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Termination of eukaryotic replication forks

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Abstract

Termination of DNA replication forks takes place when two replication forks coming from neighbouring origins meet each other usually in the midpoint of the replicon. At this stage, the remaining fragments of DNA have to be unwound, all remaining DNA replicated and newly synthesised strands ligated to produce continuous sister chromatids. Finally, the replication machinery has to be taken off chromatin and entwisted sister chromatids resolved topologically.

Over the last few decades we have learned a lot about the assembly of the helicase and replisome and the initiation stage of DNA replication. We also know much more about the ability of forks to cope with replication stress. However, only within the recent few years we gained the first glimpse of the mechanism of replication fork termination. In this chapter I will summarise the recent findings on replication termination, weigh this against the past literature and discuss relevant consequences and views for the future.

Keywords

Eukaryotic DNA replication, termination of DNA replication, ubiquitin, Cdc48 p97 segregase, cullins,

1. Introduction

To maintain genomic stability it is essential that every step of DNA replication is faultlessly executed. Mistakes during eukaryotic replication that are not efficiently repaired can lead to mutations and genome rearrangements that promote changes leading to development of cancer and other disorders.

DNA replication can be divided into three stages: initiation, elongation and termination. Initiation of DNA replication happens when licensed origins of replication fire creating two DNA replication forks, which move in opposite directions. The elongation stage involves the progression of replication forks as they unwind and replicate DNA. Finally, termination happens when two replication forks from neighbouring origins converge and the duplication of remaining fragment of DNA is neatly completed. Over the years we have learnt a lot about the mechanisms of DNA replication initiation and elongation (briefly explained below), but until recently our knowledge of replication termination was very restricted. The last few years have brought a breakthrough in our understanding of mechanisms of replication termination: we have learnt that converging replication forks can pass each other when terminating and we have also unravelled the workings of disassembly of terminated replisomes.

2. Replication fork termination occurs throughout S phase

When does replication termination take place? In our mind replication termination should happen mostly at the end of the whole replication process, so in the terms of cell cycle stages - at the end of S-phase. In reality however, DNA replication forks encounter forks from neighbouring origins throughout the entire S-phase. Forks emanating from origin clusters firing in early S-phase will also terminate in early S-phase; with average replicon size of 31 kbp (Moreno et al., 2016; Picard et al., 2014) and an average fork speed of 1.5 kb/min (Conti et al., 2007) it takes about 10 minutes for the two neighbouring forks to reach one another. In fact there is likely more termination occurring in mid S-phase than in late S-phase as the strict replication timing programme driving replication in

each cell means that only difficult-to-replicate regions are replicated in late S-phase (Gilbert, 2010).

3. Replication initiation and elongation

To ensure that all of the large eukaryotic genomes are duplicated in full before each cell division, eukaryotic DNA replication starts from multiple origins of replication. Human cells have on average about 50 thousand of them spread throughout the genome. It is also essential that DNA is replicated just once per cell cycle as re-replication of parts of the genome is a threat for the maintenance of genome integrity. To achieve this, the replicative helicase (protein complex, which unwinds double stranded DNA during replication) can be loaded onto DNA only before the onset of S-phase when CDK activity is low, and can be activated only during S-phase when CDK activity is high. Origins of replication are therefore "licensed" in late M and G1 stages of the cell cycle, by loading of the core of the replicative helicase: Mcm2-7 (Minichromosome maintenance 2,3,4,5,6,7) complexes. Double hexamers of the Mcm2-7 complex are loaded onto origins through the concerted action of ORC (Origin Recognition Complex), Cdc6 and Cdt1 factors. These double hexamers encircle the double stranded DNA and are arranged in N-terminus to N-terminus orientation with the C-terminal helicase domains on the outside. There are multiple Mcm2-7 double hexamers loaded around each origin of replication, which may be facilitated by their ability to slide on double stranded DNA (Evrin et al., 2009; Gambus et al., 2011; Remus et al., 2009).

The initiation of DNA replication requires the activity of two S-phase kinases: Cdc7/Dbf4 (DDK – Dbf4 dependent kinase) and Cdk/cyclin (CDK – Cyclin dependent kinase). DDK phosphorylates double hexamers of Mcm2-7 while CDK drives association of GINS (Go-Ichi-Ni-San, complex of Sld5, Psf1, Psf2 and Psf3 or GINS1,2,3,4) and Cdc45 with the Mcm2-7 complexes, forming the CMG complex (**C**dc45/**M**cm2-7/**G**INS), which is an active replicative helicase (Ilves et al., 2010; Moyer et al., 2006; Simon et al., 2016). The initiation process leads to rearrangement of Mcm2-7 complexes: the double hexamers split into

two CMGs and each of them now likely encircles just single stranded DNA (Costa et al., 2011; Gambus et al., 2006; Yardimci et al., 2010).

During the elongation stage of DNA replication the helicase (CMG complex) travels at the tip of the replication fork, unwinding the double stranded DNA and exposing single strands that can act as a template for DNA synthesis by DNA replication polymerases. The MCM motor of CMG belongs to the superfamily of AAA+ ATPases and is a 3'-5' DNA translocase, which encircles the leading strand of the replication fork (reviewed in (Pellegrini and Costa, 2016)). The Polα-primase complex initiates DNA synthesis with a short RNA primer that is then elongated for another 20-nt by Polα polymerase activity. The leading strand is believed to be synthesised mainly by DNA Polε (DNA polymerase epsilon) in a continuous manner, while the lagging strand is thought to be completed by DNA Polδ (DNA polymerase delta) (Daigaku et al., 2015; Georgescu et al., 2015; Pavlov et al., 2006). The latter synthesises short Okazaki fragments in the opposite direction to the movement of the fork and these fragments need to therefore be processed and ligated to produce the continuous DNA strand (maturation of Okazaki fragments). DNA Polε is therefore following the helicase and indeed has a number of connections linking it directly to the helicase to facilitate the smooth progression of the fork (see below and reviewed in (Pellegrini and Costa, 2016)).

DNA unwinding generates a compensatory increase in the intertwining of parental strands, which can be converted into helical overwinding (positive supercoiling) of the unreplicated portions of the DNA ahead of the forks (Postow et al., 2001; Wang, 2002). This mechanical strain can be transmitted to replicated DNA by rotation at the branching point of the replication fork, thus generating intertwining of the daughter duplexes (known as precatenates) (Been and Champoux, 1980) (Figure 1). Recent research in budding yeast has shown however that during normal progression of replication forks, fork rotation and precatenation are actively inhibited by components of the replisome Timeless/Tof1 and Tipin/Csm3 (Schalbetter et al., 2015). Instead, supercoils generated during replication elongation can be relaxed by both type I and type II topoisomerases (Wang, 2002). Indeed, the current view assumes that positive supercoiling is mainly relaxed by type I enzymes (Topo I, *S.cerevisiae*

Top1) anywhere in the unreplicated region (Postow et al., 2001). The replisome progression complex (RPC) built around CMG at the tip of the fork contains Top1, positioning it perfectly for its function ahead of the fork (Gambus et al., 2006) (Figure 1). Interestingly, yeast cells without Top1 and also *top2* mutants can replicate DNA, but replication is not possible when both proteins are defective (Bermejo et al., 2007; Brill et al., 1987).

Importantly, replication forks do not move through naked DNA but through a chromatin structure. Nucleosomes therefore need to be dismantled ahead of the forks and rebuilt behind the forks. The efficient repositioning of parental histones is essential for full reconstitution of epigenetic markings throughout the replicating genome. Studies of SV40 replication forks provided evidence for the existence of only 200-300 bp of apparently nucleosome-free DNA behind the replication fork (Gasser et al., 1996) and the nucleosomes in yeast were shown recently to be positioned immediately after the fork passage and restrict Okazaki fragments sizes (Smith and Whitehouse, 2012). Progressing replication forks need also to remove other proteins attached to DNA, for example the unfired Mcm2-7 double hexamers, which, loaded in excess, serve as dormant origins ready to rescue collapsed forks. Finally, sister chromatids are topologically embraced and held together until mitosis by cohesin ring complexes. This cohesion is established during DNA replication as forks progress (reviewed by (Uhlmann, 2009)).

4. Where does termination of eukaryotic replication forks happen?

The simplest answer to this question is: wherever the two neighbouring forks meet each other. Recent analysis of genome-wide replication profiles in budding yeast, both through high-resolution replication profiling (Hawkins et al., 2013) and through deep sequencing of Okazaki fragments (McGuffee et al., 2013), showed that termination generally occurs midway between two adjacent replication origins. The precise position of termination depends on the relative activation time of each of the origins and their variable efficiency. Okazaki fragment mapping in human cells (HeLa and GM06990) also confirmed such mid-point localisation (Petryk et al., 2016).

Eukaryotes not only have specific spatial patterns but also possess temporal patterns of genome replication, which are executed by regulated activation of replication origins throughout S-phase. High-throughput experiments allowed the identification of a genome-wide temporal order of replication (Gilbert, 2010). In early S-phase, "active" chromatin is replicated with origins of replication located in general in-between the genes. Not surprisingly therefore, many termination events in early S-phase were found to overlap with transcribed genes. In late S-phase however, when heterochromatin is replicated, many termination zones were found in large non-expressed regions of DNA (Petryk et al., 2016).

This sequence independent localisation of termination sites is in sharp contrast to the organisation of termination events in *E.coli* chromosome where termination takes place within a broad region containing several specialised fork barriers i.e. Tus-TER complexes, which confine fork fusion to a site of 270 kb (reviewed in (Dimude et al., 2016)). Due to these defined prokaryotic Termination Regions (TER), for a number of years termination of eukaryotic replication forks was studied only at the existing few loci within the eukaryotic genome, which contain specialised replication fork barriers (RFBs). The best characterised of such sites are the RTS1 site in *S.pombe*, which regulates mating type switching (Brewer and Fangman, 1988), and the rDNA locus within ribosomal DNA repeats of metazoa and yeasts (Dalgaard and Klar, 2000). The RFB barriers are able to arrest one of the two neighbouring forks and therefore create specific termination sites (Bastia and Zaman, 2014; Dalgaard et al., 2009). To minimise fork pausing at RFBs the protein displacement helicase Rrm3 helps to displace the barriers to allow replication passage and is required for fork termination at these sites. In yeast lacking Rrm3 tenfold accumulation of termination structures ("X" shaped DNA structures in 2D DNA gels) was observed, while only twofold accumulation of paused forks at the barrier (Ivessa et al., 2003; Ivessa et al., 2000). But Rrm3 is not required for bulk replisome unloading during normal termination (Maric et al., 2014), so it is needed only for fork convergence at rare situations when one fork is paused.

In 2010, Fachinetti et al, identified 71 termination regions (TERs) in budding yeast, through a combination of Chromatin immunoprecipitation (ChiP)

and BrdU incorporation experiments. Their work found that the majority of these regions contain fork pausing elements, such as transcription clusters, and that efficient termination at the identified sites requires activity of Rrm3 and Top2 (Fachinetti et al., 2010). However, the more recent high-resolution approaches suggest that these TERs actually represent sites with a higher than average probability of termination as they are flanked by early-firing efficient origins. Importantly, changes of origin firing pattern moved the termination positioning both in non-TER and TER replicons, indicating that it is the timing and efficiency of origin firing and not fork pausing elements that dictate the precise place of replication fork convergence (Hawkins et al., 2013; McGuffee et al., 2013).

5. How do replication forks converge?

Figure 2 summarises our current model of replication fork termination. To allow convergence of two approaching DNA replication forks all of the proteins bound to DNA between them must be evicted (Figure 2A). Unwinding of final stretches of DNA can present a problem for the forks as the torsional stress created ahead of the fork cannot be easily released due to lack of access for Top I (see below for more details) and has to be translated into precatenates, which accumulate behind the fork (Figure 2B). Two converging forks present two large protein machineries approaching one another and heading for head-on collision while unwinding the remaining DNA between them (Figure 2C). After forks converge, all of the remaining DNA needs to be replicated and the RNA-DNA primer of the last Okazaki fragment on the lagging strand needs to be processed (Figure 2D,E). Once this is complete, DNA needs to be ligated into a continuous strand and replisomes need to be disassembled (Figure 2E). Finally, the entangled sister chromatids need to be resolved into two separate strands (Figure 2G).

Recent years have brought a breakthrough in our understanding of the above processes. Beautiful work from Prof Johannes Walter's lab shed light on the mechanism by which forks converge and termination is resolved (Dewar

Walter 2015). To synchronise termination events and facilitate their analysis, they constructed plasmids with an array of *lac* repressors (LacRs) bound to *lac* operators (LacOs), which can be disrupted by IPTG. Such plasmids replicated in cell-free *Xenopus laevis* egg extract accumulated blocked forks at the edges of the array. The blocked forks were then released by addition of IPTG, and proceeded to terminate within the DNA fragment comprising the array. Using this system, Dewar et al. could monitor: unwinding of DNA as forks approach each other, synthesis of DNA, ligation of the replicated DNA and decatenation of daughter molecules. Strikingly, the rate of DNA synthesis within the array was almost perfectly linear after IPTG addition and resembled the fork progression speed reported in the same extracts. It suggests therefore that converging forks do not slow significantly before they meet; they do not collide with each other or stall but rather pass each other (Dewar et al., 2015) (Figure 2C and D). Such passage can be possible as CMGs encircle the leading strand of the replication fork and therefore approach each other on opposite strands when converging at termination (Ali et al., 2016; Costa et al., 2011; Fu et al., 2011). Interestingly, however, recent reports suggest that large protein barriers on the lagging strand can indeed slow down progression of the fork (Duxin et al., 2014; Langston and O'Donnell, 2017). Does the approaching neighbour replisome, which is on the lagging strand, not present such a barrier? Is there an active mechanism regulating the smooth passage of the replisomes? Or are the replisomes idling at the edge of the barrier, in the attempt to unwind it, especially prepared to deal with barriers laying ahead and hence better at passing each other smoothly? More work is needed to answer these questions.

The results presented by Dewar et al. also suggest, at least in the context of the plasmid template, that torsional stress building up ahead of the forks does not slow down fork convergence (Dewar et al., 2015) (see also below for role of topoisomerases) (Figure 2B). The removal of proteins (nucleosomes) ahead of the fork could not be directly addressed in this setup due to the artificial "clearing up" of chromatin ahead of the fork due to removal of the *lac* array. Interestingly, the reconstitution of eukaryotic DNA replication *in vitro* with purified budding yeast proteins revealed in fact that nucleosomal packaging does appear to inhibit replication termination. As the elongation stage of the

reaction was efficient, but termination alone was blocked, it suggests that the termination stage may be especially sensitive to the presence of chromatin structure (Devbhandari et al., 2017) (Figure 2A).

6. The completion of DNA synthesis

Data provided by Dewar at al, suggest that leading strand DNA is replicated up to a few bases away from the end of the last Okazaki fragment of the encountered lagging strand (Figure 2D and E). There is no evidence for persistent gaps between these strands upon termination (Dewar et al., 2015). These data, however, do not explain which polymerase carries on synthesis of last fragments of DNA and maturation of the last Okazaki fragment. The RNA-DNA primer of each Okazaki fragment on the lagging strand is removed by concerted action of DNA Polδ and Fen1 endonuclease (reviewed in (Balakrishnan and Bambara, 2013)). DNA Polδ can support strand-displacement re-synthesis of the DNA previously synthesised by Polα and in doing so can progress until it encounters the nucleosome or another DNA binding protein, both of which are efficiently repositioned behind the replication fork (Smith and Whitehouse, 2012). Interestingly, fragments of DNA synthesised by Polα can be detected in mature genome mostly at the junctions of Okazaki fragments, usually at the nucleosome midpoint (dyad position). In total about 1.5% of mature genome was shown to be synthesised by Polα (Reijns et al., 2015).

Is the last Okazaki fragment matured by DNA Polε? The holoenzyme of DNA Polε is unable to carry on extended strand displacement synthesis in *in vitro* reconstitution experiments, unless its 3'-5' exonuclease activity is removed, and it cannot mature Okazaki fragments on lagging strand (Devbhandari et al., 2017; Ganai et al., 2016). However, DNA Polε in the context of the replisome tightly associates with the CMG complex through the Dpb2 subunit of Polε and GINS and forms a functional unit (Langston et al., 2014; Muramatsu et al., 2010; Sengupta et al., 2013). A recent negative-stain electron microscopy reconstruction of a CMG-Polε complex visualised the close association of this complex (Pellegrini and Costa, 2016; Sun et al., 2015) and we found that the

post-replication replisome in both *C.elegans* and *X.laevis* interacts with Polε and not Polδ (Sonneville et al., 2017). This interaction of Polε with the replisome likely acts as additional processivity factor for Polε, in addition to action of PCNA (Kang et al., 2012; Langston et al., 2014; Yeeles et al., 2017). It would be interesting to investigate Polε strand-displacement activity in the context of the replisome. In support of the Polε role at termination, analysis of the genome-wide location of ribonucleotides incorporated into DNA by mutants of Polδ and Polε especially prone to such mis-incorporations discovered a substantial bias toward Polδ proximal to origins which declined toward the centre of the replicons where Polε synthesis was more evident (Daigaku et al., 2015). This would suggest that Polε carries out the replication at sites of termination.

Can Polε mature the last Okazaki fragment? Can it sustain strand displacement synthesis when supported by both PCNA and the CMG? It remains to be unravelled. Importantly, DNA Polε on its own does not interact with Fen1 (Garg et al., 2004), therefore another processing mechanism would be required to complete maturation of the last Okazaki fragment, unless Fen1 is brought in by a different component of the terminating replisome. Alternatively, Polε can slide along the last Okazaki fragment together with the post-termination replisome, making room for Polδ to displace and mature the last RNA/DNA primer. Much is to be discovered about the ability of the terminated CMG to move away from the termination site especially in the context of re-established nucleosomes. However, Polδ has been shown previously to play a role in leading strand synthesis *in vivo* (Daigaku et al., 2015; Johnson et al., 2015; Waga et al., 2001). Moreover, recent data obtained from the budding yeast *in vitro* reconstitution system of replication using purified proteins revealed that polymerase switching may be more common than expected. Polδ can play an important role in establishing leading-strand synthesis (Yeeles et al., 2017) and Polδ assembled at the leading strand was shown to be displaced if Polε was added after DNA synthesis has initiated (Georgescu et al., 2014). More research is required to show which of the polymerases finishes the replication job.

7. Role of topoisomerases during DNA replication termination

The ability of topoisomerases to act ahead of the replication forks becomes very limited as two replication forks converge (Sundin and Varshavsky, 1980). In this circumstance, fork rotation and precatenation become the primary pathway of DNA relaxing ahead of the fork. Catenated, double stranded DNA (intertwined sister chromatids) can only be resolved by type II topoisomerases (Topo II, *S.cerevisiae* Top2) (Figure 2B). Experiments with Topo II inhibitors in *Xenopus* egg extract showed that Topo II can be trapped behind, but not in front of the forks, and resolves replication intermediates in a non-redundant manner with Topo I (Hyrien, 2009; Lucas et al., 2001). Interestingly, Top2 depletion in yeast does not stop cells from completing DNA replication, nor passing through mitosis, although they do dramatically mis-segregate and break their chromosomes due to sister chromatid catenation. On the other hand, inhibition of Top2 enzymatic activity in a way that Top2 is still able to bind DNA but unable to catalyse strand breakage, causes incomplete DNA replication and induces G2/M cell cycle arrest (Baxter and Diffley, 2008). Similarly, inhibition of Topo II activity in higher eukaryotes with the small molecule inhibitor ICRF-193 was shown to block termination of DNA replication in *Xenopus* egg extract and induce G2 arrest in human cells without the high level of DNA strand breaks associated with Topo II poisons (Cuvier et al., 2008; Downes et al., 1994; Skoufias et al., 2004). ICRF-193 traps Topo II on the DNA in the form of a non-covalent intermediate named the closed clamp (Roca et al., 1994). It is unclear therefore whether replication termination defects observed upon addition of ICRF-193 to *Xenopus* egg extracts is due to inhibition of Topo II activity or some other effect of the closed clamps, such as changes to nucleosome spacing and chromatin structure (Gaggioli et al., 2013; Germe and Hyrien, 2005).

In agreement with the role of Topo II in replication fork termination, post-termination replisomes from *C. elegans* and *X. laevis* contain Topo II, unlike the budding yeast Replisome Progression Complex, which represents active helicase and contains Top1 (Gambus et al., 2006; Sonneville et al., 2017). Moreover, Dewar *et.al.* reported that site-specific termination plasmids (described above) require Topo II for decatenation of daughter plasmids, but Topo II activity is not

needed for fork convergence and DNA ligation (Dewar et al., 2015) (Figure 2B and F).

8. Replisome disassembly

The data presented by Dewar at al. suggest that the dissolution of the replisome in the plasmid-based system is the last stage of replication fork termination, executed after ligation of leading and lagging strands (Dewar et al., 2015)(Figure 2E). Work in budding yeast and *Xenopus laevis* egg extract discovered the first elements of this dissolution mechanism, which was found to be a highly evolutionary conserved process (Maric et al., 2014; Moreno et al., 2014)(Figure 3A and B). In both model organisms the Mcm7 subunit of the CMG complex becomes polyubiquitylated when forks terminate. The ubiquitin chains attached to Mcm7 are linked through lysine 48 (K48) but ubiquitylated Mcm7 is not degraded directly on chromatin as inhibition of proteasomal activity does not inhibit CMG disassembly. Instead, a protein remodeller Cdc48 (p97, VCP, segregase) recognises the ubiquitylated CMG and through its ATPase activity removes the CMG complexes from chromatin (Maric et al., 2014; Moreno et al., 2014). It is unclear at present whether the ubiquitylated Mcm7 is degraded upon removal from chromatin or de-ubiquitylated. A recent report by Fullbright *et al*. suggests that during unperturbed DNA replication in *Xenopus* egg extract ubiquitylated Mcm7 is likely to be de-ubiquitylated (Fullbright et al., 2016). Interestingly, ubiquitylation of human Mcm7 (both endogenous and exogenously expressed in cells) was reported in the past, but the fate of the ubiquitylated form of Mcm7 and the function of the ubiquitylation was not clear (Buchsbaum et al., 2007; Kuhne and Banks, 1998).

8.1 SCF^{Dia2} ubiquitin ligase in budding yeast.

In budding yeast the ubiquitin ligase, which ubiquitylates Mcm7 is SCF^{Dia2} (Maric et al., 2014). SCF^{Dia2} is a multisubunit ligase built around a Cdc53 cullin scaffold (homologue of Cullin 1 in higher eukaryotes) (Figure 3A). Dia2 is the substrate specific receptor, F-box protein, which binds through the substrate adaptor (Skp1) to the N-terminal part of Cdc53. The C-terminus of Cdc53, on the

other hand, binds RING domain factor Hrt1, connecting the ligase to the ubiquitin conjugating enzyme (E2) Cdc34 (SCF = **S**kp1+**C**ullin1+**F**-box) (Figure 4B). SCF^{Dia2} was shown to be essential for Mcm7 ubiquitylation, specifically in the context of CMG during S-phase – both *in vitro* and *in vivo*. Moreover cells lacking Dia2 (*dia2Δ*) retain CMG complexes on chromatin after S-phase until the next G1 stage of the cell cycle (Maric et al., 2014). Not surprisingly budding yeast cells lacking Dia2, although viable, are defective in S-phase progression and present high rates of endogenous DNA damage and genome instability. They are also unable to grow at low temperatures and are sensitive to DNA-damaging agents that affect replication fork progression (Blake et al., 2006; Koepp et al., 2006).

Dia2 contains a protein-protein interaction N-terminal tetratricopeptide repeat (TPR) domain, nuclear localisation signal (NLS), an F-box that connects it to the rest of the SCF ligase and a C-terminal substrate recognition domain comprising of leucine-rich repeats (LRR). The TPR domain of Dia2 was shown to interact with Mrc1 and Tof1 components of the replisome progression complex (RPC) built around the CMG helicase (Gambus et al., 2006; Morohashi et al., 2009). As a result, Dia2 was detected interacting with RPC in S-phase and this interaction was preserved when cells were treated with hydroxyurea (HU) to stall progressing replication forks (Morohashi et al., 2009). Interestingly cells lacking the TPR domain within Dia2 (*dia2-ΔTPR*) do not present the severe phenotype of *dia2Δ* cells – with the exception of synthetic lethality with rrm3Δ (a helicase supporting passage of forks past protein-DNA barriers). Cells lacking the TPR domain in Dia2 were, however, shown consequently to have a partial defect in Mcm7 ubiquitylation and CMG disassembly (Maculins et al., 2015). It seems that attaching SCF^{Dia2} to the replisome via the TPR domain increases the efficiency of CMG ubiquitylation. It may not be essential for normal CMG disassembly as the LRR domain can still recognise its substrate even without the tethering, but there may be situations when this stabilised interaction with the replisome is more vital – for example when forks struggle to pass DNA-protein barriers in the absence of Rrm3.

8.2. $CRL2^{Lrr1}$ ubiquitin ligase in higher eukaryotes

Recent research from our and two other groups discovered that in higher eukaryotes the ubiquitin ligase ubiquitylating Mcm7 at termination of replication forks is not an SCF but a Cullin2-based ubiquitin ligase with a Leucine Rich Repeat 1 protein (Lrr1) as a substrate receptor (**C**ullin-**R**ing **L**igase 2 with Lrr1 = $CRL2^{Lrr1}$)(Dewar et al., 2017; Sonneville et al., 2017) (Figure 4 C). Both in *Xenopus* egg extract and in *C.elegans* embryos, inhibition or downregulation of Cullin 1 ligase activity did not influence Mcm7 ubiquitylation nor helicase disassembly during S-phase ((Sonneville et al., 2017) and our unpublished data). On the other hand siRNA downregulation of CUL-2 / LRR-1 complex in *C.elegans* embryos and immunodepletion of $CRL2^{Lrr1}$ in egg extract blocked both phenotypes (Dewar et al., 2017; Sonneville et al., 2017) (Figure 3B). $CRL2^{Lrr1}$ was also shown to be the only cullin type ubiquitin ligase that interacts with post-termination replisomes in *Xenopus* egg extract and *C.elegans* embryos and accumulates at the sites of termination in plasmid-based termination system described above (Dewar et al., 2017; Sonneville et al., 2017). Importantly, both studies found that $CRL2^{Lrr1}$ interacts specifically with terminating CMG and not with actively unwinding helicase nor double Mcm2-7 hexamers of dormant origins. The regulated binding of $CRL2^{Lrr1}$ to post-termination replisome represents therefore the first known step of replisome disassembly. Finally, the ubiquitin ligase activity of $CRL2^{Lrr1}$ is necessary for the Mcm7 ubiquitylation and helicase disassembly, as a mutant of Cul2-Rbx1 complex, which cannot be activated by neddylation, is unable to rescue the $CRL2^{Lrr1}$ immunodepleted egg extract unlike a wild type fully functioning complex (Sonneville et al., 2017).

What is $CRL2^{Lrr1}$? Previous work has shown that *C.elegans* LRR-1 is an essential gene (Piano et al., 2002). LRR-1 is required for embryonic development but maternal rescue allows analysis of *lrr-1* loss of function in adult tissues. *lrr-1* mutants are sterile owing to severe defects in germ cell proliferation (Merlet et al., 2010; Starostina et al., 2010). Inactivation of *lrr-1* induces DNA damage, which may arise due to DNA re-replication problems (ssDNA/RPA-1 foci accumulate in *lrr-1* germ cells, which also contain greater than 4N DNA content). This in turn leads to hyperactivation of ATL-1/CHK-1 pathway (ATR/Chk1 pathway in vertebrates), which delays mitotic entry and results in embryonic

lethality. Inactivation of ATL-1/CHK-1 checkpoint components supresses the proliferation defect and fully restores *lrr-1* mutant fertility (Burger et al., 2013; Merlet et al., 2010). How the re-replication / DNA damage is induced in *lrr-1* worms is not as yet determined. Interestingly, an RNAi-based suppressor screen of *lrr-1* and *cul-2* mutants identified two genes encoding components of the GINS complex, as well as CDC-7 and MUS-101, which are needed for CMG activation (Ossareh-Nazari et al., 2016). These data suggest that reducing CMG levels on chromatin can supress the DNA damage created in *lrr-1* mutants and supress their lethality. This is in agreement with LRR-1's role in Mcm7 ubiquitylation as lower levels of CMG on chromatin would compensate for a defect in CMG unloading.

On the other hand, another study found that *C.elegans lrr-1* mutants germ cells arrest with 2C DNA content, which may be due to accumulation of CDK inhibitor CKI-1 as deletion of one copy of CKI-1 or *cki-1* RNAi treatment can rescue *lrr-1* mutant germ cells numbers. In support of the CUL-2 / LRR-1 role in targeting CKI-1 for degradation, study in human cells found that overexpressed CKI-1 was degraded faster when LRR-1 was also overexpressed (Starostina et al., 2010). Interestingly, LRR1 or CUL2 knockdown in HeLa cells did not induce a strong cell cycle arrest and LRR1 was shown to be important to regulate levels of cytoplasmic p21 (human CKI) to control actin cytoskeleton remodelling (Starostina et al., 2010). Further studies are required to analyse in depth the role of LRR1 in human cells and the interplay between different substrates of this ubiquitin ligase.

Several questions remain – what is the signal for polyubiquitylation of Mcm7 and removal of helicase? How are CMGs protected from ubiquitylation during elongation and efficiently ubiquitylated at termination (Figure 2)? Dewar et al hypothesise that it may be conformational changes within CMG upon transition from encircling single-stranded DNA to double-stranded DNA of last Okazaki fragment that provide this post-termination specificity (Dewar et al., 2015). In support of this hypothesis, it was shown that CMG is indeed able to slide on double-stranded DNA (Kang et al., 2012).

We should also keep in mind that many substrate specific receptors of CRLs recognise their substrates only when they are post-translationally modified e.g. F-box receptors of SCF often recognise phosphorylated proteins, VHL interacting with CRL2 recognises Hif1α upon its hydroxylation. It is possible therefore that terminating CMG is first modified in a yet undiscovered manner before being ubiquitylated. Budding yeast Mcm2-7 complex has been recently shown to be SUMOylated upon loading at origins in G1 stage of cell cycle before Mcm2-7 phosphorylation. The level of Mcm2-6 SUMOylation decreases during S-phase as MCM becomes phosphorylated and activated, with exception of Mcm7, which SUMOylation was retained during S-phase (Wei and Zhao, 2016). Additionally, deubiquitylating enzyme Usp7 was described recently as a SUMO-specific DUB, removing ubiquitin from SUMOylated proteins and maintaining high SUMO / low ubiquitin ratio at replication forks (Lecona et al., 2016; Lopez-Contreras et al., 2013). A theory was therefore proposed that SUMO-driven ubiquitylation could act as a signal for the termination of DNA replication (Lecona and Fernandez-Capetillo, 2016). Usp7 was also previously shown to interact with MCM binding protein MCM-BP and to cooperate with it to unload the Mcm2-7 complexes from chromatin at the end of S-phase (Jagannathan et al., 2014; Nishiyama et al., 2011). Is Usp7 DUB activity for SUMOylated proteins linked with its MCM-BP interaction? Is Mcm7 in higher eukaryotes modified by SUMO? Is SUMOylation of Mcm7 regulating its ubiquitylation at termination events? More work is needed to understand fully this complex process.

Another possibility in need of investigation is involvement of priming ubiquitin ligase. Indeed ARIH1, an Ariadne family Ring-Between-Ring (RBR) ubiquitin ligase, was shown recently to interact with a number of CRLs including CRL2s and prime their substrates (Scott et al., 2016). It is probable therefore, that such a priming ligase recognises the terminating helicase and $CRL2^{Lrr1}$ only acts on primed substrate.

8.3. The role of p97 segregase in replisome disassembly

p97, also known as VCP in metazoans, CDC-48 in *C.elegans*, Cdc48 in yeast and Ter94 in insects, is a ubiquitin-dependent segregase that plays a central role

in the regulation of protein homeostasis. Once bound to ubiquitylated substrates, this conserved hexameric AAA+ ATPase utilises the energy released from ATP hydrolysis to undergo a conformational change across its hexamer structure called interprotomer motion transmission mechanism (Huang et al., 2012; Li et al., 2012). This movement allows p97 to remove substrates from different cellular locations and complexes, likely by substrate translocation through p97's narrow central pore (Tonddast-Navaei and Stan, 2013). The separated or unfolded substrates can then be directed to the proteasome and degraded or de-ubiquitylated and recycled with the help of DUBs associating with p97. p97 carries on this segregase/unfoldase activity on a myriad of substrates participating in a large variety of cellular processes. Not surprisingly, knockdown of both p97 alleles causes early embryonic lethality in mice and siRNA-depletion of p97 in cells causes apoptosis (Muller et al., 2007; Wojcik et al., 2004).

The interaction of p97 with its many different substrates is mediated by a group of about 30 adaptor proteins that specifically recruit ubiquitylated proteins (Meyer et al., 2012; Yeung et al., 2008). The cofactors usually bind to the N-terminal domain of p97 using p97 interacting motifs. The best characterised major p97 cofactors include Ufd1/Npl4 heterodimer and p47, which bind to the p97 in mutually exclusive manner (Bruderer et al., 2004). Further, minor cofactors such as FAF1 or UBXD7 can then associate to the p97 complex with a major cofactor (Hanzelmann et al., 2011). Some of the cofactors, such as UBXD7, can also interact with various ubiquitin ligases and streamline the process of ubiquitin-dependent substrate removal/degradation (reviewed in (Meyer et al., 2012)).

The role of p97 during DNA replication was first suggested in *C.elegans* embryos. RNAi-mediated depletion of the CDC-48 complex lead to a defect in cell division: mitotic entry was delayed as a result of the activation of the DNA damage checkpoint. The severe chromatin defects observed in embryos as well as mitotic cells of the gonads included mitotic bridges and accumulated foci of RAD-51 DNA repair protein. Moreover, embryos lacking CDC-48, UFD-1 or NPL-4 are strongly reduced in DNA content (Deichsel et al., 2009; Mouysset et al., 2008). It was subsequently shown that embryos lacking CDC-48 or UFD1/NPL-4

cofactors accumulate origin licensing factor CDT-1 on mitotic chromatin and present persistent chromatin association of CDC-45/GINS after S-phase is completed (Franz et al., 2011). This process involves another p97 cofactor UBXN-3/FAF1 (Franz et al., 2016). Interestingly, inhibition of CDT-1 degradation and its accumulation on chromatin in embryos lacking CDC-48 or UFD1/NPL-4 does not lead to re-replication phenotype in these embryos but rather a strong reduction in their DNA content.

In the case of replisome disassembly, the segregase function was shown to be essential to disassemble ubiquitylated post-termination CMG in budding yeast, *C.elegans* embryos and *Xenopus* egg extract (Maric et al., 2014; Moreno et al., 2014; Sonneville et al., 2017). The ATPase activity of p97 is essential for this disassembly function as the replisome can be blocked on chromatin when two ATPase domains of p97 (D1 and D2) are mutated or the activity of p97 is blocked with a small molecule inhibitor NMS973 (Dewar et al., 2017; Moreno et al., 2014; Sonneville et al., 2017). This replisome disassembly defect phenotype is not driven through Cdt1 de-regulation, nor represents novel binding of GINS/Cdc45 to mitotic chromosomes (Moreno et al., 2014; Sonneville et al., 2017). In worm embryos, RNAi directed inactivation of *ufd-1* and *npl-4* leads to a defect in replisome unloading and the Ufd1/Npl4 heterodimer is found to interact with the post-termination replisome in *Xenopus* egg extracts (Dewar et al., 2017; Sonneville et al., 2017). Moreover, plasmids with accumulated terminating forks contain enriched Ubxn7 and Dvc1/SPRTN bound to them (Dewar et al., 2017). Future work will show whether these additional co-factors play a role in replisome disassembly.

8.4. Back-up pathway for replisome disassembly

Importantly, work in *C.elegans* embryos revealed that if the removal of CMG complexes is not accomplished during S-phase due to defective $CRL2^{Lrr1}$ then they can be removed from chromatin at the beginning of mitosis, in late prophase (Sonneville et al., 2017)(Figure 3C). This back-up mitotic pathway of replisome disassembly also requires p97/Ufd1/Npl4 (worm CDC-48/UFD-1/NPL-4) segregase, but to accomplish it p97 requires yet another cofactor: Fas-associated factor 1 FAF1 (worm UBXN-3) (Sonneville et al., 2017). FAF1 is an

evolutionarily conserved proapoptotic factor that contains multiple protein-interaction domains: ubiquitin-associated UBA, ubiquitin-like UBL1 and UBL2, Fas-interacting domain FID, death effector domain-interacting domain DEDID, Ubiquitin-associated UAS, ubiquitin regulatory X UBX (Lee et al., 2013; Menges et al., 2009). FAF1 is an essential gene (Adham et al., 2008), an established modulator of apoptosis, regulates NFκB and is involved in ubiquitin-mediated protein turnover (reviewed in (Menges et al., 2009)). FAF1 was also shown to bind to p97-Ufd1-Npl4 complex via the UBX domain and polyubiquitylated proteins via the UBA domain to promote endoplasmic reticulum associated degradation ERAD (Lee et al., 2013). Finally, recent work from the Thorsten Hoppe lab showed that FAF-1/UBXN-3 is required for cell cycle progression in *C.elegans* embryo due to the problem with CDT-1 degradation and its inappropriate maintenance on chromatin during mitosis, together with CDC-45 and GINS (Franz et al., 2016). Moreover, Franz *et al*. has shown that downregulation of FAF1 by siRNA in human cells causes a pronounced replication stress phenotype: defective fork progression, fork stalling, dormant origin firing and activation of both S-phase checkpoint (ATR/Chk1) and DNA damage checkpoint (ATM/Chk2) (Franz et al., 2016). It remains to be investigated whether this observed replication stress is the result of Cdt1 induced re-replication, a defect in unloading of the post-termination replisomes or one of the many other FAF1 functions.

Intriguingly, the back-up mitotic pathway of replisome disassembly in *C.elegans* embryos is modulated by the activity of the SUMO protease ULP-4: co-depletion of ULP-4 with LRR-1 delayed the release of CMG components from chromatin (Sonneville et al., 2017). ULP-4 is a major mitotic SUMO protease in worms and is present at mitotic chromosomes and at the spindle midzone (Pelisch et al., 2014). The ULP-4 analogous proteases in human cells are SENP6-7. It remains to be unravelled whether SUMO plays a regulatory role in the back-up process or whether ULP-4 functions in another way e.g. by bridging some important interactions and allowing p97 complex recruitment. It would be very interesting to investigate the existence of such a potential back-up pathway in human somatic cells.

9. The importance of faultless termination

Does deregulation of termination contribute to genomic instability and human disease? Cancer chromosomal instability (CIN) is observed in most solid tumours and is associated with poor prognosis and drug resistance (McGranahan et al., 2012). CIN leads to increased rate of changes in chromosomal numbers and structure, and generates intra-tumour heterogeneity. Recent data implicate a central role for replication stress in the generation of CIN (Burrell et al., 2013). Can faulty termination provide a source of replication stress, which then contributes to the generation of genomic instability and CIN? What are the ways in which problems during replication fork termination could lead to genomic instability? At present we have restricted experimental data on consequences of problems with replication fork termination but we can speculate based on what we know.

We know that failure to decatenate newly replicated sister chromatids upon termination of replication forks does not tend to be detected by G2/M checkpoint but leads to dramatic missegregation of chromosomes during mitosis (Baxter and Diffley, 2008). What about other stages of termination process?

What would happen if forks cannot converge properly? What if their passing each other at the termination stage is blocked? We can imagine that problems during convergence of replication forks could lead to similar torsional stresses as these created by lack of Topoisomerase I during elongation. Inhibition of Topo I activity in human cells, mouse embryonic fibroblasts and *Xenopus laevis* egg extract frequently induces replication fork reversal (reviewed in (Neelsen and Lopes, 2015)). Fork reversal can have physiological roles during replication but can also have pathological consequences, contribute to genome instability in neurogenerative syndromes and cancer. A small but reproducible number of reversed forks was detected also in various unchallenged human cell lines, whilst deregulation of poly(ADP-ribose) metabolism, which regulates fork reversal and restart, induces high level of reversed forks even in the absence of genotoxic replication stress (reviewed in (Neelsen and Lopes, 2015)). Fork reversal is also very frequent in mouse embryonic stem cells (Ahuja et al., 2016). Where do these reversed forks come from? Could problems with termination of

replication forks be one of the sources of such reversed forks? Interestingly, transient over-replication, fork reversal and end-processing by exonucleases were recently associated with completion of replication termination in *E.coli* (Wendel et al., 2014). More research and visualisation of converging forks either unchallenged or upon termination perturbations is needed to elucidate the possibility of fork reversal at sites of troubled replication fork termination.

Can failure to complete DNA synthesis at termination sites create genome instabilities? It has been shown recently, that not all of the DNA is always replicated in human cells during S-phase – unreplicated segments resulting from double fork stalling in large replicons are frequently present in G2. They can be partially resolved during mitosis, create ultrafine bridges during segregation in mitosis and are subsequently recognised in the G1 stage of the cell cycle by DNA repair protein p53-binding protein 1 (53BP1) to be resolved in this new cell cycle (Moreno et al., 2016). Failure to complete of DNA synthesis at termination sites would likely lead to a similar scenario.

What about inhibiting disassembly of the replisome? This is the part of the termination process that we understand best at present. If disassembly of the replisome constitutes the last step of replication termination, then the failure to remove it should not leave unligated DNA nor unusual DNA structures (Dewar et al., 2015). It would leave however a DNA helicase on a DNA substrate. Tested on synthetic *in vitro* substrates CMG can translocate on double stranded DNA and then start unwinding DNA if a fork structure is present (Kang et al., 2012). One can imagine therefore that the second-to-last Okazaki fragment, which may be in a mid-maturation stage with a flap created by Polδ, could be such a substrate for the approaching post-termination CMG to start de-novo unwinding. In bacteria, recent data suggest that in termination zones 3' ssDNA flaps are created that, if not removed by RecG nuclease (in RecG mutants), can provide substrates for *de novo* replication, leading to re-replication and creating pathological DNA structures. Tus termination sequences limit the extent of such re-replication initiated in termination zones (Rudolph et al., 2013). What about eukaryotic cells? They do not have Tus terminating sequences. Can faulty termination of replication forks initiate re-replication?

Moreover, CMG complexes left behind on chromatin would disturb proper chromatin re-establishment and pose a problem to processes for which DNA is a substrate, such as transcription and next replication. As mentioned above, CMGs can translocate on double stranded DNA (Kang et al., 2012), by moving along DNA they could displace other proteins bound to DNA. At present we do not know whether CMG sliding on dsDNA can displace nucleosomes or if they will be trapped by them.

A final potential problem arising from lack of efficient disassembly of the CMG complexes at the termination of replication forks is entrapment of Cdc45 and GINS within these post-termination complexes. Cdc45 was shown to be a rate limiting factor for DNA replication in mammalian cells. It was proposed that regulated expression levels of Cdc45 enforces reutilisation of existing Cdc45 during S-phase, which in turn can limit and stagger origin activation throughout the S-phase (Kohler et al., 2016; Wong et al., 2011). A lack of Cdc45 available for recycling can therefore potentially slow S-phase progression and inhibit DNA synthesis. Primary untransformed human cells with reduced levels of GINS components present all the phenotypes of replication stress and accumulation of DNA damage (Barkley et al., 2009). Future studies of replisome disassembly in human somatic cells is essential to shed light at this possibility as so far this process was investigated only in embryonic systems (*Xenopus laevis* egg extract and *C.elegans* embryos) which have higher levels of Cdc45 and GINS.

Is there experimental evidence that faulty disassembly of the replisome can lead to genome instability? *S. cerevisiae* cells lacking Dia2, which are unable to remove post-termination CMG from chromatin, are viable but present very high levels of genomic instabilities (described above). LRR-1 – the CRL2 Mcm7 specific receptor in higher eukaryotes is an essential gene in *C.elegans*, most likely due to also other than Mcm7 substrates, as CMG becomes unloaded by a back-up system in *lrr-1* embryos. However, partial downregulation of LRR-1 together with downregulation of the back-up pathway factors: FAF-1/UBXN-3 or ULP4 results in synthetic lethality, suggesting that inhibition of CMG removal by partial blocking of both pathways results in non-viable worms (Sonneville et al., 2017). FAF1 itself is a factor often downregulated or mutated in multiple cancers. It may be its proapoptotic function that drives this downregulation, but

in consequence these cancers could exhibit higher levels of genomic instability due to their replication fork termination problems. It is crucial therefore that we investigate the process of replisome disassembly in human cells to confirm its analogy.

Factors that drive replication initiation and the assembly of CMG, such as Cdc7 kinase and TopBP1 (Cut5) initiation factor, are currently being explored as potential anti-cancer therapy targets in tumours that present defects in chromosome replication (Chowdhury et al., 2014; Montagnoli et al., 2010). Can CMG disassembly also serve as a potential target for future therapies? Could we target the S-phase pathway of CMG disassembly in cancers with mutated or downregulated FAF1? For this we need to understand the CMG disassembly process in much more detail and crucially confirm its conservation in human cells. It seems likely that ubiquitylation is rate limiting for CMG disassembly, although it needs to be demonstrated by mapping the ubiquitylation sites and creating an un-modifiable mutant. It is clear, however, that Mcm7 ubiquitylation is regulated in a precise fashion on many levels, both spatially and temporally.

Finally, many factors implicated in DNA replication fork termination and replisome disassembly, such as p97 segregase and Usp7 are also targets of small molecule inhibitors used or being tested for antitumour therapies (Magnaghi et al., 2013; Reverdy et al., 2012). A better understanding of CMG disassembly pathway and replication fork termination in human cells might help us to explain the mode of action of these inhibitors in clinic.

Figure legends

Figure 1. Topoisomerases at replication fork. Topo I relaxes the positive supercoiling building up ahead of the fork. Sometimes this supercoiling can lead to rotation of the fork and intertwining of the daughter strands of DNA behind the fork (precatenates). These are resolved by Topo II.

Figure 2. Model of termination of eukaryotic replication forks. When two neighbouring replication forks approach each other from opposite directions, all of the proteins organising DNA in between the forks (nucleosomes and others)

have to be removed, while Topo I relaxes the torsional stress (positive supercoiling) (A). When two terminating forks converge the supercoiling of DNA between them cannot be resoled by Topo I due to lack of space for it to act. Instead, terminating forks depend on transmission of this torsional stress behind the forks creating precatenates resolved by Topo II (B). During convergence two replisome approach each other moving on opposite strands of DNA (leading strand of each fork) (C). The replisomes can pass each other and most likely CMG slides onto the double stranded DNA of last Okazaki fragment (D). The synthesis of DNA needs to be completed, the last Okazaki fragment matured and DNA ligated. Replisome is then ubiquitylated and removed by p97/VCP/Cdc48 segregase (E). Intertwined sister chromatids need to be resolved by Topo II (F). The final product: two individual sister chromatids with reconsitituted chromatin structure (G).

Figure 3. Model of replisome disassembly at the termination of replication forks. In budding yeast *S.cerevisiae* the Mcm7 subunit of the terminating replisome is ubiquitylated by SCF^{Dia2} and removed from chromatin by Cdc48 segregase (A). In *Xenopus* egg extract and *C.elegans* embryos $CRL2^{Lrr1}$ ubiquitylates Mcm7 during termination of replication forks and CDC-48/p97 segragase removes it from chromatin with help of Ufd1/Npl4 cofactors (B). If the mechanism of removal of the replisome during termination of forks in S-phase does not work *C.elegans* embryos have a back-up mechanism removing replisomes in prophase in mitosis. This mechanism requires CDC-48/p97 and Npl4/Ufd1 but also UBXN-3/FAF1 cofactor and is regulated by ULP-4/Senp6,7 (C).

Figure 4. Model of cullin ligases ubiquitylating Mcm7 during termination of replication forks. General model of organisation of cullin family members (A). Model of SCF^{Dia2} ubiquitylating Mcm7 in *S.cerevisiae* (B). Model of $CRL2^{Lrr1}$ ubiquitylating Mcm7 in *C.elegans* embryos and *Xenopus* egg extract (C).

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Figure 1. Gambus

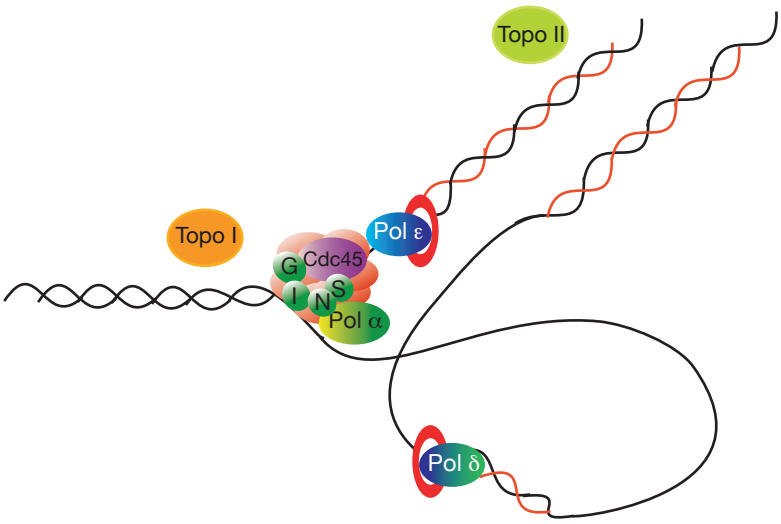


Figure 2. Gambus

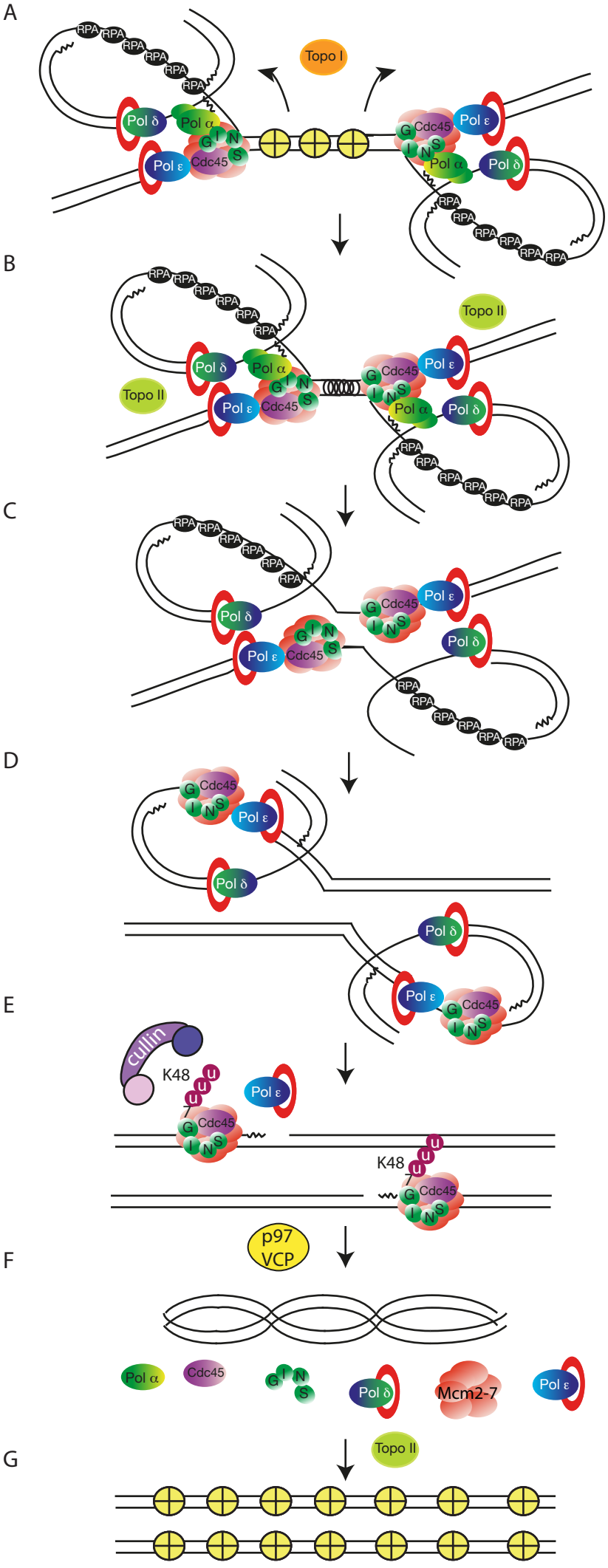


Figure 3. Gambus

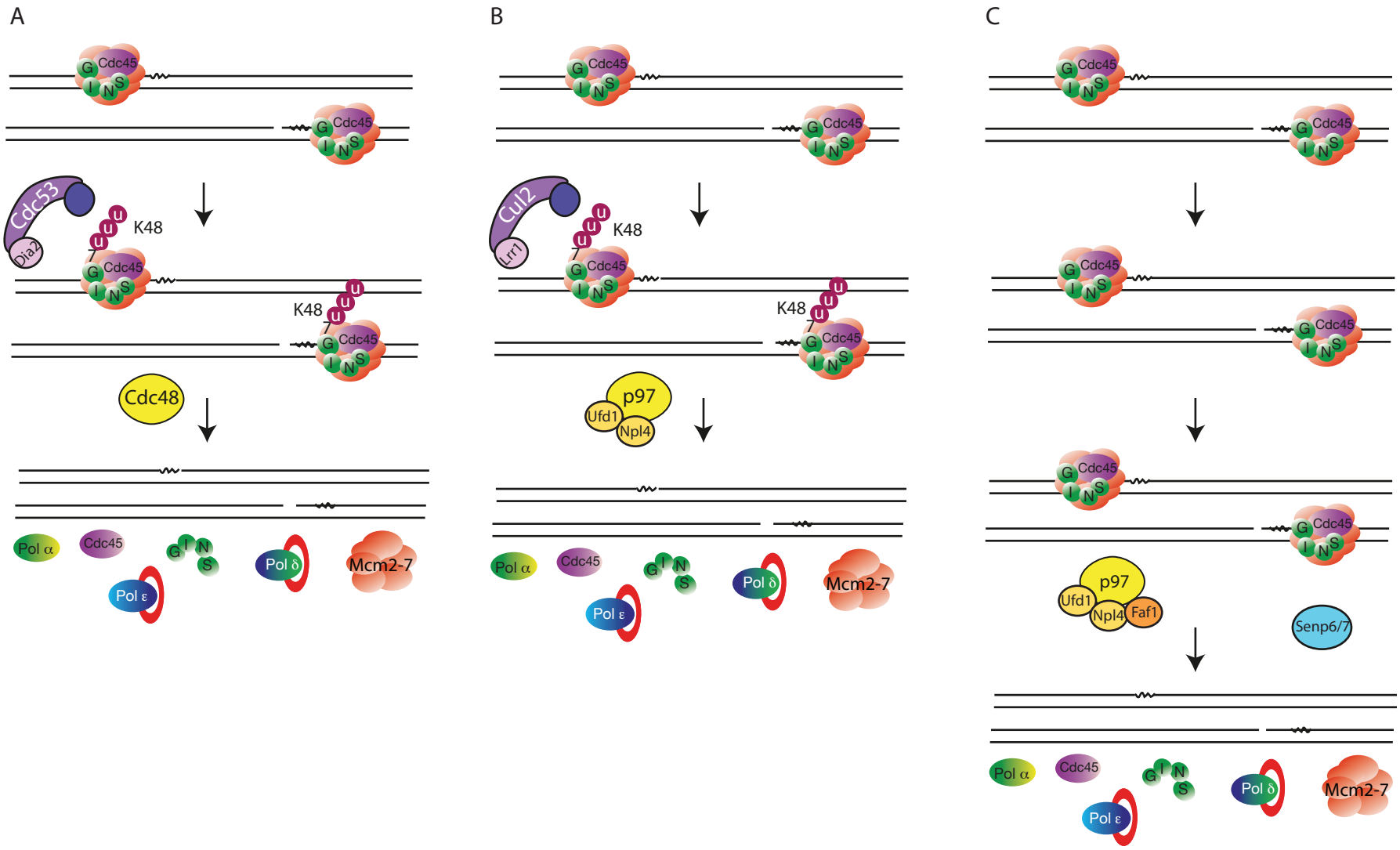
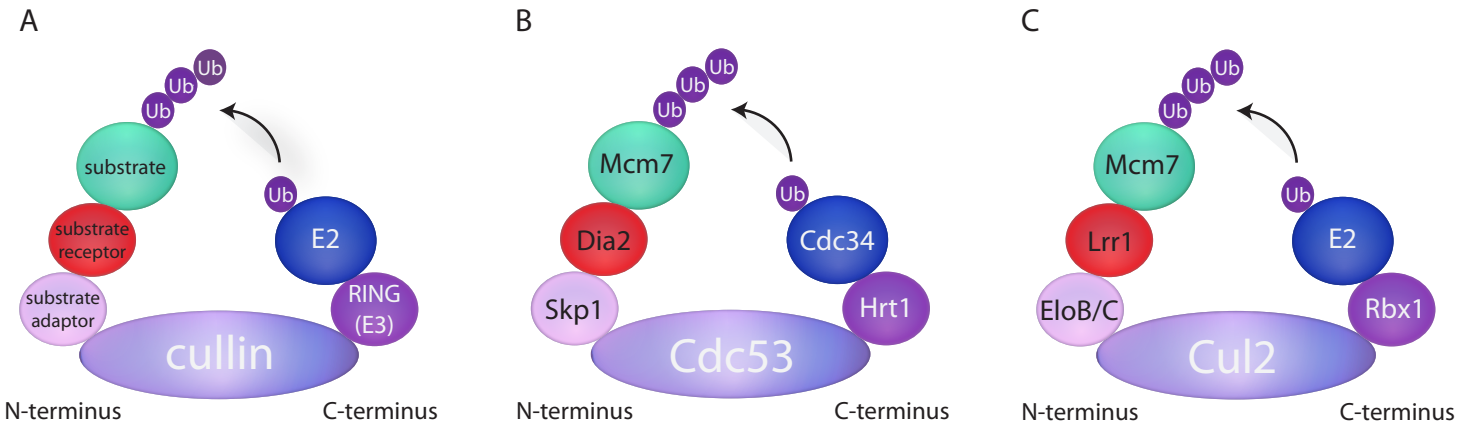


Figure 4. Gambus



cullin E3 ligase complexes
in higher eukaryotes

S. cerevisiae
SCFDia2 complex

metazoan
$CRL2^{Lrr1}$ complex