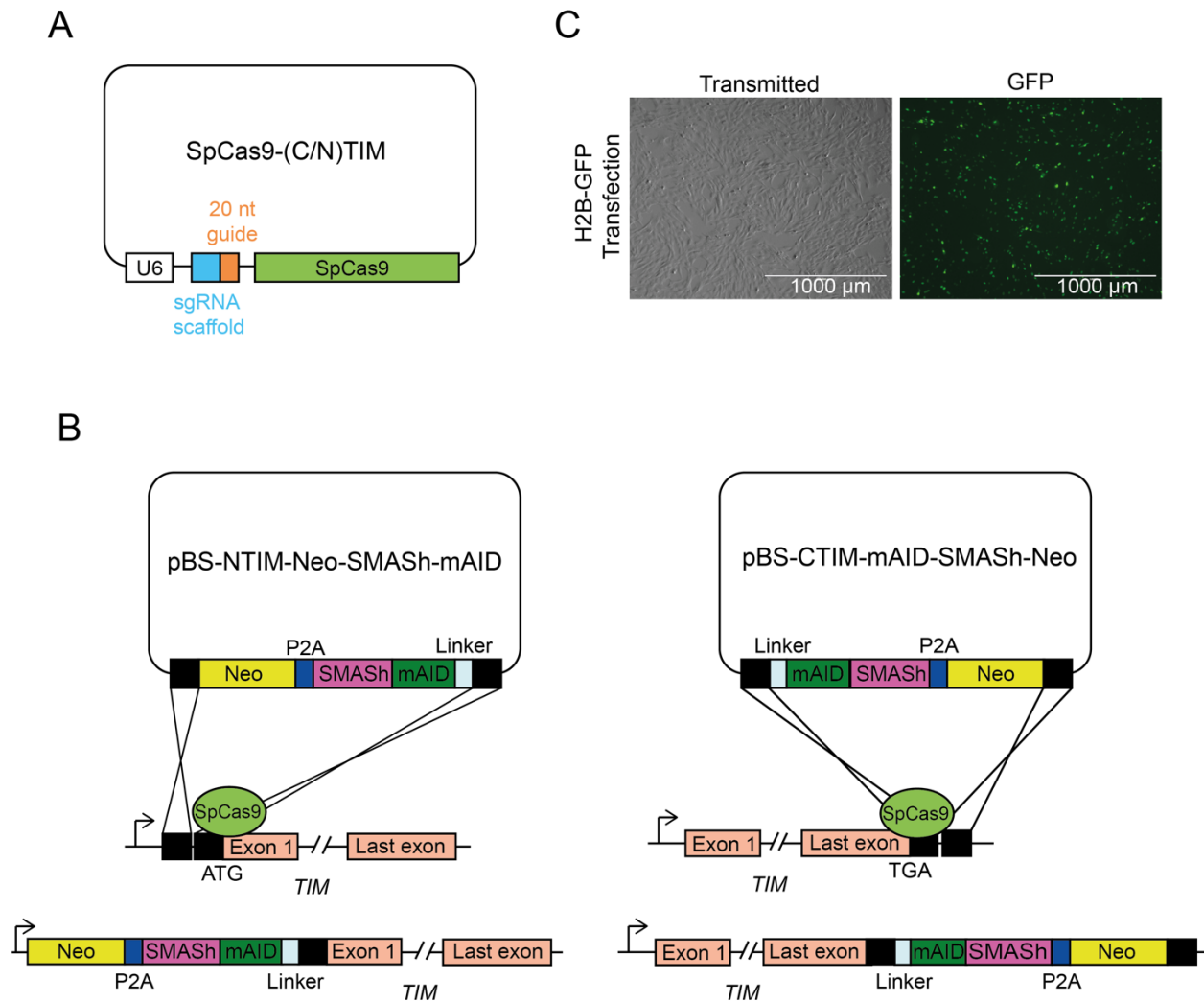


Figure 6.2: Schematic of SMASH-mAID degron knock-in strategy at the endogenous *TIM* locus on chromosome 12

A) Vectors SpCas9-CTIM and SpCas9-NTIM were generated by incorporating a 20-nucleotide guide sequence (orange) with homology to either the initiation (for N-terminal tagging) or termination (for C-terminal tagging) codons of the endogenous *TIM* gene. These sequences were cloned immediately upstream of the scaffold RNA (blue), which when translated together produce a single gRNA (sgRNA) capable of targeting the SpCas9 nuclease to the specific guide sequences chosen within the genome.

B) Schematic of knock-in strategy by co-transfection of plasmids containing N- or C-terminal SMASH mAID degron tags along with SpCas9 plasmids described in (A). pBS-NTIM-Neo-SMASH-mAID and pBS-CTIM-mAID-SMASH-Neo act as homology donors to integrate the degron sequence into the endogenous *TIM* locus (at either the N- or C-terminus) by HR-mediated repair of the SpCas9 induced break. Black squares represent regions of homology between vectors and regions at the endogenous *TIM* locus on chromosome 12.

C) Representative images of RPE-1 OsTIR1 cells 24 hours after transfection with a vector expressing GFP-tagged H2B to assess transfection efficiency.

Following generation of the cloning vectors required to target the endogenous *TIM* locus, these vectors were introduced into RPE-1 OsTIR1 cells by electroporation. As the outcome of the gene targeting relies heavily on the efficiency of transfection, a control in which a vector expressing GFP-tagged histone H2B was also transfected to RPE-1 cells, followed by visualisation of the cells using fluorescence microscopy 24 hours post transfection (Figure 6.2C). After allowing cells to recover from the electroporation for 48 hours in non-selective media, I then selected for cells which had successfully integrated the SMASh-mAID-Neomycin construct by transferring cells to growth medium containing the antibiotic G-418. Cells were grown in selective media for at least 3 weeks before checking for colonies. Despite several attempts, transfection of the N-terminal targeting construct yielded no colonies, potentially due to poor efficiency of the gRNA chosen. However, transfection of the C-terminal construct yielded between 20-30 colonies per 96-well plate using this method, indicating that the gene knock-in was highly efficient. 23 clones were selected for initial screening and further characterisation. A mixture of smaller (slower growing) and larger (faster growing) colonies were selected, as it was reasoned that the slower growth observed in some colonies could be due to successful gene targeting resulting in a mild growth defect.

6.2.1.2. Characterisation of C-terminally SMASh-mAID tagged Tim clones by PCR screening

After selection and expansion of 23 colonies, these cells were screened for stable insertion of the SMASh-mAID-Neo cassette into the endogenous *TIM* locus. Initially, genomic DNA extracted from these clones was screened by PCR using two sets of primers. The first of these was designed to anneal within the last exon of the *TIM* gene (on the – strand) and within the SMASh sequence (on the + strand), producing a 662 bp product over the 5' insertion site of the knock-in sequence (Figure 6.3A, primers 1 and 2). The second set was designed to anneal within the Neomycin resistance gene (on the – strand) and in the genomic DNA downstream of the *TIM* gene (on the + strand), producing a 885 bp product across the 3' insertion site (Figure 6.3A, primers 3 and 4). As expected, amplification of genomic DNA from untargeted RPE-1 OsTIR1 parental cells produced no bands, confirming the specificity of the primers chosen for the knock-in sequence (Figure 6.3B). Amplification of genomic DNA extracted from clones 1-11 resulted in both bands of the expected sizes in 9 out of 11 clones (clones 3-11) (Figure 6.3B), suggesting that the knock-in had worked with high frequency.

However, the use of these primer sets did not distinguish between clones that had successfully integrated the SMASh mAID sequence into just one or both *TIM* alleles and so the PCR genotyping approach was modified. To allow for this distinction, PCR was repeated with the two primers designed to anneal to the regions 138 bp upstream and 381 bp downstream of the endogenous *TIM* termination codon. Upon amplification using this primer set, two possible products can be generated: a 422 bp product corresponding to the wild-type *TIM* allele, or a longer, 2480 bp product indicating the stable integration of the SMASh-mAID cassette at this locus (Figure 6.3A, primers 1 and 4).

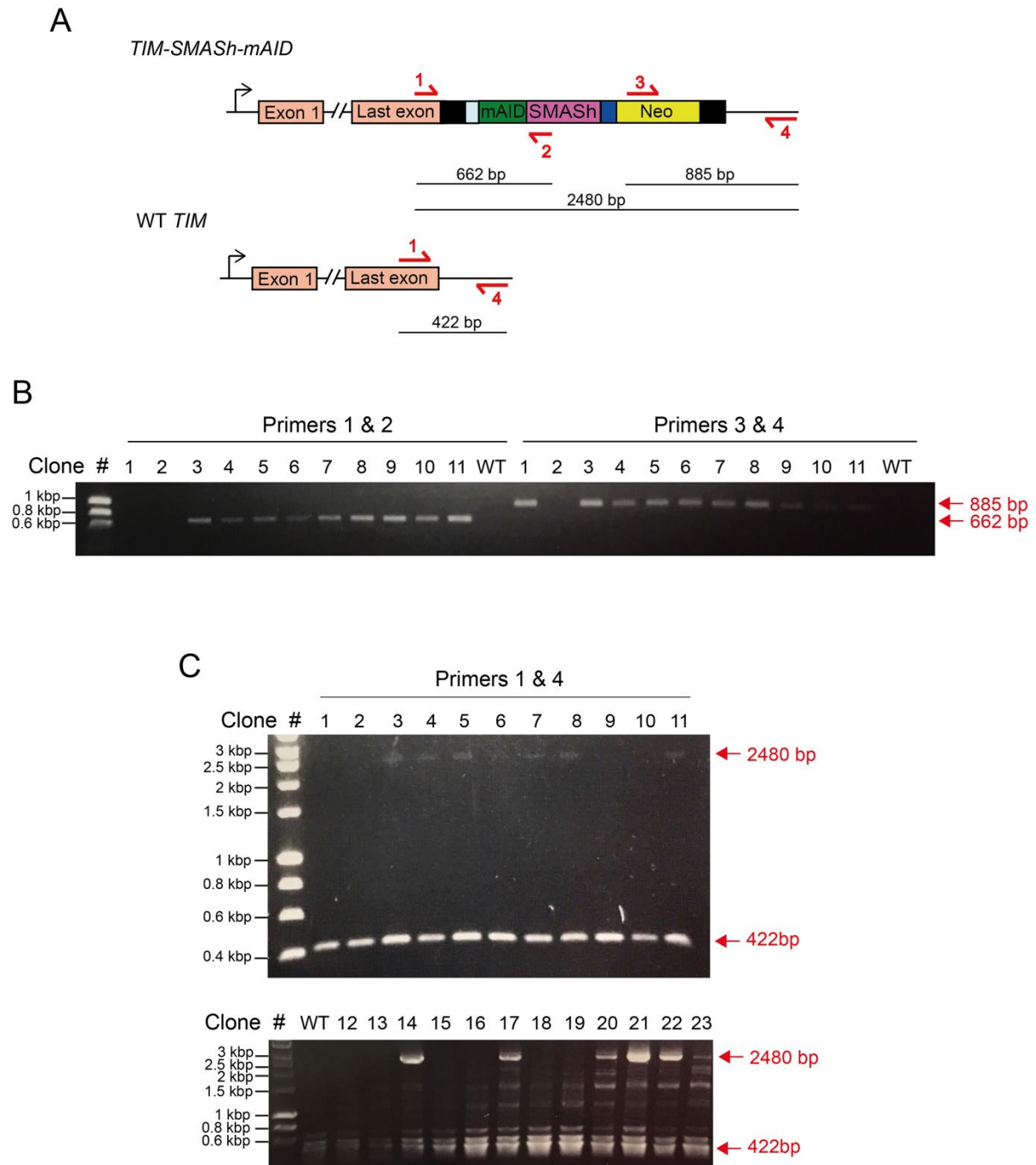


Figure 6.3: PCR genotyping of RPE-1 OsTIR1 clones targeted with C-terminal SMASH mAID degon tags

(A) Schematic of PCR approach to genotype the clones generated from SMASH-mAID knock-in at the Timeless C-terminus as well as at the untargeted wild-type *TIM* gene. Primers used indicated in red and the resulting fragment sizes expected from amplification of the tagged or wild-type *TIM* gene are shown.

(B-C) PCR products generated from amplification of genomic DNA from RPE-1 OsTIR1 (WT) cells or C-terminally SMASH-mAID tagged clones using primer sets: (B) 1 & 2 and 3 & 4 or (C) 1 & 4.

Genomic DNA extracted from all selected clones was used for PCR amplification using this primer set. As expected, only the 422bp band corresponding to the wild-type *TIM* allele was seen in both untargeted RPE-1 OsTIR1 cells and in clone no. 2 which had been identified as negative from the first PCR step (Figure 6.3C). Despite being positive for gene knock-in from the first genotyping PCR, in clones 1, 9 and 10, only the 422 bp wt band could be seen. This was potentially due to poor PCR amplification in these clones using the second primer set (Figure 6.3C). However, in 13 out of the 23 clones screened overall (clones 3-8, 11, 14, 17 and 20-23), the larger band corresponding to the 2480 bp product generated from amplification of the SMASh-mAID tagged locus was consistently observed (Figure 6.3C). This confirmed the stable integration of the SMASh-mAID tag construct at the endogenous *TIM* C-terminus in these cells. However, in all 13 of these clones, the 422 bp band corresponding to the wild-type allele was also observed, suggesting that the knock-in had only been successful at one of the two endogenous *TIM* alleles, resulting in heterozygously tagged cells.

6.2.1.3. Characterisation of heterozygously-tagged *TIM* clones by western blotting

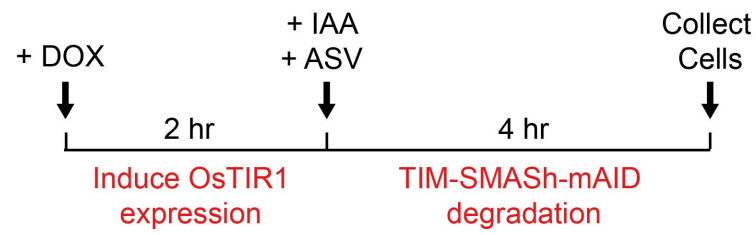
Given that all of the positive clones identified by PCR were heterozygously tagged, this suggested that homozygous knock-in of the SMASh-mAID degron tag at the Timeless C-terminus was not tolerated by cells. Alternatively, introduction of the SMASh-mAID tag into both *TIM* alleles may be viable but rare, in which case many more colonies would have to be screened. Unfortunately, due to time constraints I did not have time to expand and screen more colonies to see whether this was the case. However, I decided to visualise Tim protein levels in the heterozygous SMASh-mAID knock-in clones I had already generated, as potentially these cell lines could be useful for analysis of a partial Tim depletion phenotype. Specifically, I used western blotting to compare Tim protein levels in wild-type and in three Tim SMASh-mAID clones both in endogenous conditions and upon addition of DIA. If the Tim-SMASh-mAID fusion protein is unstable in cells, potentially the levels of visible Tim in these clones would be reduced even without DIA treatment. Alternatively, Tim-SMASh-mAID may be degraded specifically upon DIA induction.

RPE-1 OsTIR1 (wt) cells and three of the heterozygously tagged Timeless SMASh-mAID clones (14, 20 and 22) were treated with DOX for 2 hours to induce OsTIR1 expression, followed by treatment with IAA and ASV for 4 hours to deplete SMASh-mAID tagged Timeless (Figure 6.4A). Cells were collected and their lysates subjected to western blotting using antibodies against the endogenous Timeless protein. As an additional control, SMASh-mAID tagged Cyclin A2 cells that have been previously characterised (Hégarat et al., 2020) were used, with antibodies against Cyclin A2 to confirm that addition of DIA was resulting in efficient protein depletion. As expected, addition of DIA to Cyclin-A2-sMASh-mAID cells resulted in complete loss of visible protein after 4 hours, confirming the efficacy of the knockdown.

Importantly, in -DIA conditions the removal of the SMASh tag results in the remaining mAID tag being the only degron still fused to Timeless. The mAID tag is small, being only 68 amino acids, and therefore contributes only ~7.4 kDa to the overall molecular weight of the protein. Hence, it is not expected that the wild-type Timeless protein (138 kDa) will be distinguishable by size from the tagged protein (~145.4 kDa). As expected, in RPE-1 OsTIR1 cells a band running just above 130 kDa corresponding to endogenous Timeless

(~138 kDa) was observed both in untreated and DIA treated cells (Figure 6.4B). Interestingly, two additional bands were also consistently observed across cell lines running just below 130 kDa and just below 100 kDa (Figure 6.4B). However, in all three of the heterozygously tagged clones analysed (14, 20 & 22), Timeless protein levels were comparable with those seen in RPE-1 OsTIR1 cells both in normal conditions and after addition of DIA (Figure 6.4B). These results suggest that introduction of the SMASh-mAID tag into one *TIM* allele in RPE-1 OsTIR1 cells does not alter Tim protein levels, neither under normal conditions nor upon addition of DIA. Unfortunately, for these reasons, it was decided not to continue with further characterisation of the heterozygously tagged Timeless clones generated.

A



B

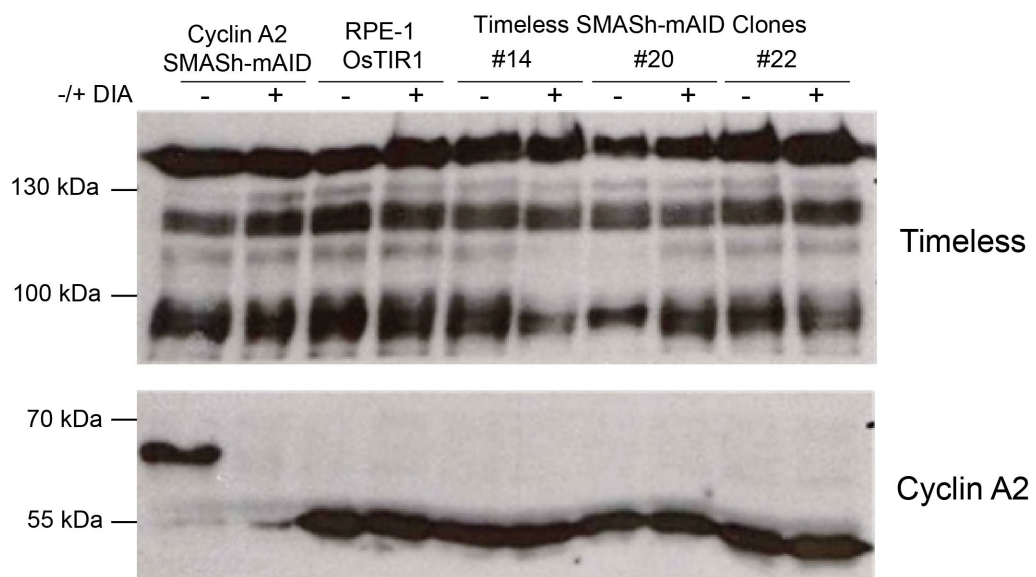


Figure 6.4: Western blotting analysis of Timeless and Cyclin A2 levels in parental RPE-1 OsTIR1 cells and in Tim-SMASH-mAID clones

(A) Outline of experiment performed in panel B. RPE-1 OsTIR1 cells were treated with DOX for 2 hours to induce OsTIR1 expression, before addition of IAA and ASV to induce SMASH-mAID mediated depletion of degron tagged proteins. Cells were then collected for lysis and subject to western blotting using antibodies against Timeless or Cyclin A2

(B) Western blot using Timeless or Cyclin A2 antibodies in RPE-1 OsTIR1, Cyclin A2 SMASH-mAID cells or in heterozygous Timeless SMASH-mAID clones 14, 20 and 22, before and after DIA treatment. Representative western from 3 independent experiments is shown.

6.2.2. Timeless exists as three distinct isoforms in RPE-1 cells

Whilst screening the SMASh-mAID Timeless clones by western blotting, I had consistently observed three distinct bands when probing with antibodies to endogenous Timeless (Figure 6.4). In human cells, expression of the *TIM* gene produces a protein of 1208 amino acids (Sangoram et al., 1998) corresponding to a molecular weight of approximately 138 kDa. However, smaller isoforms of Tim have previously been reported in mice (Li et al., 2000). Potentially, the three bands observed in RPE-1 cells could be different isoforms of Tim. Alternatively, non-specific antibody binding could explain the presence of these bands. To distinguish between these two possibilities, I collected lysates from cells siRNA-depleted of Timeless, and subject lysates from these cells to western blotting. Specifically, RPE-1 OsTIR1 cells were treated with either control or Timeless-targeting siRNA and cells were collected 48- and 72-hours post-transfection with the siRNA. The resulting lysates were then used for western blotting using the same anti-Timeless antibody previously used for clone characterisation. Surprisingly, I observed that all three major bands visualised by probing for endogenous Timeless disappeared upon siRNA depletion of the Timeless protein, indicating that each of these three bands are protein products of the *TIM* gene (Figure 6.5). Interestingly, this suggests that in human RPE-1 cells, Timeless exists in 3 isoforms which could be produced by alternative splicing or potentially via proteolytic degradation.

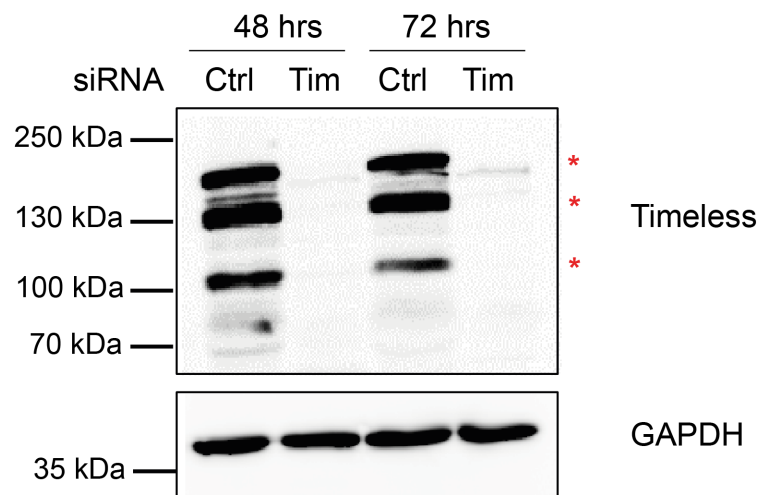


Figure 6.5: Timeless exists as three distinct isoforms in RPE-1 cells

Western blot using antibodies against endogenous Timeless in lysates collected from RPE-1 OsTIR1 cells treated with either control or anti-Timeless siRNA. Cells were collected 24, 48 or 72 hours post-transfection with siRNA. Red asterisks mark the 3 bands corresponding to hTim. Representative western of 2 independent experiments is shown.

6.3. Discussion

The role of Timeless proteins in maintaining genome stability is conserved across eukaryotes, including humans. However, there is still a significant gap in our understanding of how human Timeless achieves these functions and previous studies of Timeless in human cells have solely utilised siRNA mediated depletion of the protein to investigate its multiple roles. Depletion of protein targets using RNA interference presents several problems, including off-target effects and often a slow depletion of the target protein. Therefore, there is a need for a more direct, specific and rapid way of investigating Timeless function in human cells. In this chapter I have attempted to address this problem by attempting the construction of human cell lines in which the Timeless protein can be conditionally degraded. Specifically, I worked on a system whereby Timeless can be targeted by a dual degron SMASh-mAID tag to induce rapid depletion of the protein upon the addition of several small molecules.

To do this I have used CRISPR-Cas9 genome editing to introduce double-stranded breaks at the endogenous *TIM* locus, and designed and introduced plasmids containing homology donors to knock-in the SMASh-mAID degron tag at this locus. As described, I targeted both the Tim N- and C-terminus using this approach but found that when targeting the N-terminus, no positive clones were generated. The N-terminus of human Timeless has been successfully tagged with other small epitopes in previous studies, including STREP-tags (Young et al., 2015, Holzer et al., 2017). In addition, as outlined in chapter 3 of this thesis I have successfully tagged both full-length and truncated Tof1 proteins with N-terminal HA tags in *S. cerevisiae*. Given this, it is unclear why N-terminal targeting of Timeless with a SMASh-mAID tag was unsuccessful. However, the lack of any positive clones after selection suggests that potentially the gRNA chosen for Cas9-mediated cleavage at the *TIM* N-terminus was not efficient. Whilst SpCas9 can be directed to essentially anywhere in the genome preceded by a 5'-NGG-3' protospacer adjacent motif (PAM), the efficacy of different gRNAs can be variable. Certain bases at defined positions of the gRNA sequence, for example a guanine at position 20 next to the PAM sequence can increase guide efficiency (Doench et al., 2016). Due to the popularity of CRISPR-Cas9 genome editing in recent years there are multiple tools available online to choose optimal guide sequences. Such tools generally give a 'score' out of 100 to predict both on-target efficiency and off-target effects, with a higher score being more desirable for candidate gRNA selection. When choosing my gRNA sequences for

genome editing, I made use of such online tools. However, the best gRNA available for targeting the *TIM* N-terminus had a score of 43 out of a possible 100, potentially suggesting poor on target cutting efficiency. To determine whether this was the case, there are a number of assays available to determine Cas9 cutting efficiency such as the surveyor assay that exploits the T7 endonuclease 1, or targeted next-generation sequencing on a pool of targeted cells (Sentmanat et al., 2018). However, due to the fact that I had managed to select many C-terminally tagged clones at the time, this was not carried out.

In this chapter I have shown using genotyping PCR that I have been able to successfully integrate the SMASh-mAID double degron tag at the *TIM* C-terminus, validating both the sgRNA sequence and the donor vector used for degron tag integration at this locus. It is assumed genome editing occurred with a high frequency using this method, due to the very high number of colonies growing after antibiotic selection of transfected cells (>20 positive clones per 96-well plate). However, by using PCR across the knock-in insertion site, I observed that none of the clones screened had successfully integrated the degron sequence into both *TIM* alleles, resulting in only heterozygously targeted cell lines.

A previously published study used a similar approach to target various proteins in human and mouse cells lines with mAID tags (Natsume et al., 2016). In this study, targeting the essential *MCM2* gene in mouse embryonic stem (ES) cells by incorporation of a mAID tag resulted in 50% of clones (where n= 32) after selection having “at least one” allele tagged. I found that after screening 23 clones, 61% of these were tagged at least one allele but tagging at both alleles did not occur in any. The absence of even just 1-2 homozygously tagged clones after this initial round of selection and screening suggests that this particular degron tag at the Tim C-terminus may not be tolerated by cells. As previously demonstrated in this thesis, *S. cerevisiae* Tof1 can be destabilised by the disruption of the C-terminus, either by premature truncation at specific regions of the protein (see chapter 3) or by insertion of large epitope tags into the C-terminus (see chapter 4). Potentially, introduction of the SMASh-mAID tag into the C-terminus of human Timeless may have a similar effect in destabilising the protein. As *TIM* is embryonic lethal in mice (Gotter et al., 2000) and essential in human cells (Hart et al., 2015), this could explain the reason for the inability to generate homozygous knock-in of the double degron tag at the *TIM* locus, if the tagged *TIM* allele results in an unstable protein in cells. To be more confident that this is indeed the case, however, it would be necessary to screen

more than 23 clones. Alternatively, to increase the number of clones tagged at both Tim alleles, it could be considered to try introduction of two separate donor vectors containing different antibiotic resistance markers. Natsume and colleagues (2016) adopted this approach to target the RAD21 gene with a mAID tag and found that by introduction of two separate targeting vectors containing the mAID tag and either Neomycin or Hygromycin resistance genes, more than 70% of clones were homozygously tagged. However, isolating, expanding and screening clonal cell lines is a laborious and time-consuming task, and due to increased work-load from the parallel study on *S. cerevisiae* Tof1 (as described in chapters 3-5), further work on cell line development was not carried out.

Finally, in this chapter I have found by western blotting that three distinct and consistent bands correspond to Tim protein products in RPE-1 cells, which were confirmed by depletion of each of these protein products by RNA interference. This suggests that Tim may undergo either post-transcriptional or translational regulation in RPE-1 cells to produce multiple protein isoforms. The evidence for several Tim isoforms in different organisms is varied. A 'short' isoform of Timeless has been identified in mice (termed mTim-s), corresponding to the last 475 residues of the protein (Li et al., 2000). Interestingly, the longer Tim isoform with additional N-terminal residues, but not the short isoform, appears to oscillate within the suprachiasmatic nucleus (SCN) in the brain and interacts with the core circadian clock protein CRY1 in cultured cells, potentially linking the larger mTim isoform to a role in circadian regulation (Barnes et al., 2003, Engelen et al., 2013). However, a definitive role for Tim proteins in mammalian circadian rhythm is still poorly defined, primarily hindered by the inability to generate Tim knockout mice due to early embryonic lethality.

The evidence for multiple Tim isoforms in human cells is lacking. Previous analysis of Tim mRNA in 16 different human tissue types has identified only one ~4.5 kb transcript (although retinal epithelium from which RPE-1 cells are obtained was not tested) (Sangoram et al., 1998) and to date there are no published reports of multiple isoforms identified at the protein level in humans. The appearance of three distinct Tim bands in RPE-1 cells raises several further questions. First, it is unclear whether these isoforms are generated by alternative splicing of mRNA or targeted proteolysis. Using northern blotting, it would be simple and interesting to determine whether Tim mRNA in RPE-1 cells undergoes alternative splicing to produce multiple transcripts, particularly as this would indicate a tissue-specific regulation of Tim in the retinal epithelium as compared to

the singular transcript present in other tissue types. Alternatively, the three isoforms may be generated from a single mRNA transcript, in which case it would be assumed that post-translational, targeted proteolysis would explain the presence of the distinct Tim isoforms. In this case the identity of these proteolytic isoforms could be established using methods designed to label and identify terminal amines in peptide sequences (known as 'Terminal amine isotopic labelling of substrates' or 'TAILS' (Doucet et al., 2011)).

In summary, in this chapter I have generated and characterised cell lines containing heterozygously tagged *TIM* alleles. However, these cell lines have not proved useful for conditional depletion of the protein and instead suggest that introduction of degron tags, specifically the SMASh-mAID degron tag, at the Timeless C-terminus is not tolerated in human cells. Consequently, this adds weight to the argument that *TIM* is an essential gene in humans and mice, as the failure to isolate cells tagged homozygously suggests that introduction of the mAID tag into the *TIM* C-terminus leads to an unstable protein product, in which case it would be predicted that homozygously tagged clones would be inviable. Therefore, the task of creating mammalian cells in which Timeless can be conditionally depleted still remains and will likely be complicated by this fact. However, such a tool would be highly beneficial for future studies into the numerous roles of this functionally diverse protein, especially in human systems where little is known about how the protein accomplishes its roles. In addition, further characterisation of alternative splicing and/or proteolytic regulation of Tim in various human cell types may give insight into how Tim is able to be differentially regulated to perform its multiple reported functions.

7. Discussion

7.1. Overview

To date, virtually all studies on Timeless family proteins have utilised gene deletion or RNAi to investigate the roles of these proteins in maintaining genome stability. As a consequence, it is not known how Tim proteins carry out their numerous reported roles in S-phase, and whether these roles are linked to the same function or can be separated from one another. This thesis has addressed this problem by carrying out a structure-function analysis of a Timeless family protein, namely Tof1 in the budding yeast *S. cerevisiae*. This work has defined several distinct domains within the Tof1 protein that carry out different replisome-associated functions in response to different replication stress events. In addition, this thesis includes preliminary data pertaining to the construction of human cell lines in which Timeless can be conditionally depleted.

7.2. Structure-function analysis of Tof1 in *S. cerevisiae*

The key findings of this thesis have revealed that distinct functional domains of the Tof1 C-terminus carry out different roles during DNA replication. Specifically, I have investigated the contributions of the Tof1 C-terminus in the response to topological stress, replisome pausing and coupling, in the intra-S phase checkpoint and in fork restart after replication stress. A summary of phenotypes of the C-terminal Tof1 truncation mutants *tof1-627*, *tof1-762*, *tof1-830*, *tof1-997* and *tof1-1182* can be found in Table 7.1 (page 176).

7.2.1. The relationship between Tof1 structure and its interactions within the replisome

In chapter 3 of this thesis I have characterised various C-terminal Tof1 truncation mutants in budding yeast cells. I started this analysis by determining the effect of Tof1 truncation on its interaction with Csm3 and have found that *tof1-627* and *tof1-762* truncation mutants do not form a stable complex with Csm3 (Figure 3.5). This interaction was stabilised in the *tof1-830*, *tof1-997* and *tof1-1182* mutants showing that residues 762-830 of Tof1 are crucial for formation of the Tof1/Csm3 heterodimer (Figure 3.5). Interestingly, recent structural analysis has demonstrated that Csm3 is recruited to the replisome entirely by Tof1 (Baretić et al., 2020). Specifically, several key hydrophobic residues of Tof1 between amino acids 759-765

form a key interface for Csm3 binding (Baretić et al., 2019). Truncation at residues 627 or 762 of Tof1 is likely to disrupt this interaction interface. Therefore, I propose that the mid C-terminus of Tof1 is required to stably recruit Csm3 into the replisome, and breaking this interaction by truncating within this region results in a replisome structure lacking Csm3 entirely. However, the implications of breaking this interaction for Tof1 function are not clear. Previous analyses have shown that Tof1 levels are stable in *csm3Δ* cells, whereas Csm3 levels are reduced in *tof1Δ* cells (Bando et al., 2009). In line with this, I have found that Csm3 levels are reduced in *tof1-627* and *tof1-762* cells, suggesting that Tof1/Csm3 heterodimer formation is key to Csm3 stability in cells (Figure 3.5). Potentially, the primary role of Tof1 in Tof1/Csm3 related activities (e.g. replisome pausing) is simply to recruit Csm3 to the replisome where it can promote these functions, rather than a Tof1-specific mechanistic function. To investigate this in more detail it would be interesting to generate cells in which Csm3 could be recruited to replication forks in the absence of Tof1, for example by generation of fusion proteins to force Csm3 tethering to the replisome.

In addition to characterising Tof1/Csm3 binding, the analyses from chapter 3 have shown that disruption of a specific domain in the C-terminus of Tof1 can lead to its destabilisation in cells. Truncations or internal deletions within the C-terminal domain situated between 810-950 of Tof1 can lead to loss of Tof1 protein and/or function. Specifically, this thesis shows that truncation of Tof1 at aa 901 results in its destabilisation in cells as visualised by western blotting (Figure 3.6). Furthermore, truncations at residues 870, 880 and 890 lead to loss of Tof1 function as determined by CPT sensitivity assays (Figure 3.8). Interestingly these mutations reside within a region of Tof1 that is structurally similar to the human Timeless PARP-binding domain (PAB) (Xie et al., 2015). Whilst removal of the entire PAB domain (aa 810-950) of Tof1 did not disrupt its function, removal of regions within the PAB domain resulted in loss of Tof1 function (Figure 3.7). These results may be explained by a model in which this region of Tof1 regulates its stability by exposure of a destabilising domain located in the C-terminus of the protein (Figure 7.1).

The model described above gives rise to several other questions. Which proteins and/or processes regulate Tof1 destabilisation, and what physiological function does it serve? Tony Oliver, a structural biologist at the GDSC, informed us during this structure-function study that the PAB domain of Tof1 threaded well to another conserved protein domain known as the 'PUB' (peptide N-glycanase and UBA or UBX-containing) domain. The PUB domain was first functionally characterised in the human protein peptide N-glycanase where it was demonstrated to bind the ATPase p97 (Sc Cdc48) (Allen et al., 2006), a key component of the ubiquitin-proteasome system in cells which can remove polyubiquitinated proteins from the

chromatin in an ATP-dependent manner prior to their destruction by the proteasome. (Dantuma and Hoppe, 2012, Meyer and Wehl, 2014). Other replisome factors have been demonstrated to be regulated by the ubiquitin-proteasome system, for example at termination events where removal of CMG is mediated by Mcm7 ubiquitination and the subsequent action of p97 (Maric et al., 2014, Moreno et al., 2014). Potentially the region of Tof1 between aa 810-950 may function as a p97 binding module to regulate removal of Tof1 from the replisome and facilitate its subsequent proteasomal degradation. To test whether p97-mediated unfolding of the *tof1-901* truncation is the cause for its destabilisation in cells, *cdc48* degron mutants could be utilised to see whether loss of Cdc48 in cells results in *tof1-901* stabilisation. Such a mechanism may be required to unload stalled replisomes that accumulate as a result of replication stress, in a mechanism analogous to that of replisome unloading at termination. As Tof1 plays multiple roles in replisome pausing and DRC activation, it is possible that it undergoes structural changes in response to various types of replication stress. Such structural changes could “expose” a destabilising patch in the mid C-terminus, promoting replisome disassembly at stalled or collapsed forks by the p97-mediated mechanism outlined above. This could be particularly important to allow various remodelling and repair enzymes access to collapsed forks, promoting their repair in cells.

An important consideration is the potential requirement for the Tof1 C-terminus in efficient recruitment of Tof1 protein to the replisome. As this has not been directly addressed in this thesis, it cannot be ruled out that the Tof1 truncation mutants generated are not recruited to replisomes during chromosome duplication, which would likely be a factor in explaining the various phenotypes observed throughout this structure-function study. However, structural data suggests that Tof1 interacts with the CMG helicase primarily via distinct N-terminal features, including an “omega-loop” and “MCM-plugin”. These structural elements span residues 215-249 and 295-432 of the Tof1 protein respectively and make direct contacts with Mcm4, Mcm6 and Mcm7 (Baretić et al., 2020). Whilst both of these regions will still be present in the smallest Tof1 truncation described in this thesis (*tof1-627*), it is possible that truncation of the protein at aa 627 disrupts these features in the overall tertiary structure and results in loss of these key contacts with CMG. To address this, it would be necessary to use a readout of Tof1-CMG interaction, which could be achieved using co-IP experiments conducted in yeast strains containing tagged subunits of the CMG complex.

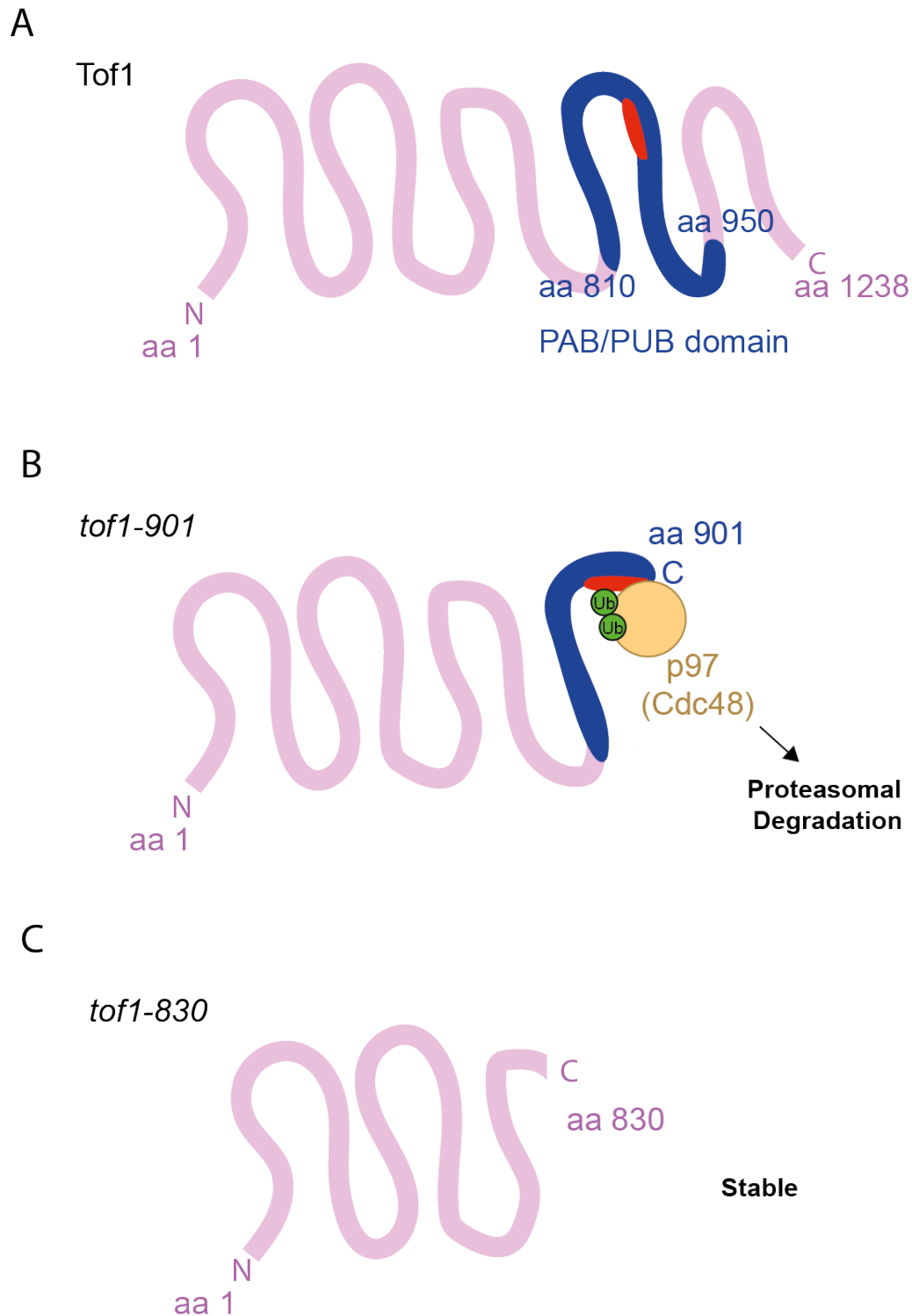


Figure 7.1: A model for Tof1 destabilisation by a C-terminal domain

A) Within the C-terminus of Tof1, aa 810-950 (dark blue) share structural similarity to the human PAB domain as well as the p97-binding PUB domain. Within this PAB/PUB domain a p97-binding motif (red) may normally be buried by the protein tertiary structure.

B) Upon truncation at aa 901 the p97-binding motif in the Tof1 C-terminus is exposed. When ubiquitinated (green circles) this leads to p97-targeted proteasomal degradation of Tof1.

C) Truncation of Tof1 at aa 830 removes the PAB/PUB domain and thus the p97-binding motif, resulting in stable expression of this protein in cells.

7.2.2. The role of Tof1 in resolving topological stress

Initially, the generation of Tof1 truncations focused specifically around the C-terminus of the protein stemmed from the reported interaction of this region with Top1. Residues 981-1238 of Tof1 are required to interact with Top1 (Park and Sternglanz, 1999, Shyian et al., 2019). Furthermore, recent ChIP-seq data suggests that *tof1Δ* cells show reduced Top1 enrichment at early replicating regions (Shyian et al., 2019) implicating that this interaction serves as a general mechanism for Top1 recruitment to the replisome during normal CMG progression. However, the relevance of this interaction in the response to topological stress had not previously been addressed directly. By using an assay to directly visualise replication fork rotation events in cells, this thesis has confirmed that the far C-terminus of Tof1, specifically between the region of 997-1182 of the protein, is required for suppression of replication fork rotation (Figure 4.2). These data therefore support a model in which this region of Tof1 recruits Top1 to the replisome to resolve topological stress during normal replisome progression (Figure 7.2). Recently published structures of Tof1 fail to resolve this far C-terminal region of the protein, suggesting that it is flexible (Baretić et al., 2020, Grabarczyk, 2020). Such flexibility may be an important feature of the Tof1-Top1 interaction, as it is likely some plasticity in this region would be required to allow the bulky Top1 enzyme to physically access and cleave DNA ahead of the fork.

The data presented in this thesis suggest that Csm3 recruitment alone to the replisome is not sufficient to restrict replication fork rotation, as *tof1-830* cells which are capable of binding Csm3 still show unrestricted levels of fork rotation (Figure 4.2). However, *csm3Δ* cells are unable to restrict replication fork rotation (Schalbetter et al., 2015), arguing that Csm3 is required for this function. Therefore, I would argue that although Top1 recruitment to the replisome by the far C-terminus of Tof1 is the primary mechanism of resolving topological stress during CMG unwinding, Csm3 is required to facilitate this interaction at replication forks, either by directly binding to Top1 or indirectly by properly co-ordinating the C-terminus of Tof1.

Given that the *tof1-997* truncation mutant is capable of performing all other roles tested in this thesis, it appears that the primary role of the far Tof1 C-terminus is to act as a platform for Top1 recruitment to the replisome. This suggests that the presence of a Top1-recruiting factor is an integral component of eukaryotic replisomes, highlighting the importance of continual resolution of topological stress during genome duplication. Indeed, cells lacking Tof1 and depleted for Top2 show elevated markers of DNA damage such as phosphorylated H2AX, a marker of double-stranded breaks (Schalbetter et al., 2015). A key question that remains unanswered is how unresolved topological stress generates genome instability in cells;

mapping when and where DNA damage arises in cells experiencing elevated topological stress will provide novel insights into this problem.

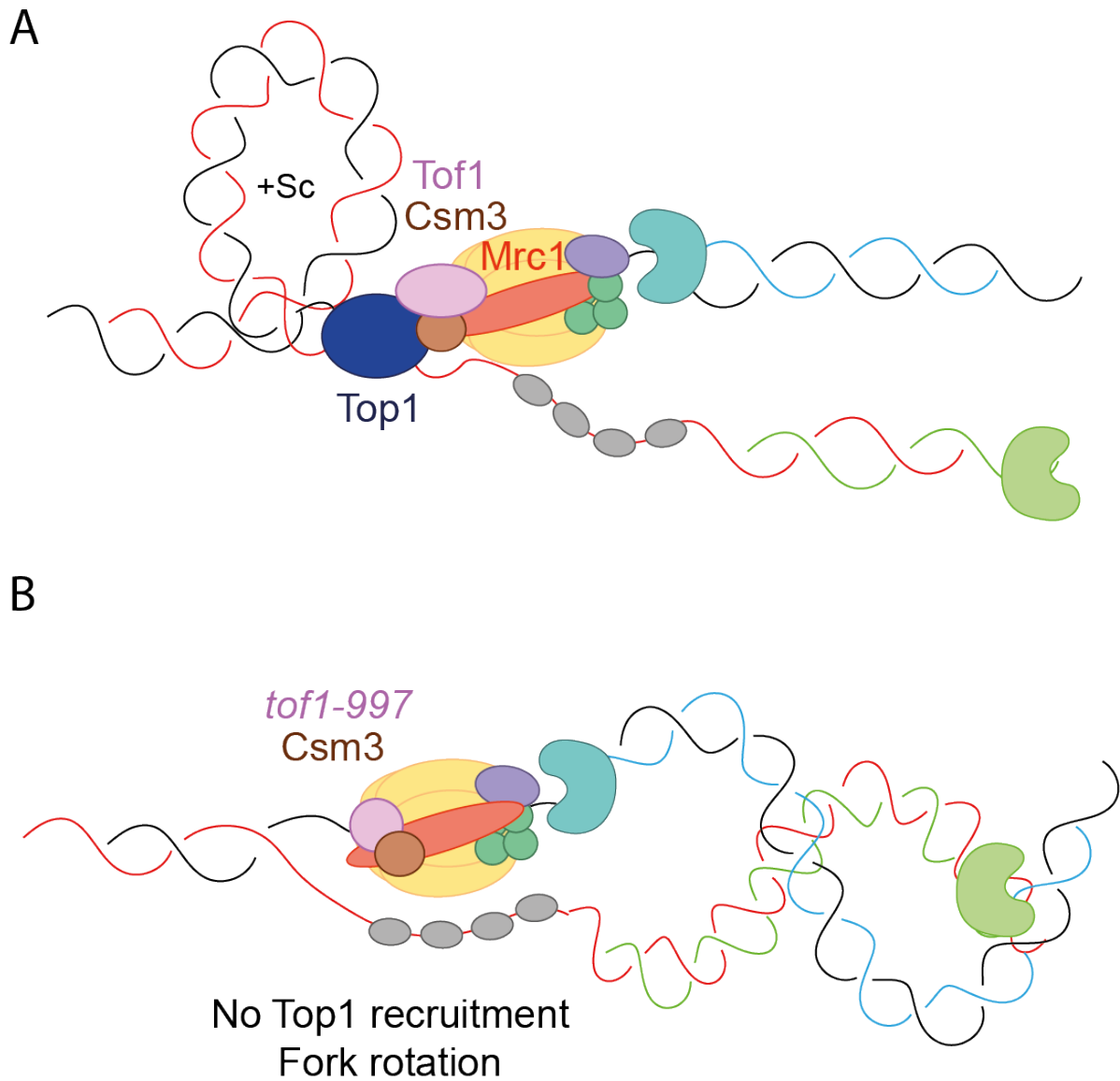


Figure 7.2: Model for how Top1 restricts replication fork rotation

A) In wild-type cells Top1 directly recruits Top1 to the replisome allowing for direct resolution of positive helical stress ahead of CMG. Csm3 is required to co-ordinate this interaction either directly or indirectly. B) Truncation of Top1 at residue 997 disrupts the Top1-Top1 interaction domain. Top1 is not recruited to elongating forks, resulting in increased fork rotation events to resolve helical stress accumulating ahead of CMG. Csm3 is unable to recruit Top1 to the replisome in the absence of Top1-997-1182.

7.2.3. The role of Tof1 in promoting replisome stability

Next, this thesis has defined the region of Tof1 responsible for its most well characterised role in replisome pausing and stabilisation in response to different types of replication stress. Initially, I focused on the role of Tof1 in programmed fork pausing at the Fob1 RFB and its role in responding to Top1-cc's. Polar fork blocking at the rDNA by programmed fork barriers is conserved across eukaryotic species and serves to prevent collisions between replication forks and the transcription machinery at the highly transcribed rDNA genes. Such collisions are toxic in nature and represent a potentially frequent source of DNA damage and genome instability in cells (Prado and Aguilera, 2005, Helmrich et al., 2011). In *S. cerevisiae*, both Tof1 and Csm3 are required for this function, whilst Mrc1 is not (Tourrière et al., 2005, Calzada et al., 2005, Mohanty et al., 2006). Similarly, cells lacking Tof1 or Csm3 are hypersensitive to the Top1-cc inducing drug CPT, whereas *mrc1Δ* cells are not (Redon et al., 2006). These studies suggest that Tof1/Csm3 have a checkpoint-independent role in fork pausing and stabilisation, although the domains responsible for these differing roles were previously undefined.

By using 2D gel electrophoresis and Southern blotting I have shown that a mid-C-terminal domain of Tof1, between residues 762-830 of the protein, are required to support replication fork pausing at the RFB (Figure 4.4). This same domain is also required to suppress the sensitivity of *tof1Δ* cells to CPT (Figure 4.5). The requirement for the same region of Tof1 to pause at both Fob1 and Top1 protein barriers suggests that Tof1's function at these blocks represents a general mechanism for fork pausing in response to stable protein-DNA barriers. Furthermore, this implies that the sensitivity of *tof1Δ* cells to CPT stems from an inability to pause replisomes at these lesions, favouring a model in which stable fork pausing at Top1-cc's is required to prevent replication fork runoff and the generation of double-stranded breaks (Strumberg et al., 2000).

Importantly, data presented in chapter 3 and 4 of this thesis together show that the same region of Tof1 required to stabilise the replisome at the RFB and at Top1-cc's is the same region required for stable Csm3 interaction in cells (aa 762-830) (Figure 3.5). This suggests that the role of Tof1 in replisome pausing is linked to its ability to stably recruit Csm3 to the replisome. Interestingly, recent structural data has revealed that Tof1/Csm3 are positioned at the front of the replisome and make several contacts with the DNA duplex ahead of progressing replication forks (Baretić et al., 2020). Whilst the exact functions of the Tof1/Csm3 DNA binding motifs are not yet clear, it is tempting to speculate that these motifs 'sense'

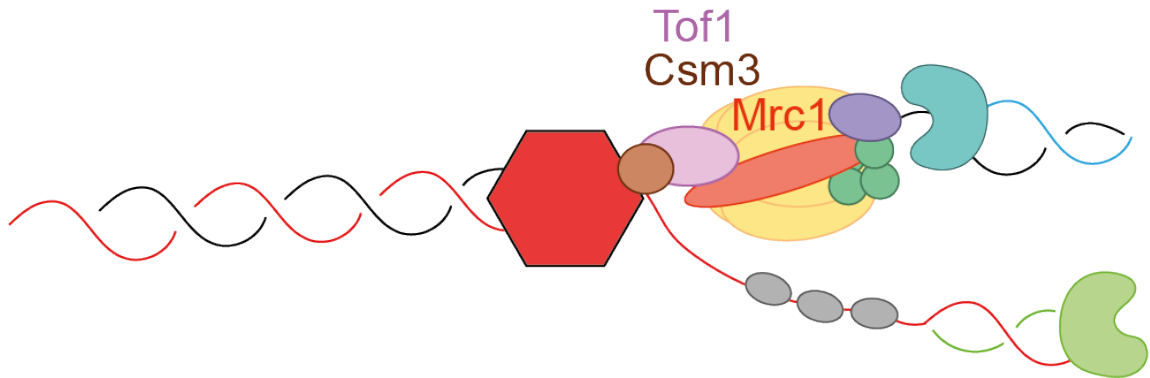
protein blocks ahead of the replisome to pause forks before they collide and result in potentially toxic replication intermediates and breaks.

In addition to stabilising replication forks at physical blocks to CMG progression (i.e. stable protein-DNA barriers), this thesis has also investigated the contribution of Tof1 to replisome stabilisation in HU. Cells lacking Tof1 show an 'uncoupling' phenotype in HU, whereby CMG progression continues in the absence of nascent strand synthesis (Katou et al., 2003). Unlike fork pausing at the rDNA RFB and at Top1-cc's, this replisome function is dependent on Mrc1 as well as Tof1/Csm3 (Katou et al., 2003). This thesis has experimentally confirmed the prediction that uncoupling in *tof1Δ* and *mrc1Δ* cells in HU results in accumulation of RPA-coated ssDNA (Figure 4.6). Furthermore, this analysis has shown that residues 762-830 of Tof1 are required to suppress this phenotype, showing that the coupling function of Tof1 is linked to the mid C-terminus of the protein. Interestingly this argues that the Tof1 mid C-terminal domain promotes functions that are both Mrc1-independent (in the case of fork pausing) and Mrc1-dependent (i.e. in replisome coupling).

Importantly, this thesis cannot rule out that additional residues N-terminal to the region between aa 762-830 may also be required for the aforementioned functions in Csm3 recruitment and replisome stabilisation. To determine whether regions between aa 1-762 are also required for these functions, it would be necessary to generate additional mutants truncated from the N-terminus of the protein.

A

Csm3 senses block ahead of fork
Fork pausing



B

Csm3 not recruited to fork
Replisome does not pause

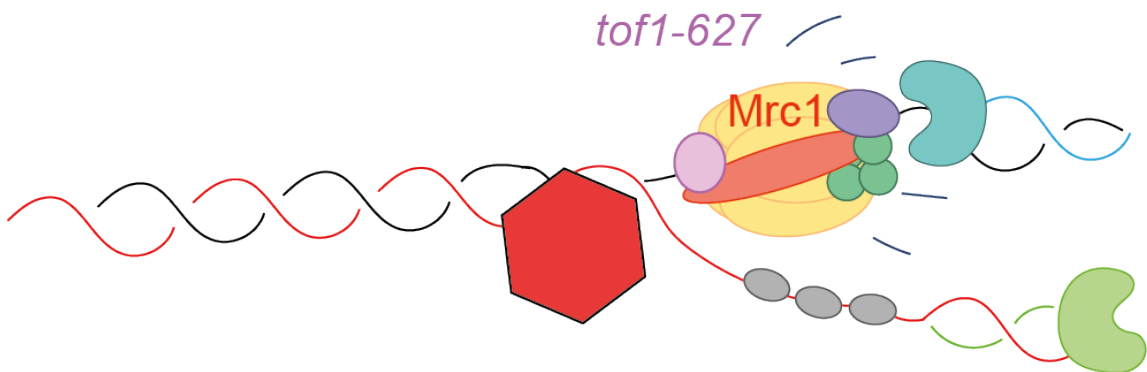


Figure 7.3: Model for how Tof1 promotes fork pausing at fork barriers

A) Csm3 is recruited to the replisome solely by its interaction with Tof1 (Baretić et al., 2020). In wild-type cells this interaction promotes Csm3 activities ahead of the fork including 'sensing' protein barriers (red hexagon) to enact replisome pausing.

B) In *tof1-627* cells the Tof1/Csm3 interaction is broken, resulting in a failure to recruit Csm3 to the front of the replisome. This results in a loss of fork pausing activity.

7.2.4. The role of Tof1 in intra-S checkpoint activation and replication fork restart

S. cerevisiae cells lacking Tof1 are only checkpoint-deficient when lacking the DNA damage checkpoint (DDC) mediator Rad9 (Foss, 2001), indicating that Tof1 functions to activate Rad53 in a separate, S-phase specific pathway. In chapter 5 of this thesis I have explored the role of Tof1 in activation of the DNA replication checkpoint (DRC). By using western blotting to visualise Rad53 activation, I have shown that the N-terminus alone of Tof1, specifically residues 1-627, are sufficient to elicit DRC activation in response to HU (Figure 5.1). Interestingly, this has defined *tof1-627* as a separation-of-function mutant, as *tof1-627* cells are unable to support replication fork pausing at protein blocks, cannot couple the replisome in response to HU and cannot restrict replication fork rotation.

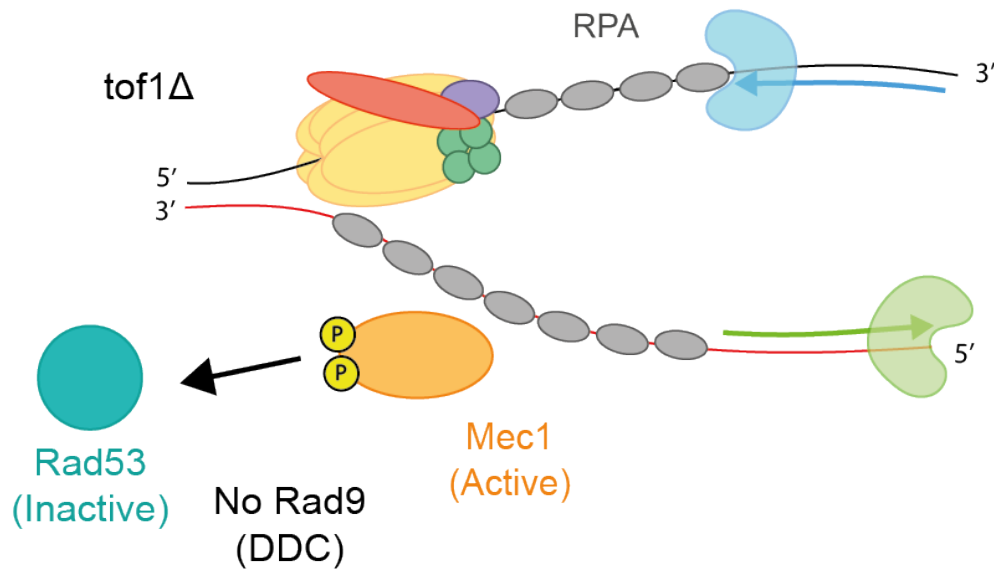
The roles of Tof1 at the replisome can generally be divided into two functional groups, showing either a Mrc1-like phenotype, as is the case for intra-S checkpoint activation, or not Mrc1-like, in the case of replisome pausing and responding to topological stress. With the exception of replisome coupling in HU (Katou et al., 2003), cells lacking Csm3 are deficient in the functions associated with the latter group that are Mrc1-independent, and thus do not appear to be linked to Mrc1 function. This thesis shows that Csm3 does not appear to play a significant role in DRC activation as *tof1-627 rad9Δ* mutants unable to bind Csm3 are DRC proficient. These data support a model in which Tof1 has a key role in supporting checkpoint activities at the fork, whilst Csm3 does not. Cells lacking Mrc1 and Rad9 are inviable (Alcasabas et al., 2001), suggesting that the role of Mrc1 in DRC activation is essential. Therefore, although I was not able to complete pulldowns to assess whether *tof1-627* and Mrc1 interact, it is hard to see how *tof1-627 rad9Δ* cells would be capable of mediating DRC activation without Mrc1 stably bound at the replisome. Whilst it cannot be ruled out that Tof1 and Mrc1 may be able to promote DRC activity independently of replisome recruitment, for example via interactions with RPA (Witosch et al., 2014), evidence for a non-replisome associated batch of FPC components is lacking. Therefore, I would argue that the N-terminus of Tof1, specifically residues 1-627, are sufficient to promote Mrc1 function at the fork to mediate DRC activation (Figure 7.4). In line with this, the recently published cryo-EM structure of the FPC bound to CMG suggests that the N-terminal half of Tof1 makes contacts with both CMG and Mrc1 (Baretić et al., 2020). However, had time allowed it would have been interesting to find out the exact domain(s) required for Tof1 and Mrc1 to bind, and whether these are required for DRC activation, as this is yet to be elucidated.

Using FACS analysis and Sync-seq to monitor fork progression after release from a HU-arrest, I have been able to show that *tof1-627 rad9Δ* cells are capable of completing bulk DNA replication and can resume DNA replication from forks stalled in HU. This is in line with evidence suggesting that checkpoint activation is required for resumption of replication following fork stalling. In budding yeast, *rad53Δ* mutants treated with HU accumulate abnormal fork structures after release from the HU arrest (Lopes et al., 2001). However, whilst the N-terminal half of Tof1 is sufficient to mediate Rad53 phosphorylation in response to HU, *tof1-627 rad9Δ* cells are unable to survive both chronic and acute HU treatment. This argues that Tof1 has an additional role in stabilising the replisome following replication fork restart outside of DRC activation. Specifically, this thesis has shown that replisomes restarted from a HU-induced arrest require the key stabilising region of Tof1 between residues 762-830 to maintain genome stability following restart. As shown by Sync-Seq, mutants lacking this mid-C terminal domain of Tof1 are unable to maintain stable fork progression following restart. Given the decreased cell survival of *tof1-627 rad9Δ* mutants after acute HU treatment, this loss of fork stability is likely to result in unrecoverable lesions.

A key remaining question from the analyses in chapter 5 is which lesion(s) are toxic to cells lacking the mid-C-terminus of Tof1 following fork arrest. Importantly, *tof1-627* cells themselves are only very mildly sensitive to HU, suggesting that the lesions generated after fork restart in *tof1-627 rad9Δ* cells would normally be prevented or repaired by a Rad9-dependent mechanism, potentially linked to Rad9's role in the DDR. Rad9 is known to play a key role in preventing Sgs1 and Dna2 mediated resection of DSBs, via an interaction between its Tudor domain and methylated histone H3 (Pardo et al., 2016, Bonetti et al., 2015, Lazzaro et al., 2008). Potentially, replisome destabilisation may result in the generation of DSBs. Rad9 may then be essential to ensure these lesions are not erroneously processed, which could potentially result in large deletions and genome rearrangements.

A

No *Tof1* = *Mrc1* function in DRC impaired?



B

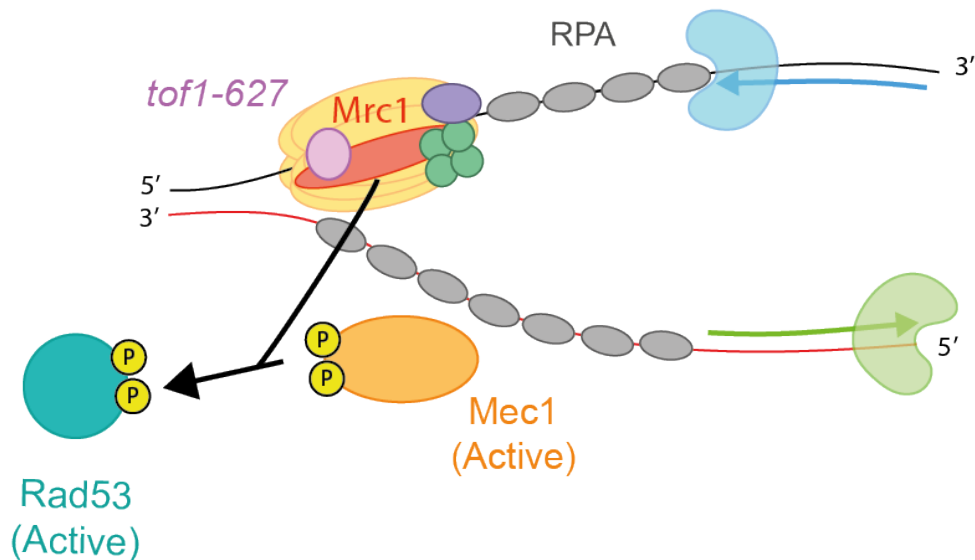


Figure 7.4: A model for how *Tof1* promotes DRC activation in response to replication stress

A) RPA-coated ssDNA acts as the signal for Mec1 activation, but this signal requires either the DRC or the DDC to mediate activation of the effector checkpoint kinase Rad53. In *tof1Δ* cells lacking the Rad9-dependent DDC, Rad53 phosphorylation cannot be mediated by the DRC mediator *Mrc1*, potentially as a result of failure to stably recruit or position *Mrc1* in the replisome.

B) In *tof1-627* cells, the N-terminus of *Tof1* is sufficient to stabilise *Mrc1* in the replisome to promote its mediator activity in the DRC. The inability to recruit *Csm3* to the replisome does not significantly impair *Mrc1*-dependent DRC activity.

Mutant	Stable protein product	Tof1-Csm3 Binding	Suppresses CPT sensitivity	Suppresses fork rotation	Pauses forks at RFB	Helicase/polymerase coupling in HU	Rad53 phosphorylation *	Suppresses sensitivity to chronic HU exposure (in <i>rad9Δ</i> background)	Restarts replication after acute HU exposure (in <i>rad9Δ</i> background)	Suppresses lethality after acute HU exposure (in <i>rad9Δ</i> background)
<i>tof1Δ</i>	N/A	N/A	X	X	X	X	X	X	X	X
<i>csm3Δ</i>	N/A	N/A	X	X	X	X	Partial	NT	NT	NT
<i>mrc1Δ</i>	N/A	NT	✓	✓	✓	X	Inviabile	Inviabile	Inviabile	Inviabile
Tof1 wt	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
<i>tof1-627</i>	✓	X	X	X	X	Partial	✓	X	Partial	Partial
<i>tof1-762</i>	✓	X	X	X	X	Partial	✓	X	---	---
<i>tof1-830</i>	✓	✓	✓	X	✓	✓	✓	✓	✓	✓
<i>tof1-997</i>	✓	✓	✓	X	✓	✓	✓	✓	NT	NT
<i>tof1-1182</i>	✓	✓	✓	✓	✓	✓	✓	✓	NT	NT

Table 7.1: Summary of the phenotypes observed in Tof1 truncation mutants described in this thesis compared with the phenotypes of *tof1Δ*, *csm3Δ* and *mrc1Δ* mutants.

7.3. Generation of human conditional Timeless mutant cell lines

In chapter 6 I have outlined my efforts to generate human cell lines in which Timeless can be conditionally depleted. Currently all published analyses of hTim have used siRNA depletion to investigate the function of this protein in mammalian cells. This is most likely due to the inability to generate *TIM* knockout cell lines, as *TIM* knockout mice are embryonic lethal (Gotter et al., 2000) and *TIM* has been described as a core fitness gene in genome-wide analyses (Hart et al., 2015). For this reason, I attempted to generate cell lines in which Tim protein could be conditionally depleted. However, the method used to generate these conditional Tim mutants generated only heterozygously tagged Tim clones, suggesting that introduction of the SMASH-mAID degron at the endogenous Tim C-terminus at both *TIM* alleles results in cellular inviability and further supporting the argument that *TIM* has adopted an essential role in mammals.

This in turn gives rise to an interesting question – what is the essential mammalian function of *TIM*? A simple explanation may be the inherent complexity of mammalian genomes. The human genome contains approximately 3 billion base pairs (Lander et al., 2001), compared with the ~12 million base pair genome of *S. cerevisiae* (Goffeau et al., 1997). Replication of every base is thus a huge undertaking and the frequency of fork stalling events during a single S-phase is thus likely to be much higher when duplicating a larger template. In addition, the increased length of mammalian genes is likely to introduce heightened levels of collisions between replication and transcription processes. Indeed, in human cells the identification of ‘common fragile sites’ (CFSs) as regions of increased replication-transcription collisions highlights the importance of maintaining replisome stability in mammals (Helmrich et al., 2011). The role of Timeless in suppressing chromosomal instability at such collisions has not yet been investigated. However, the replisome stabilising function of Timeless proteins may be the essential function required to duplicate mammalian genomes, even in the absence of exogenous replication stress. Depletion of Timeless from human cells using siRNA results in markers of DNA damage such as Rad51 and γH2AX foci, as well as chromosomal breakage, even in the absence of additional genotoxic stress (Chou and Elledge, 2006, Urtishak et al., 2009). These observations are in line with the need for Timeless to suppress genome instability even during normal cellular processes in higher eukaryotes.

Lastly I have shown that in RPE-1 cells, Timeless exists as three distinct isoforms as visualised by western blotting. To date, no published studies have observed more than one Tim isoform in human cells, and so this finding is enigmatic. Whilst I have not had time to follow up this

finding, it will be interesting to further characterise these isoforms and their function within cells. Specifically, it will be interesting to determine whether this regulation of hTim is tissue specific to retinal epithelial cells by characterising hTim expression in different cell lines.

7.4. Conclusion

All organisms must be able to fully and faithfully replicate their genomes every S-phase. If one considers only the basic enzymatic functions of strand separation and nascent strand synthesis, this task appears relatively straight-forward. In reality, however, genome duplication is much more complex, requiring the replisome to overcome numerous challenges that arise during the process of DNA replication. Failure to respond to these challenges can damage the DNA and consequently lead to genome instability and mutagenesis (Lambert and Carr, 2013). Importantly, evidence suggests that replication stress is a key driver in the development of many cancers, highlighting the importance of pathways to overcome these challenges in higher eukaryotes (Macheret and Halazonetis, 2015).

However, despite these numerous challenges, cells manage to accomplish the task of genome duplication without major errors every S-phase. The FPC, consisting of Tof1/Csm3 and Mrc1 are the key drivers in maintaining stability of the genome during the process of genome duplication. The presence of FPC proteins in eukaryotic cells but not in bacteria, as well as the essential nature of Tim in mammals likely reflects the need to stabilise replisomes on increasingly complex templates and maintain genome stability during this process.

This thesis has dissected the functions of Tof1 in budding yeast and shown that distinct domains of the protein carry out different roles all pertaining to replisome stability during S-phase. The N-terminus of Tof1 is sufficient for DRC activation but is unable to support activities pertaining to the physical stabilisation of replisomes or in response to topological stress. These functions additionally require mid- and far-C terminal domains of the protein, respectively. Together these results suggest that Tim family proteins have evolved in eukaryotes as factors capable of supporting multiple distinct processes at the replisome to facilitate DNA replication. Specifically, this work suggests that Tof1 promotes Mrc1-linked activities and Csm3-linked activities via the N- and C- terminus, respectively (Figure 7.5). The physical separation of N-terminal and C-terminal Tof1 activities gives a ready explanation for the long-standing observation that Tof1 functions appear to be linked either to Mrc1 or Csm3 activity, but rarely both.

Given Timeless is frequently overexpressed to protect cancer cells from increased levels of replication stress (Tourrière et al., 2005), it is evident that understanding the ways in which this conserved family of proteins maintain genome stability may have direct implications for human health. Thus, more research is needed to fully elucidate the mechanisms by which Tim proteins function, particularly in mammals. This may then open up new avenues to therapeutically target cancer cells exploiting the function of FPC proteins in resolving replication-stress induced genome instability. In addition, the development of better tools to conditionally deplete Timeless in mice and other mammals may give some insight into the long-standing debate over the role of mammalian Tim proteins in circadian rhythm regulation.

Tof1

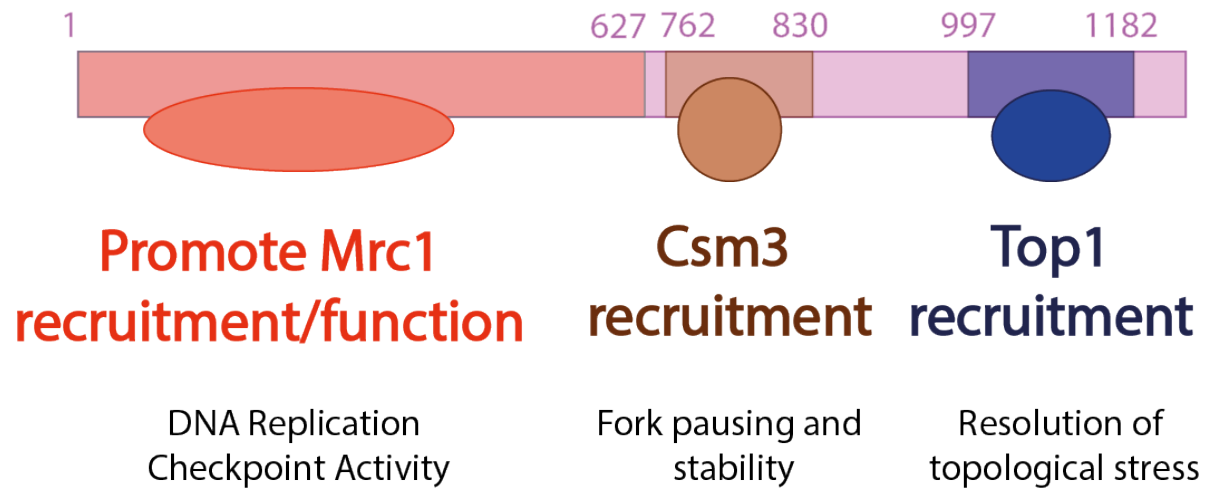


Figure 7.5: Summary of the Tof1 functional domains required for distinct replisome processes during DNA replication

The N-terminal half of Tof1, specifically residues 1-627, promote DRC activities potentially by recruitment and/or co-ordination of Mrc1 function in checkpoint mediation. The mid C-terminus of Tof1, specifically residues 762-830, are required for Csm3 binding and promote replisome pausing and stabilisation functions, most likely by properly co-ordinating Csm3 ahead of the replisome. A far C-terminal domain between residues 997-1182 of the protein promotes resolution of supercoiling ahead of the progressing CMG by its interaction with Top1, which recruits Top1 to the replisome.

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Appendix

Materials and Methods Relating to ChIP-Seq, Sync-Seq and Bioinformatic Analysis of Sequencing Data

RPA1 ChIP-seq

For RPA ChIP experiments cells were grown in YP Raff to mid-log phase at 25°C, before being arrested with 10 µg/ml alpha factor peptide. After 1hr 45 min 2% galactose and an additional 5 µg/ml alpha factor was added. After 2hr, when cells were >90% unbudded, 25 µg/ml doxycycline was added. 15 minutes after doxycycline addition temperature was switched to 37°C and incubated for 1hr. Cells were then released by washing 3 times with pre-heated YP 2% raffinose 2% galactose with 25 µg/ml doxycycline and resuspended in the same media supplemented with 200 mM HU. Time 0 was taken as the time from the first wash. Samples were then incubated for 1 hr at 37°C before being fixed by resuspending in YP + 1% formaldehyde (Merck 104003) for 45 min at 25°C. 125 mM glycine was then added followed by a 5 min incubation at 25°C. Cells were washed with PBS before being pelleted and snap-frozen in liquid nitrogen.

Pellets from 75 ml cultures were resuspended in 500 µl SDS buffer (1% SDS, 10 mM EDTA, 5M Tris HCl, cOmplete Tablets Mini EDTA-free EASYpack (Roche), PhosSTOP (Roche)). 200 µl of 0.5 mm zirconia/silica beads were added to samples and cells were lysed using the FastPrep-24 (MP Biomedicals) on max speed (6.5 m/s), with 5 rounds of 1 min each. Lysate was spun out and IP buffer (0.1% SDS, 1.1% Triton-X-100, 1.2 mM EDTA, 16.7 mM TRIS HCl (pH8), cOmplete Tablets Mini EDTA-free EASYpack (Roche), PhosSTOP (Roche)) was added to a final volume of 1 ml. Samples were sonicated using the Focused-Ultrasonicator (Covaris) (Average incident power – 7.5 Watts, Peak Incident Power – 75 Watts, Duty Factor – 10 %, Cycles/Burst – 200, Duration – 20 min). The sample was centrifuged for 20 min at 13000 rpm at 4°C. Supernatant was then diluted to 7.5 ml with IP buffer. 75 µl protein A Dynabeads (Invitrogen) and 75 µl protein G Dynabeads (Invitrogen), were washed 3 times in IP buffer before adding to the sample and incubating for 2 h at 4°C. 2 ml of the supernatant was taken to 15 ml falcon tubes, and the rest was kept at -20°C as an input sample. To the 2 ml sample RPA1 antibody (1:10000, Agrisera, AS07214) was added followed by overnight incubation on a rotating wheel at 4 °C.

A mix of Dynabeads, Protein A (30 µl) and Protein G (30 µl), was washed 3 times in IP buffer. This was added to each sample and incubated at 4°C for 4 h. Supernatant was removed and beads were washed at 4°C for 6 min in TSE-150 (1% Triton-X-100, 0.1% SDS, 2 mM EDTA, 20 mM Tris HCl (pH8), 150 mM NaCl), followed by TSE-500 (1% Triton-X-100, 0.1% SDS, 2 mM EDTA, 20 mM Tris HCl (pH8), 500 mM NaCl), followed by LiCl wash (0.25 M LiCl, 1% NP-40, 1% dioxycholate, 1 mM EDTA, 10 mM Tris HCl (pH8)) and finally Tris-EDTA (TE pH8). Elution was carried out in 400 µl elution buffer (1% SDS, 0.1M NaHCO₃, for 30 min at room temperature. At the same time 50 µl from the input sample was added to 150 µl of elution buffer. 20 µl of 5 M NaCl and 10 µl of 10 mg/ml proteinase K (Invitrogen) was then added to the input, and 40 µl and 20 µl to the IP samples respectively. These were incubated at 65°C overnight. Then 10 µl of DNase-free RNase (Roche) was added to the input and 20 µl to the IP samples, and they were left at 37°C for 30 min. All DNA was purified with a Qiagen PCR purification kit and eluted in 40 µl H₂O. 34 µl from the RPA1 samples and 1 ng DNA in 34 µl water from the input were used for library preparation. 5 µl 10 x NEB2.1 buffer and 5 µl of random primers (8N, 3 mg/ml stock) were added and the samples were boiled at 95°C for 5 min and immediately placed on ice for 5 min. 5 µl 10 x dNTPs with dUTP instead of dTTP (2 mM each) and 1 µl T4 polymerase (NEB) were added and the mixture was incubated at 37°C in a thermal cycler for 20 min, and 5 µl 0.5 M EDTA (pH 8) was immediately added to stop the reaction. The resulting dsDNA was used to create libraries using the Ultra II library kit (NEB) as per the manufacturer's instructions with 13 cycles at the amplification step.

Paired end sequencing was performed using the MySeq (75bp reads from each side) or NextSeq 500 (42 bp reads from each side) systems to result >2 million reads.

ChIP Seq Analysis

FASTQ files were generated by Illumina basespace (<https://basespace.illumina.com/home/index>). The resulting sequences were aligned to a reference genome (R64-1-1, *Saccharomyces cerevisiae* S288c assembly from Saccharomyces Genome Database) using Bowtie 2 generating a SAM output file for each sample (<http://bowtie-bio.sourceforge.net/bowtie2/index.shtml>). Reads from MySeq were trimmed 25 bp from 3' and 1 bp from the 5' end, while reads from NextSeq were not trimmed.

Command for MySeq reads:

```
bowtie2 -p 14 -x [path to index folder] --trim3 25 --trim5 1 -1 [Path and name of R1 fastq file] -2 [Path and name of R2 fastq file] -S [name of the resulting .sam file]
```

Command for NextSeq reads:

```
bowtie2 -p 14 -x [path to index folder] --trim3 0 --trim5 0 -1 [Path and name of R1 fastq file] -2 [Path and name of R2 fastq file] -S [name of the resulting .sam file]
```

SAM files were then converted into sorted BAM files by using SAMtools (<http://samtools.sourceforge.net/>):

```
samtools sort [name of the .sam file generated with bowtie2] -o [name for the resulting .bam file] -O bam -T [name for resulting .bam file wo .bam]
```

Duplicates were then removed using picard (<https://broadinstitute.github.io/picard>)

```
java -jar ~/picard/picard-tools-1.138/picard.jar MarkDuplicates I= [name for the resulting .bam file] O= [name for the resulting without repeats.bam file] M= [name of metrix file.txt] REMOVE_DUPLICATES=true
```

BAM files were used for Model-based Analysis of ChIP-Seq (MACS2). We used the 'call peak' function which also generates genome wide score data. These were used to generate fold enrichment tracks. Example command:

```
macs2 callpeak -t [sorted BAM file from yh2a data]-c [sorted BAM file from h2a data]-f BAMPE -g 12100000 -n [name for output file] -B -q 0.01 --SPMR
```

The data then was sorted into 50 bp bins, normalized to have a mean value of 1, and used for meta data analysis using custom-made R programs.

Sync-Seq

Pellets from 2 ml cultures were resuspended in 500 µl SDS buffer (1% SDS, 10 mM EDTA, 5M Tris HCl, cOmplete Tablets Mini EDTA-free EASYpack (Roche), PhosSTOP (Roche)). 200 µl of 0.5 mm zirconia/silica beads were added to samples and cells were lysed using the FastPrep-24 (MP Biomedicals) on max speed (6.5 m/s), with 5 rounds of 1 min each. Lysate was spun out and IP buffer (0.1% SDS, 1.1% Triton-X-100, 1.2 mM EDTA, 16.7 mM TRIS HCl (pH8), cOmplete Tablets Mini EDTA-free EASYpack (Roche), PhosSTOP (Roche)) was added to a final volume of 1 ml. Samples were sonicated using the Focused-Ultrasonicator (Covaris) (Average incident power – 7.5 Watts, Peak Incident Power – 75 Watts, Duty Factor

– 10 %, Cycles/Burst – 200, Duration – 20 min for G1 samples and 13.5 for HU and released samples). 200 µl of sample was removed and 10 µl of DNase-free RNase was added and incubated at 37°C for 30 min. DNA was then purified with a Qiagen PCR purification kit and eluted in 50 µl H₂O. 50 ng of DNA in 50 µl water was used for library preparation using the Ultra II library kit (NEB) as per the manufacturer's instructions with 6 cycles at the amplification step.

Paired end sequencing was performed using NextSeq 500 (42 bp reads from each side) systems to result >2 million reads.

Sync-SEQ analysis

Sync-seq analysis was done by using LocalMapper shell script and Repliscope R package from (Batrakou et al., 2020)

```
localMapper.sh -g [path to index folder] [Path and name of R1 fastq file] -2 [Path and name of R2 fastq file] -s [name of the output files] -w 3000 -c 14
```

The resulting .bed files were then read in to R:

```
repBed <- loadBed(file name for the replicating sample)
nrepBed <- loadBed(file name for the non-replicating sample)
```

Outliers were removed:

```
repBed<-rmOutliers(repBed, "median", loLim = 0.25)
repBed<-rmOutliers(repBed, "max", n = 2)
nrepBed<-rmOutliers(nrepBed, "median", loLim = 0.25)
nrepBed<-rmOutliers(nrepBed, "max", n = 2)
```

Ratio between non-replicated and replicated samples were calculated:

```
ratio <- makeRatio(repBed,nrepBed)
```

And normalised:

```
ratio <- normaliseRatio(ratio, [rFactor])
```

Where rFactor was empirically determined to fit the lowest replicating regions to 1.

The resulting ratios were smoothed by a moving average of 2 and plotted using custom-made R programs.