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The many facets of the Tim-Tipin protein families' roles in chromosome biology

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Failures in DNA replication are a potent force for driving genome instability. The proteins which form the replisome, the DNA replication machinery, play a fundamental role in preventing replicative catastrophes. The Tim (TIMELESS/TIMEOUT) and Tipin proteins are two conserved replisome associated proteins which have functions in preventing replication fork collapse and replicative checkpoint signalling in response to factors which slow the progression of the replisome. Intriguingly, TIMELESS family members have been implicated in the regulation of the biological clock, giving a tantalising pointer to a possible link between DNA replication and circadian rhythm control. Here we report on our current understanding of the many facets of these protein families in maintaining genome stability and replication checkpoint control.

Key words: DNA replication, recombination, checkpoints, timeless, tipin, genome stability

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DNA replication is required for living cells to divide. The DNA replication machinery must replicate DNA in the context of complex chromosomal structures and will encounter an array of DNA regions which provide less favourable substrates. Replicative stresses can arise from a range of sources including depletion of nucleotide pools, DNA damage, disruption to the replication machinery and DNA regions which are refractory to replication. Failures in DNA replication can result in chromosomal breakage or the disengaging of the replicative helicases from the DNA polymerases, resulting in regions of unreplicated single-stranded (ss)DNA. Both these structures are triggers for checkpoint systems which delay the progress of the cell division cycle until they are appropriately ameliorated and replication is completed correctly. Moreover, perturbations in DNA replication can result in unprogrammed genetic changes, such as gross chromosomal rearrangements, which, in some cases, can result in deleterious outcomes; indeed, in humans, DNA replicative stress has been implicated as a major oncogenic force.[1-3]

Two proteins, Tim and Tipin, associate both physically and functionally with each other and the replication machinery, the replisome.[4-9] Whilst there is compelling evidence linking the function of these proteins to DNA replication, replication stalling, replicative checkpoints and genome stability maintenance, elucidation of their exact role in each of these pathways is complex. Here we provide an overview of our current understanding of the roles of Tim-Tipin in these processes.

The Biological Function of Tim-Tipin: A Puzzle from the Insect World

Tim and Tipin are conserved in all eukaryotes with orthologues identified in organisms as diverse as humans and the unicellular yeasts. In the budding and fission yeasts the orthologues of Tim and Tipin are Tof1/Csm3 and Swi1/Swi3 respectively. Tim is named after the *TIMELESS* gene from Drosophila sp. and Tipin acquired its name as it was identified as a TIMELESS interacting protein using yeast two hybrid.[4] Drosophila TIMELESS is involved in the regulation

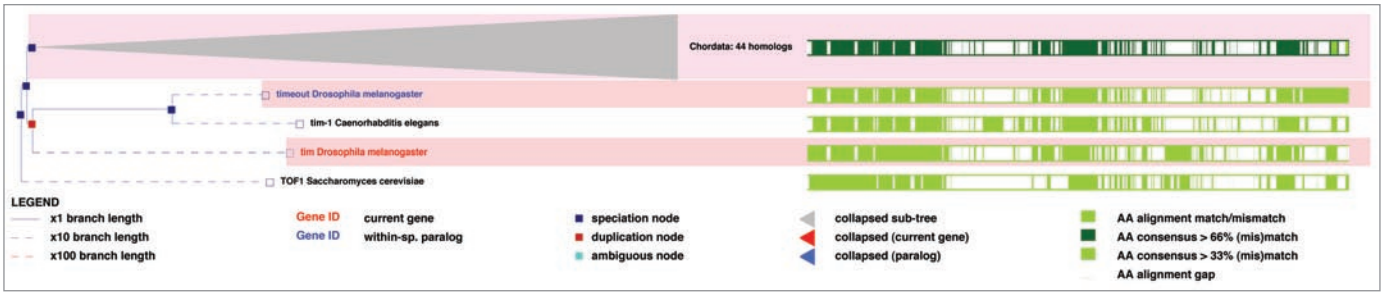

Figure 1. Phylogenetic relationship of the Timeless family of proteins. A phylogenetic tree (left) shows that insects acquired a paralogous pair, Tim/Timeless (Drosophila—red) and Timeout (Drosophila—blue) prior to the rise of the bilateral animals (http://www.ensembl.org/*Drosophila melanogaster*/Gene/Compara Tree?g=FBgn0014396). This places the budding yeast prior to the evolutionary development of the paralogous pair. The alignment (green and white bars, right) shows highly conserved motifs, regions, and domains in this protein family. The TIMELESS domain is located towards the N-terminus; the TIMELESS-C domain is located in the central part of the protein.

of the circadian clock and this has resulted in the proposal that there is an intimate association between genome maintenance mechanisms and the control of biological time.[10] However, at some point in evolution the insects acquired a paralogue pair of Tim-related proteins. In Drosophila these are TIMELESS and its close relative TIMEOUT.[11,12] A multiple sequence alignment of the protein family suggests that of the two paralogues, TIMEOUT is the more likely to be the orthologue of Tim-like proteins. However, there is some uncertainty because a phylogentic tree places the paralogue branch point prior to the emergence of Bilateria and the yeast genes (**Fig. 1**). Compared to Tim, TIMEOUT remains poorly characterised so comparative studies of their expression pattern during development and with age, subcellular location, and so on, might prove informative.

The evolutionary duplication event in the insects, and the closer homology between TIMEOUT and orthologues in other organisms, might suggest insect TIMELESS has evolved to specifically regulate circadian rhythms and the TIMEOUT protein functions in DNA replication, bringing functional distinction to this paralogue pair. Addressing the question of whether TIMELESS in other eukaryotes controls biological rhythms is confounded by the fact that deletion of TIMELESS orthologues lead to an embryonic lethal phenotype in key model systems such as the mouse and *Caenorhabditis elegans*, most likely due to a fundamental role associated with DNA replication.[12,13] However, depletion of mammalian mTim (TIMELESS) from the suprachiasmatic nucleus (SCN), the region of the mammalian brain responsible for the master circadian clock in mammals, results in circadian regulatory processes becoming arrhythmic, indicating that there is a key role for mTim in the regulation of the biological clock.[14] This leads to the proposal of two possibilities: firstly, mTim switches functional activity between distinct roles in DNA replication in proliferating cells and circadian regulation in the SCN, possibly switching during development (in support of this developmental functional split microarray data indicates Drosophila TIMEOUT exhibits slightly higher expression during metamorphosis than TIMELESS; http://flybase.org); alternatively, these functions are not mutually exclusive and the role of mTim in DNA replication directly impinges on the regulation of the biological clock in the SCN. Whilst the former seems more likely, given that cells in the SCN are not proliferative, this remains an area for conjecture and further investigation.

The Tim-Tipin pairing have been shown to interact with components of the replisome in organisms as diverse as yeast and higher metazoans, indicating the primary role of the key family members relates to control of DNA replication. Indeed, these proteins have been linked to the DNA replication checkpoint system and to the maintenance of chromosome stability both in the absence and presence of replicative perturbation. Here we will look at these features of Tim-Tipin function.

A Fundamental Role in DNA Replication Checkpoint Control

Saccharomyces cerevisiae Tof1 was the first member of the TIMELESS family of proteins that was shown to have a checkpoint role.[15] In *S. cerevisiae* there are two parallel checkpoint pathways both dependent on the central kinase Mec1 for function; one cell cycle wide pathway is dependent on Rad9 and one S-phase-specific pathway. Because of the existence of these parallel pathways Rad9-deficient cells are not as sensitive as a Mec1 mutant strain to DNA damage. The *TOF1* gene was isolated in a genetic screen for mutant factors that increased the MMS sensitivity of a *rad9* mutant strain, so it became as sensitive as a *mec1* mutant strain.[15] To verify that the S-phase-specific branch of the checkpoint pathway was affected in a *tof1* mutant background it was shown that the *rad9 tof1* double mutant was unable to slow DNA replication in response to MMS treatment. Mec1, and to a lesser degree Tel1, the two central kinases involved in checkpoint activation in budding yeast, are required for the activation of the effector kinase Rad53 by phosphorylation in response to DNA replication stress. Tof1 and Rad9 act downstream of Mec1 in this pathway where they are redundant for the phosphorylation of Rad53. The *tof1 rad9* double mutant is unable to phosphorylate Rad53 in response to hydroxy urea (HU). These data suggested that Tof1 has a key role in activation of the intra-S phase checkpoint in *S. cerevisiae*.

Due to the role of Tof1 in the inter-S phase checkpoint in *S. cerevisiae*, it was

investigated whether *S. pombe* Swi1, the Tof1 orthologue, also possessed a similar checkpoint function.[16] In *S. pombe* the central checkpoint kinase Rad3 acts upstream of two kinases Cds1 and Chk1. Cds1 has a role in intra-S phase checkpoint while Chk1 acts in the G_2 to M checkpoint. Here, Cds1 is the functional orthologue of *S. cerevisiae* Rad53. In this system, Swi1 is required for the full activation of Cds1. The kinase activity of can be assessed by assaying the ability of immunoprecipitated Cds1 to phosphorylate myelin basic protein.[16] Only weak activity was observed using *swi1* mutant cells compared to wild-type cells when Cds1 was purified and assayed from HU treated cells. Similarly, a kinase called Mik1 that accumulates in HU-treated cells in a Cds1-dependent manner failed to accumulate in *swi1* mutant cells.[16] Finally, overexpression of Cds1 was able to partly complement the sensitivity of a *swi1* mutant. Thus, these data are consistent with Swi1 having a role in activating Cds1 in response to replication stress. A subsequent study demonstrated that Swi3 has a similar role in Cds1 activation, with a significant decrease in the ability of a *swi3* mutant to activate Cds1.[17] Again, overexpression of Cds1 partly complements the DNA damage sensitivity defect of a *swi3* mutant. Interestingly, the role of Swi1 in the intra-S phase checkpoint is complicated by several studies that suggest that Swi1 acts together with another kinase complex called Hsk1/Dfp1, which affects the intra-S phase checkpoint.[18] Hsk1/Dfp1 is homologous to the essential *S. cerevisiae* and metazoan Cdc7/Dbf4 kinase complex involved in the initiation of DNA replication. *swi1* was shown to genetically interact with the temperature sensitive mutant *hsk1-89*. This interaction was further supported by the detection of a directly interaction with Dfp1 by yeast two-hybrid analysis, and the co-immunoprecipitation with Dfp1 and Hsk1.[19] Another study showed that while *swi1* and *swi3* mutants have additive check point defects (the *cds1 swi3* double mutant seems to lack an appropriate G_2-M checkpoint) and increased sensitivity to alkylation damage to DNA when combined with a *cds1* mutant, both *swi1-111* and *swi3-146* mutations were epistatic with *hsk1-89* and $dfp1_{1\text{-}376}$ mutations in their response to alkylation damage.[18] However, it should be noted here that other studies detected additive effects when *hsk1-89* was combined with *swi1*Δ and *swi3*Δ mutants, suggesting an effect of the alleles used.[19,20] Importantly, the three mutant strains, *swi1, swi3* and *hsk1-89* displayed a similar inability to arrest cells within S-phase when they were exposed to alkylation damage.[18] A recent paper might explain these observations; here it was shown that Swi1 and Swi3 were required for the chromatin association of Mrc1, the functional homologue of the metazoan Claspin.[20] Mrc1 is required of activation of Cds1. The study showed that Mrc1 interacts with Swi3 and Hsk1 thought a SQ/TQ cluster segment, and that this segment is sufficient for the checkpoint reaction. How Swi1, Swi3, Mrc1 and the two kinases Hsk1 and Cds1 act in consort to mediate the functional inter-S phase check point or checkpoints remains to be understood. Interestingly, in some genetic studies Hsk1 seems to act upstream of Cds1 while in others downstream, suggesting some form of cross-talk between these two kinases.[21,22]

In metazoans, TIMELESS and Tipin also have been shown to have a similar checkpoint role. Here there are two kinases, Chk1 and Chk2, which act downstream of the central kinase ATR (a homologue of *S. pombe* Rad3 and *S. cerevisiae* Mec1), where Chk1 has a similar role to *S. pombe* Cds1 and *S. cerevisiae* Rad53, acting in the intra-S phase checkpoint response, while Chk2 acts in the G_2-M checkpoint, similar to *S. pombe* Chk1. Firstly, in human cells hTipin and hTim were shown to be required for an efficient cell cycle arrest in response to DNA damage mediated by gamma radiation and siRNA depletion of hTipin and hTim cause sensitivity to gamma radiation and HU.[5] Several studies showed that hTipin and hTim are required for Chk1 phosphorylation; siRNA depletion of hTipin and hTim inhibits phosphorylation of Chk1 when cells are treated with HU, H_2O_2 or UV.[6,8,23,24] Similarly, depletion of Tipin in *Xenopus laevis* extracts inhibits Chk1 phophorylation.[25] Interestingly, siRNA of hTipin or hTim prevents the nuclear accumulation of Claspin when treated with HU, while Claspin depletion has no effect on hTipin or hTim localization suggesting a potential mechanism for the role of hTim and hTipin in the intra-S phase checkpoint.[23] Another mechanistic explanation for hTim and hTipin involvement in the inter-S phase checkpoint response comes from the observation that the hTim-hTipin complex interacts directly with the 34 kDa subunit of the replication protein RPA and can be co-immunoprecipitated with Claspin.[6,8] Thus, the hTim-hTipin complex could act as a bridge between the proteins involved in ssDNA recognition and checkpoint activation. Finally, hTim has been shown to interact with Chk1 by co-immunoprecipitation and this interaction is damage (HU and UV)-dependent.[24] hTim also co-immunoprecipitates with ATR, in the absence and presence of HU.[24]

Together these datasets strongly suggest that Tipin and Timeless role in the intra-S phase checkpoint is phylogenetically conserved.

A Non-Checkpoint Role in Maintaining Genome Stability

Whilst the DNA replication checkpoint function has been established for the Tim-Tipin protein families, there are other, non-checkpoint functions. This is revealed by the observation that fission yeast double mutants defective in either Swi1 (Tim) or Swi3 (Tipin) and the DNA damage checkpoint pathway (*swi1/3*Δ *chk1*Δ) exhibit higher levels of cytogenetic defects than double mutants defective in the DNA replication and DNA damage checkpoint pathways (*cds1*Δ *chk1*Δ).[17] It has been demonstrated that depletion of Swi1(Tim1)-Swi3 (Tipin) function results in extensive ssDNA.[17,18] This has resulted in the proposal that Tim-Tipin form a functional conjoining between the replicative helicase and polymerases and that the loss of Tim-Tipin disengages these processes from one another resulting in the helicase running ahead of new strand synthesis generating extensive ssDNA (**Fig. 2**, top). This model is supported by evidence demonstrating the interactions made by the Tim-Tipin pairing with replisome components, including the replicative polymerases and helicases.[7,8] Indeed distinct groups have proposed that *S. pombe* Swi1-Swi3 specifically associate with the

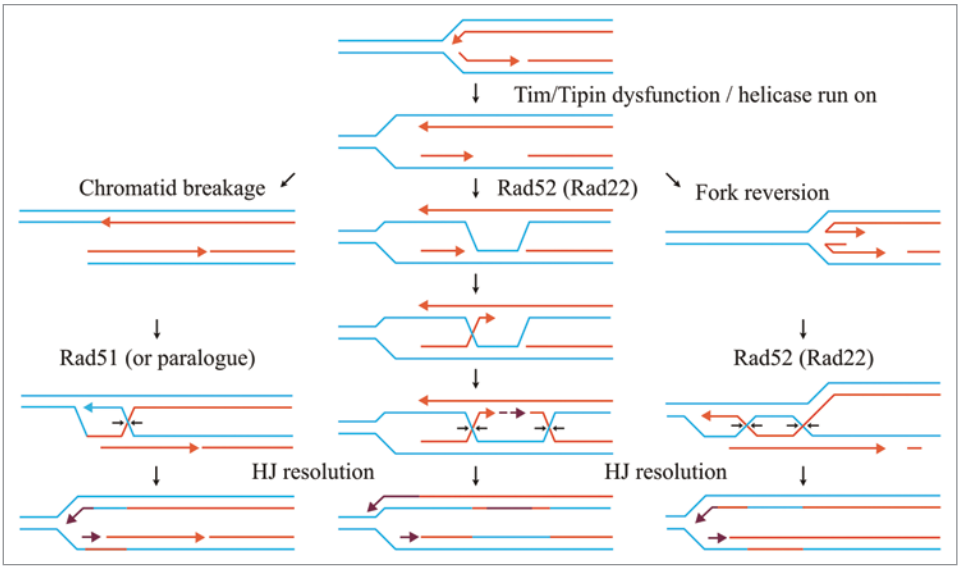

Figure 2. Model for the generation of recombinogenic lesions following Tim-Tipin dysfunction and possible pathways for subsequent DNA replication fork re-establishment. Top: failure in Tim-Tipin function results in uncoupling of DNA polymerase and helicase activities resulting in helicase run on which generates regions of single-stranded DNA. The model proposes a number of routes to process these failed forks to re-establish a functional replication fork. Firstly, the collapsed fork can be processed into a one-sided DNA double-strand break (left), by unidentified activities. The broken arm is processed and provides the substrate for Rad51 (and BRAC2) in humans and Rad51 and possibly other Rad51 paralogues in other organisms, such as *S. pombe*. These mediate strand invasion and re-establishment of the replication fork following Holliday junction (HJ) resolution. Two alternative, Rad51-independent routes could rely upon the single-stranded annealing capabilities of Rad52 (Rad22) (middle and right). Firstly, a single-stranded region generated in the lagging strand could anneal with the other parental strand; this would ultimately result in the formation of a double HJ which could be resolved to re-form a functional replication fork. Alternatively, (right) reversion of a failed fork could result in the so called 'chicken foot' structure which could form the substrate for a Rad52-mediated strand invasion/annealing reaction ahead of the strand convergence point; this would result in a double HJ which could be resolved to a functional replication fork. Adapted from Urtishak et al. (2009) and Noguchi et al. (2004).[17,27]

lagging strand polymerase and so couples leading and lagging strand DNA synthesis.[17,18,26] This coupling role is thought to be of importance at replication barriers, where both leading and lagging strand replication is stalled in a Swi1(Tof1)- and Swi3(Csm3)-dependent manner.[26]

Consistent with observations in the fission yeast, mouse cells depleted for mTim exhibit increased sister chromatid exchanges (SCEs).[27] One explanation for this, which is strongly supported by the association of Tim-Tipin with replisome components, is that the loss of Tim-Tipin function results in an increase in recombinogenic lesions, such as single ended broken chromosomes (**Fig. 2**). Indeed, in mouse cells, depletion of Tim results in an increase in the number of cytologically measurable histone H2AX phosphorylation events, indicators of DNA broken ends, and increases in the number of spontaneous foci of recombination proteins are observed in fission yeast *swi1/3* mutants.[5,16,17,27] More direct evidence for recombinogenic lesions comes from the fact *swi1/3* mutants exhibit elevated levels of spontaneous inter-repeat recombination.[18] Downregulation of the activity of the BLM helicase, which is mutated in Bloom's syndrome, results in a similar elevations in SCEs.[28] However, reduction in mTim levels in *BLM*$^{-/-}$ mouse cells results in additive levels of SCE events, suggesting that these two proteins function independently in pathways required for suppression of genome instability.[27] Further analysis using knockdown technologies demonstrates that the likely route to repair of breaks generated by depletion of mTim is via a homologous recombination reaction mediated by the RecA-like strand invasion protein Rad51 in conjunction with Brac2, a Rad52-like Rad51-associated protein (**Fig. 2**).[27] These studies were extended to demonstrate that Mus81, one of the proteins capable of a resolution of Holliday junctions,[29,30] is required, indicating that a Holliday junction intermediate is formed during the recovery from breakage caused by mTim depletion (**Fig. 2**).[27] Noguchi and co-workers (2004) have also demonstrated that loss of Swi3 resulted in a dependence upon Mus81 for cellular survival, implying that Holliday junctions are conserved features in the pathway employed to recover from the replicative defects induced by loss of Tim-Tipin.[17] Contrary to the proposal that recovery from Tim depletion in mouse cells requires Rad51, Nogouchi and co-workers (2004) found no dependence upon the canonical Rad51-mediated homologous recombination pathway components in fission yeast *swi3* mutants, suggesting that recombinogenic lesions generated by loss of Swi3 were processed to form Holliday junctions by a novel Rad51-independent route (**Fig. 2**, middle/right). This may suggest that the lesions generated by Tim-Tipin depletion in mammals and yeast are distinct. However, they did demonstrate that the fission yeast Rad52 homologue, Rad22, was required, indicating potentially common features of the lesions generated and the pathways required for their repair. Rad52 (Rad22) has been demonstrated to have ssDNA annealing capabilities and they proposed a model for fission yeast which is distinct from that proposed for mouse cells (**Fig. 2**, middle/right). In

this model loss of Swi3 function does not result in double-stranded breakage at failed replication forks, rather the uncoupling of the replicative helicase and polymerase results in tracts of ssDNA. These tracts of ssDNA provide the substrate for Holliday junction formation via the ssDNA annealing activity of Rad22 (Rad52) (**Fig. 2**).[17] Both this model and the Rad51-dependent model proposed from the work with mouse cells could result in elevated SCEs on loss of Tim(Swi1)-Tipin(Swi3) activity (dependent upon the HJ resolution direction). However, a further scenario remains untested, which leave open the possibility that lesions created by Tim-Tipin perturbation in mouse and yeast are similar in nature. The fission yeast has five paralogues of the *rad51* gene, including one, *rlp1* (recA-like protein 1) which has been demonstrated to be required for recovery from agents which perturb DNA replication during the mitotic cell division cycle.[31] It may be possible that processing of recombinogenic lesions formed due to abrogation of the replisome may require Rlp1 in fission yeast and not Rad51. This may mean that double-strand breaks due to single-stranded regions generated at the replication fork are formed in both fission yeast and mouse in response to depletion of Tim(Swi1)-Tipin(Swi3), but that mouse is Rad51-dependent and fission yeast is Rlp1-dependent (or Rlp1 and Rad51 are redundant).

Recent work has demonstrated a role for Tof1 in suppressing gross chromosomal rearrangements specifically involving segmental duplication which are likely to be driven by homologous recombination.[32] Loss of Swi1/3 function in fission yeast results in spontaneous elevations in recombination.[18] This observation has been extended to demonstrate that this is more prevalent at genomic sites, such as *tRNA* genes,[33] which slow the progression of the replication machinery.[34] This brings to light an intriguing feature of Swi1 (and most likely Swi3). Pryce and co-workers (2009) demonstrated that recombination activity increased at a replication barrier generated by *tRNA* genes in a *swi1* mutant, but that the intensity of the barrier remained indistinguishable from wild-type levels. In stark contrast, mutants in both *swi1* and *swi3* are defective in generating a replication fork block at a specific replication barrier, *RTS1*, at the fission yeast mating type locus, which functions to ensure the region is replicated in a unidirectional fashion,[35-37] demonstrating Swi1/3 are actually required to generate the blockage. Moreover, replication fork barriers in the *S. pombe* rDNA locus can be distinguished as some are Swi1/3-dependent and others not.[38] A similar barrier-specificity is apparent in *S. cerevisiae*. Both Tof1 and Csm3 are required for full barrier activity at Fob1-binding sites and replication fork barriers in the rDNA locus, whilst there is no negative effect on replication fork barriers generated by hairpin structures arising from artificially inserted inverted *Alu* repeat sequences or expanded CGG repeats in a *tof1* mutant;[39-41] interestingly, the requirement for Tof1-Csm3 for Fob1-dependent barrier activity is independent of their checkpoint role or the budding yeast Claspin homologue, Mrc1.[40] A similar site-specific activity is observed for recombination. When the *S. pombe RTS1* element is placed ectopically within recombination reporter cassettes it generates recombinogenic lesions at a high frequency in the presences of Swi1/3.[42,43] However, when Swi1/3 functional loss results in the loss of the replicative barrier activity, then recombination is suppressed. These contradictory roles of Swi1-Swi3/Tof1-Csm3 in yeast demonstrate an important feature of Swi1-Swi3/Tof1-Csm3, which is that they have the capability to respond differentially to distinct genetic elements, having both positive and negative influences on genome stability. Potentially, Swi1 and Swi3 might be required to prevent ssDNA regions forming at the replication fork when there is structure in the DNA template such as at *tRNA* genes. This feature of these conserved proteins has not been demonstrated in other eukaryotes, but differential responses to genetic elements for Swi1/3 have also been reported for elements within the rDNA locus in fission yeast.[38]

Other Roles in Genome Stability

The Tim-Tipin family of proteins also play other roles in genome stability regulation. Genetic studies have demonstrated that Tof1-Csm3 are required for trinucleotide repeat stability.[44] Here structure in the template DNA could also play a role. It has also been proposed that Tim-Tipin may function in both CRY-associated photolyase repair of (6-4) photoproducts and response to oxidative stress,[10] although both of these proposals require direct experimental investigation.

Sister chromatid cohesion is an important feature of chromosome dynamics which ensures sister chromatids are appropriately aligned in mitosis and meiosis and can provide a co-localised partner for homologous recombination repair of chromosomal breakages.[45] DNA replication and sister chromatid cohesion are intimately linked.[15] Mutants in budding and fission yeast Tim-Tipin (Tof1-Csm3/Swi1-Swi3) exhibit mild defects in sister chromatid cohesion, but a functional link between Tim and cohesion was first made in *C. elegans*, where depletion of *C. elegans* TIM-1 resulted in mitotic and meiotic cohesion defects.[13] Genetic studies in yeast have also linked Tim-Tipin to cohesion function.[46-49] Other genetic studies have linked Tof1-Csm3 to proposed specialised cohesion structures during the response to chromosomal lesions induced by topoisomerase I.[50] However, the exact nature of the functional link with cohesins remains unclear.

Closing Remarks

From studies in a wide range of organisms, it is now clear that Tim and Tipin (and their orthologues) play a fundamentally important role in maintaining and monitoring the integrity of the DNA replication fork in eukaryotes. Moreover, when these proteins become dysfunctional recombinogenic intermediates are generated, which can drive genetic change, most likely due to uncoupling of the replicative helicase and polymerase activities. Given this, it is not unreasonable to argue that detrimental augmentation of Tim-Tipin function, either through mutation or non-genetic perturbation, may have oncogenic potential. This has important implications for studying and treating tumours, as one might anticipate that tumours carrying dysfunctional Tim-Tipin may be exquisitely sensitive to therapeutic agents which perturb DNA replication.

However, some enigmatic and fundamentally important questions hang over this protein pair, not least of which is their relationship to circadian rhythm regulation. If, in organisms such as humans, the single Tim-Tipin pairing functions both in replisome monitoring and circadian regulation, then this has far reaching implications for how we view these disparate processes and the link to human health and well being.

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