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Telomeres: Structures in need of unwinding

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Abstract

Telomeres protect the ends of eukaryotic chromosomes from being recognized and processed as double strand breaks. In most organisms, telomeric DNA is highly repetitive with a high GC-content. Moreover, the G residues are concentrated in the strand running 3′–5′ from the end of the chromosome towards its center. This G-rich strand is extended to form a 3′ single-stranded tail that can form unusual secondary structures such as T-loops and G-quadruplex DNA. Both the duplex repeats and the single-stranded G-tail are assembled into stable protein–DNA complexes. The unique architecture, high GC content, and multi-protein association create particularly stable protein–DNA complexes that are a challenge for replication, recombination, and transcription. Helicases utilize the energy of nucleotide hydrolysis to unwind base paired nucleic acids and, in some cases, to displace proteins from them. The telomeric functions of helicases from the RecQ, Pi., FANCJ, and DNA2 families are reviewed in this article. We summarize data showing that perturbation of their telomere activities can lead to telomere dysfunction and genome instability and in some cases human disease.

Keywords

Telomere; Telomerase; Helicase; G-quadruplex; Telomere replication

1. Helicases

Helicases are best known for their ability to harness the energy of nucleotide triphosphate (usually ATP) hydrolysis to catalyze the unwinding of duplex nucleic acids. Helicases can unwind a variety of different structures including DNA, RNA, and DNA/RNA duplexes as well as more exotic molecules, such as forked or bubbled replication intermediates, Holliday junctions, and non-Watson Crick base paired structures such as G-quadruplexes. In addition to unwinding base paired nucleic acids, some helicases can translocate along an RNA or DNA substrate and others can displace proteins from nucleic acids. Owing to this great versatility, helicases are required for virtually all biological processes involving nucleic acids including DNA replication, repair and recombination, and RNA transcription, splicing and translation. This wide variety of cellular functions may explain why organisms encode so many different helicases. For example, 134 (2%) of the open reading frames in the *Saccharomyces cerevisiae* genome encode predicted helicase proteins [1]. In this review we focus on DNA helicases with demonstrated functions at telomeres, the ends of eukaryotic chromosomes (see Fig. 1 for helicase families with roles at telomeres).

The majority of helicases are classified into two superfamilies (SFI and SFII) defined by the presence of seven conserved amino acid motifs. The helicase motifs are found in a domain of approximately 300–500 amino acids and are observed by structural analysis to cluster in

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space (review [2]). Helicases are placed into families based on higher levels of sequence similarity, both within and outside of the helicase motifs. In this review we discuss helicases in the RecQ, Pif1, FANC-J, and DNA2 families.

Helicases are divided into DNA or RNA helicases depending on the chemical identity of the strand onto which they load (all of the helicases discussed in this review are DNA helicases). They are further divided by their direction of unwinding. Helicases typically unwind duplexes in a unidirectional manner, moving either 3′ to 5′ (RecQ family helicases) or 5′–3′ (Pif1, FANC-J, DNA2 families) along the strand onto which they load. The number of nucleic acid base pairs unwound before the helicase dissociates from its substrate defines its processivity. Helicases vary in their in vitro determined processivity, which may reflect the enzyme's preferred substrate and/or necessary co-factors. For example, the *S. cerevisiae* Pif1 DNA helicase is poorly processive on conventional duplex DNA but is highly processive on forked RNA/DNA duplexes [3] and G-quadruplex structures (KP and VAZ, in preparation) (discussed in more detail in Section 4.1).

2. Telomeres

This section reviews the properties of telomeres with an emphasis on those aspects of telomere biology that are likely to involve DNA helicases. Telomeres are protein–DNA structures that distinguish natural chromosome ends from double strand breaks (DSBs). Because telomeres protect chromosome ends from processing events that result in degradation and/or end-to-end fusions, they are essential for chromosome integrity. Telomeric DNA is replenished by an unusual replication mechanism that involves a telomere-dedicated reverse transcriptase called telomerase that compensates for the inability of DNA polymerases to replicate the 5′ ends of linear chromosomes [4]. In addition, from yeasts to humans, telomeres are specialized sites for gene expression, as structural genes positioned near telomeres are transcriptionally repressed (reviewed in [5]).

Telomeric DNA consists of a tandem array of short repeated sequences in which the strand running 5′–3′ from the centromere towards the chromosome end is usually guanine-rich. The amount of duplex telomeric DNA per chromosome end varies enormously from organism to organism. For example, the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* have ~300 bps of telomeric repeats per end while many mammals have ~10 kb or more. Extension of the guanine-rich strand forms a 3′ single stranded overhang (G-tail). This G-tail is a conserved feature of telomere structure and is essential for telomere function [6–10]. Because duplex telomeric DNA is G–C rich, its thermal stability is high, which may explain why multiple helicases have roles in telomere dynamics. Because of the high concentration of guanines, telomeres are able to form stable G-quadruplex structures (discussed in detail below).

As part of their end-protection function, telomeric DNA is bound constitutively by a core group of proteins called shelterin. Shelterin components include both duplex (TRF proteins in mammals, Rap1 in *S. cerevisiae*, Taz1 in *S. pombe*) and single strand (Pot1 in mammals and *S. pombe*, Cdc13 in *S. cerevisiae*) binding proteins as well as proteins that associate via protein–protein interactions [8,11–13]. The overall composition of the shelterin complex varies between species in respect to protein content and higher order structure, but the general design is conserved [14]. The role of the shelterin complex in telomere function and regulation includes distinguishing natural ends from DNA breaks and recruiting telomerase to DNA ends [12].

In addition to the shelterin complex, higher-order DNA structures are thought to contribute to telomere functions. A well documented example of such structures is the T-loop that was first identified in human and mouse cells by electron microscopy [15] and later observed in a

variety of other organisms, including trypanosomes, ciliates, and nematodes [15–23]. T-loops are duplex lariat structures formed when the single stranded telomeric G-overhang invades the double stranded telomeric region of the same chromosome [24]. Unwinding T-loops may require a specialized helicase. By sequestering chromosome ends, T-loops are proposed to protect telomeres against checkpoint recognition, DNA repair, and telomerase-mediated extension [15,25,26]. Since T-loops are structurally similar to Holliday junctions, they may also be important for telomere recombination [27,28]. It is not yet known if T-loops exist at each telomere, how they are regulated in the cell cycle, or how they are displaced to allow both semi-conservative and telomerase-mediated telomere replication.

G-quadruplex structures are another secondary DNA structure that can affect telomere function. G-quadruplexes involve the association of four guanines into a cyclic Hoogsteen hydrogen bonding arrangement in which each guanine shares a hydrogen bond with its neighbor (N1–O6 and N2–N7) (reviewed in [29,30]). The G-rich single stranded telomeric overhang can form intra- and intermolecular G-quadruplex structures in vitro. The in vivo occurrence of telomeric G-quadruplex structures has so far been demonstrated only in ciliates [31–33]. In ciliates, two telomere-binding proteins regulate and promote the formation of telomeric G-quadruplex DNA in vitro and in vivo [31,32,34]. Some DNA helicases with well documented effects on telomeres, such as WRN and Pif1, can unwind G-quadruplex structures in vitro [35,36]. G-quadruplexes have the potential to regulate telomerase activity: intramolecular G-quadruplexes block telomerase activity in vitro [37,38], and telomerase RNA exhibits a G-quadruplex motif, which might be regulated by an as yet unidentified RNA helicase [39]. The formation and regulation of secondary structures such as T-loops and G-quadruplexes may contribute to telomere function, but these structures have to be tightly regulated as they also present a problem for telomere maintenance. Secondary DNA structures are an obstacle for both semi-conservative and telomerase-mediated replication and must be resolved prior to these events.

Telomeres become shortened during every cell division due to incomplete replication of the lagging strand (the so called “end replication problem”). Additional loss of telomeric DNA occurs due to post-replicative degradation of the 5′ strand that generates long 3′ G-rich overhangs [40,41]. In most species, the loss of telomeric DNA is balanced by the action of telomerase that uses its RNA component to template extension of the 3′ G-tails [42–44]. The complementary C-strand is then synthesized by conventional RNA-primed DNA replication [4,45].

The importance of telomerase to genome integrity is best illustrated by situations where telomerase is not expressed, which occurs naturally in most human somatic cells or by mutation in genetically tractable organisms. Due to incomplete replication, telomeres progressively shorten in telomerase deficient cells: human telomeres lose ~100 bps of telomeric DNA in each cell division, while yeast telomeres lose ~4 bps. Although telomerase is not essential and its absence is tolerated for many cell divisions, extreme telomere shortening causes telomere dysfunction, which leads to chromosome instability, end-to-end fusions, and checkpoint-mediated cell cycle arrest and/or apoptosis (reviewed in [46,47]). The ability of human cells to divide in culture is finite. However, if the catalytic subunit of telomerase is introduced into these cells, its expression confers an unlimited division potential upon them [48]. Although telomerase is not expressed in most human somatic cells, inherited mutations in telomerase components or certain telomere structural proteins result in short telomeres and reduced life expectancy, probably as a result of stem cell failure [49,50]. Finally, telomerase is up regulated in the vast majority of human cancers [50–52], and this heightened activity contributes to the increased division potential of malignant cells. Thus, both down and up regulation of telomerase are associated with human disease.

Semi-conservative replication of duplex telomeric DNA is usually unidirectional, moving from an internal origin of replication towards the chromosome end. Because of this unidirectional replication, if replication forks stall within telomeric DNA they cannot be rescued by a converging replication fork. Moreover, because telomeric DNA is always replicated in the same direction, the telomeric G-rich strand is always the template for lagging strand synthesis [53–55]. During the unwinding of the parental duplex to allow the start of a new Okazaki fragment, the lagging strand is transiently single stranded, which at least theoretically provides an opportunity for it to form stable secondary structures, such as G-quadruplex DNA. The occurrence of such structures could impede replication fork progression through telomeric regions. Indeed, replication fork stalling within telomeres has been seen in diverse organisms using a variety of methods, although it is not known if G-quadruplex structures cause these replication problems.

Replication forks move slowly through *S. cerevisiae* telomeres as seen from both two-dimensional (2D) gel and genome-wide DNA Polymerase 2 association analyses [56,57] (see Section 4.2 for a discussion of the role of the Rrm3 helicase in semi-conservative replication of *S. cerevisiae* telomeres). 2D gels also reveal that semi-conservative replication of *S. pombe* telomeres is slowed when cells lack Taz1, the duplex telomere binding protein, in a manner that is independent of the direction of replication through the repeats [58] (see Section 3.2 for role of the RecQ helicase Rqh1 in *S. pombe* telomere replication). Moreover, in *S. pombe*, the leading strand polymerase arrives at the telomere before the lagging strand DNA polymerases, suggesting that the telomeric template for lagging strand synthesis may have a longer half-life as single stranded DNA than lagging strands elsewhere in the genome, a situation that is expected to exacerbate replication problems [59]. A situation seemingly similar to *S. pombe* Taz1 deficient cells exists in mammalian cells depleted for the Taz1 ortholog, TRF1. Using DNA combing, which allows the examination of replication at the single molecule level, replication forks move about half as fast through mouse telomeric DNA when it lacks TRF1 [60]. Moreover, when examined by FISH (fluorescence in situ hybridization), telomeres of metaphase chromosomes in TRF1 depleted cells have an aberrant structure in which telomeres are no longer discrete entities but often have a bipartite appearance that is reminiscent of structures at chromosomal fragile sites upon replication stress [60]. Both RecQ (BLM, WRN) and FANCJ (RTEL) family helicases are implicated in replication of mammalian telomeres (see Sections 3.3 and 5).

Given that telomeres from yeasts to humans are regions where transcription of structural genes is repressed [5], the finding that telomeric repeats and subtelomeric regions are transcribed was quite surprising [61–63]. This telomeric transcribed region, called TERRA, produces a non-coding G-rich RNA transcribed from the C-rich strand that is often telomere-associated. Although TERRA was only recently discovered, it may be a wide spread feature of telomeres, as it has been detected in *S. cerevisiae* [61] and mammals [62,63]. In yeast increased TERRA results in telomere shortening in cis, suggesting that TERRA interferes with telomerase-mediated telomere lengthening [64,65]. So far no helicases are identified that function during TERRA biogenesis, but it would not be surprising if helicases are required for TERRA synthesis, regulation, and/or removal from telomeric DNA.

Although telomerase is the major mechanism for telomere maintenance, homologous recombination (HR) provides an alternative (ALT) method for maintenance of telomeric DNA, especially in telomerase deficient cells. In *S. cerevisiae*, most cells lacking telomerase ultimately die, although a subset of cells emerge as survivors if the strain is recombination proficient. Yeast has two distinct ALT pathways, called type I and type II recombination [66]. The two pathways use different telomeric sequences as substrates for recombination and depend on different recombination proteins [66–68]. In type I recombination, the sub-

telomeric Y′ elements are expanded into large tandem arrays at individual chromosome ends. In contrast, in type II recombination, the simple G-rich repeats at chromosome ends are expanded with some telomeres bearing 10–100 times more telomeric DNA than at wild type telomeres, whereas other telomeres are very short [67,68]. The heterogenous lengths and telomere dynamics in yeast type II survivors are very similar to what is seen in human tumors that maintain telomeres by ALT [69].

Because telomeric DNA is G-rich, it is particularly stable. This stability is true for standard B-form duplex telomeric DNA as well as unusual secondary structures, such as T-loops, G-quadruplex DNA, and RNA/telomeric DNA hybrids that form during TERRA and telomerase extension. Given this high inherent thermal stability, as well as the many shelterin components with which telomeric DNA is associated, it is probably not surprising that helicases play a large role in telomere biology. In the remainder of this review we focus on helicases with demonstrated roles in telomere metabolism. The telomere functions of helicases discussed in this review are summarized in Table 1.

3. RecQ family helicases

The SFII RecQ family of 3′–5′ DNA helicases, named for its prototypical member from *Escherichia coli*, is conserved from bacteria through humans. As described in more detail below, RecQ helicases have important roles in various aspects of DNA metabolism and seem to be particularly important in preventing illegitimate recombination, repairing stalled replication forks, and processing DNA breaks to generate the 3′ single strand tails that initiate HR.

Most unicellular organisms including *E. coli* (RecQ), *S. cerevisiae* (Sgs1), and *S. pombe* (Rqh1) encode a single RecQ family member (Fig. 1). The null phenotypes of cells lacking their single RecQ homolog is complex, suggesting that these proteins act at multiple steps in DNA replication, recombination, and repair. Multicellular organisms including *Caenorhabditis elegans*, *Xenopus laevis*, *Drosophila melanogaster*, *and Homo sapiens* encode multiple RecQ family members. The human genome encodes five RecQ homologs called RECQ1, BLM (Bloom’s syndrome), WRN (Werner’s syndrome, WS), RECQ4 (Rothmund–Thomson syndrome), and RECQ5. Mutations in three of the human RecQ helicases result in inherited diseases (disease names are in parentheses) that affect genome integrity and cause an increased propensity to cancer (Bloom’s, Rothmund–Thomson) and/or accelerated aging (WS, Rothmund–Thomson). Although it is not clear if the five human RecQ proteins provide complementary or independent cellular roles, their different substrate specificities as defined in vitro allow for the possibility of their having specialized functions as does their distinguishable disease phenotypes [70–75].

Other metazoans also encode multiple RecQ proteins, and in most cases they are clearly homologs of one of the human proteins (Fig. 1). For example, the four *C. elegans* RecQ proteins can be identified as homologs of the human RECQ1, BLM (called HIM-6), WRN (WRN-1), and RECQ5 [76–80]. However, the number of distinct RecQ family proteins does not correlate directly with evolutionary position. For example, *X. laevis* encodes only two RecQ family members, Xl BLM (BLM) and FFA-1 (WRN) (Fig. 1).

RecQ family members range in size from 610 amino acids in *E. coli* to 1447 amino acids in *S. cerevisiae*. Although the N-termini are not well conserved [81,82], the middle and C-terminal regions have high similarity in three conserved regions: the helicase domain, the RecQ carboxy-terminal (RQC) domain, and the Helicase and RNase D C-terminal (HRDC) domain. Although different RecQ helicases have different substrate specificities, most act preferentially on structured substrates that resemble replication and recombination

intermediates such as replication forks, Holliday junctions, D-loops, and 5′ flaps [71,83]. Additionally, several RecQ helicases are able to unwind G-quadruplexes, including the *S. cerevisiae* Sgs1 and the human WRN and BLM proteins [35,72,75]. Consistent with their more general roles in recombination, Sgs1, mouse WRN, and human BLM function in ALT, where telomeres are maintained by homologous recombination (Table 1) [69,84–86].

3.1. Telomere functions of the S. cerevisiae RecQ homolog Sgsl

Sgs1 has roles in the two major pathways for telomere maintenance, telomerase and ALT. Sgs1 participates in degradation of the C-strand of newly replicated telomeres to generate long 3′ overhangs, which are the presumed DNA substrate for telomerase [87]. This function is not specific to telomeres, as Sgs1 plays a similar role in resection of DSBs, which generates the 3′ single strand tails that initiate HR [88–91]. At telomeres Sgs1 may also help in promoting the formation of telomeric G-tails by unwinding G-quadruplex structures, as Sgs1 is able to efficiently unwind these structures in vitro [75,87].

In telomerase deficient cells, telomeres slowly shorten until most cells die. The rate of death in these senescing cultures is heightened by the absence of Sgs1. In cells lacking both telomerase and Sgs1, recombination-dependent X-shaped structures accumulate [92]. These structures are interpreted as late intermediates during telomere recombination that cannot be resolved in the absence of Sgs1. The interpretation that Sgs1-mediated recombination in telomerase deficient cells prevents cell death is supported by sequencing analysis that reveals reduced telomere recombination in *sgs1* telomerase deficient cells [92].

A small fraction of the cells in senescing telomerase deficient cultures give rise to type I or II survivors in which telomeres are maintained by recombination. Sgs1 helicase activity, its interaction with topoisomerase III, and its modification by sumoylation at the C-terminus are required for generation and maintenance of type II survivors [84,85,92]. Remarkably, expression of mouse WRN helicase in yeast suppresses the slow growth and G2/M arrest observed in telomerase negative *sgs1* yeast cells, while expression of human BLM allows telomerase deficient *sgs1* cells to form type II survivors [85,93]. These findings suggest that Sgs1, mouse WRN, and human BLM have conserved functions in telomere recombination. Finally, in cells deficient for both telomerase and recombination, Sgs1 inhibits the generation of rare survivors that arise by recombination-independent mechanisms [94].

3.2. Telomeric functions of the S. pombe RecQ homolog Rqh1

Rqh1 is important to help cells recover from impaired DNA replication by stabilization and restart of stalled replication forks and activation of checkpoints in response to replication stress [95–97]. Several lines of evidence indicate that Rqh1 also has telomere functions, although these may be extensions of its more general role in DNA replication. Telomere length is at best modestly shorter in cells lacking Rqh1 [98,99]. However, mutations in *rqh1+* affect telomere maintenance in certain mutant backgrounds where telomere length is already compromised. For example, cells lacking the telomeric double strand DNA binding protein Taz1 have exceptionally long telomeres that render them cold sensitive [100,101]. If *taz1*-cells are deficient in telomerase [98] or if they express a partial loss of function allele of an RPA subunit (*rad11*-D223Y; RPA, replication factor A, is a sequence non-specific single strand DNA binding protein that is essential for DNA replication, repair, and recombination) [102], they lose telomeric DNA extremely rapidly. In both *taz1*-cell types, telomere loss is suppressed if mutant cells also lack Rqh1.

As noted in Section 2, semi-conservative replication of *S. pombe* telomeric DNA is impaired in cells lacking Taz1 [58]. When telomeres are replicated in Taz1 defective cells, telomeres do not separate properly in mitosis, especially at low temperatures, a process called telomere

entanglements. These entanglements result in DNA breaks, chromosome missegregation, and loss of viability [98,101]. This telomere dysfunction is promoted by sumolyated Rqh1 [98]. Rqh1 sumolyation appears to be fairly specific for telomeres as non-sumolyated Rqh1 is proficient for non-telomeric functions. Rqh1 sumoylation is proposed to affect its localization to telomeres or to allow unwinding of telomere-specific structures such as stalled replication forks or G-quadruplexes [98].

S. pombe encodes multiple telomere-linked helicase (tlh) genes that are reported to have significant sequence homology between residues ~1180 and ~1820 with RecQ helicases [103]. Tlh genes, evolutionarily conserved in fungi, are normally transcriptionally repressed by telomeric silencing. However, in telomerase deficient cells, these genes are transcriptionally activated once telomeres become very short, and cell division is slowed. Moreover, this activation is important for the emergence of cells able to maintain telomeres by the ALT pathway [104,105]. Indeed, if a wild type domain of one of the tlh1 genes is over-expressed in telomerase deficient cells, these cells exit from the growth crisis faster than controls [103]. The helicase activity of the Tlh1 proteins may be important for exit from crisis, as over-expression of a presumed helicase dead version of this domain does not hasten this process. Similarly, *S. cerevisiae* subtelomeric Y′ elements encode several helicases called Y′-helicase protein 1 (Y′Help1). The expression level of Y′-help is higher in telomerase deficient survivors cells [106]. Our analysis reveals no significant similarity of these full-length helicases to other helicase families discussed in this paper (KP and KRM, unpublished results).

3.3. Telomere functions of mammalian RecQ helicases

Two human RecQ helicases WRN and BLM are also implicated in telomere maintenance. Primary fibroblasts from WS patients recapitulate a tissue culture version of the most dramatic phenotype of WS patients, premature aging, as these cells have a dramatically reduced division potential in vitro (reviewed in [107]). In addition, cultured WS cells grow slowly and exhibit accelerated telomere shortening and genome instability [108,109]. These deleterious phenotypes are likely associated with telomere dysfunction as over-expression of the catalytic subunit of telomerase rescues the reduced division potential, slow growth, and chromosome instability phenotypes of WS cells [110,111]. Moreover, wrn knockout mice show premature aging only in a telomerase minus background [112]. As shown by live cell imaging, chromatin immunoprecipitation, and immunostaining, WRN associates with telomeres during S phase [109,113] and thus likely affects telomeres and their replication directly.

In primary fibroblasts of WS patients, WRN helicase activity is necessary during replication of the G-rich telomeric lagging strand [109]. Using FISH, lack of WRN results in a preferential loss of telomeres from the sister chromatid of the lagging strand; a phenotype referred to as sister telomere loss (STL) [109]. STL is observed in cells expressing helicase-deficient WRN but not in cells expressing wild-type WRN or nuclease-deficient WRN, suggesting that WRN helicase but not exonuclease activity is necessary for its function in telomere replication [109]. Furthermore, expression of telomerase rescues STL [109]. WRN could act by promoting semi-conservative replication through duplex telomeric DNA, perhaps by unwinding G-quadruplex structures [72] that form on the lagging strand. Alternatively or in addition, WRN could resolve telomeric D-loops to allow passage of the replication fork and/or telomerase access [109].

Although the WRN nuclease activity may not be important for telomere replication, there are suggestions that it plays a role in processing telomeric DNA to activate a DNA damage response. In support of this idea, if telomeric oligonucleotides with free 3′ ends are introduced into cells, they elicit a WRN exonuclease-mediated DNA damage response

[114]. Moreover, this response does not occur if the 3′ ends are nuclease resistant, providing additional support for an important function of the WRN exonuclease activity in checkpoint activation in response to telomere perturbations.

In Section 2 we summarized data showing that replication of mammalian telomeres is impaired in cells lacking TRF1, as demonstrated by a reduced rate of fork movement through telomeric DNA and by the occurrence of fragile telomeres [60]. Depletion of the RecQ BLM helicase also generates fragile telomeres, while surprisingly depletion of WRN does not. Thus, while both BLM and WRN are implicated in telomere maintenance, their exact role at telomeres are likely different. However, WRN and BLM both unwind G-quadruplex structures in vitro [72], and it is possible that both promote telomere replication via this activity.

A variety of studies indicate that BLM and WRN interact biochemically and functionally with several shelterin components, providing further evidence that the two helicases affect telomeres. For example, both helicases interact with TRF1 and TRF2, the two sequence specific duplex telomere binding proteins, as well as with POT1, the sequence specific single strand telomere binding protein. TRF2 stimulates WRN and BLM helicase activity in vitro, especially during the unwinding of long telomeric substrates [115,116] while POT1 stimulates WRN and BLM activity on long telomeric forked duplexes and D-loop structures [117].

As with the *S. cerevisiae* Sgs1, WRN and BLM are also implicated in the recombination dependent ALT pathway. Both proteins localize to ALT telomeres and to ALT-associated promyelocytic leukemia bodies (PML), a nuclear structure whose exact function is not known but that is associated with DNA repair and recombination [83,118–120]. In the presence of TRF1 and TRF2, WRN unwinds artificial telomeric D-loops, which are thought to be intermediates in ALT [113]. Perhaps the most direct evidence for a role for RecQ helicases in ALT is that RNAi-mediated reduction in BLM expression results in shortening of ALT telomeres [121].

4. Pif1 family helicases

The SFI PIF1 family of 5′–3′ DNA helicases, named for its prototypical member from *S. cerevisiae* [122], is found in almost all eukaryotes (Fig. 1). Eukaryotic PIF1 helicase family proteins share sequence similarity in all pair-wise combinations over the 400–500 amino acid helicase domain (reviewed in [123]). In contrast, the N- and C-termini of Pif1 family helicases differ in size and sequence. *S. cerevisiae* Pif1 has low sequence similarity (~16%) to the bacterial RecD helicases with which it shares three additional motifs [124]. Phylogenetic comparisons show that a small subset of the prokaryotic RecD-like proteins cluster with the most divergent eukaryotic Pif1 family proteins, rather than with prokaryotic RecD proteins and that organisms with a Pif1/RecD-like gene also encode a more canonical RecD protein. Thus, this analysis suggests that the similarity between RecD and Pif1 proteins reflects a common evolutionary origin [123]. Eukaryotic Pif1 family proteins contain a highly conserved 21-residue signature motif between helicase motifs II and III that is not found in the prokaryotic RecD proteins [123].

S. cerevisiae, in which Pif1 family helicases were first discovered and where they have been the most extensively studied, encodes two Pif1 family members, Pif1 itself and a second protein called Rrm3, which is 40% identical to Pif1 over the helicase domain (Fig. 1). As described below, the two *S. cerevisiae* family members have quite different functions. Several other fungi also encode two distinct Pif1 family proteins, one that is clearly a homolog of the *S. cerevisiae* Pif1 and one that is clearly Rrm3-like. In contrast, all

metazoans encode a single Pif1 family helicase that has roughly equal similarity to the two *S. cerevisiae* proteins (Fig. 1). At this time, Pif1 family helicases have been studied in detail only in *S. cerevisiae* (Pif1, Rrm3), *S. pombe* (Pfh1), mouse (mPIF), humans (hPIF), and parasites (which can encode up to seven family members, the majority of which reside in mitochondria). In all of these organisms except mouse, where the question has not been addressed, Pif1 family helicases are expressed as both nuclear and mitochondrial isoforms [125–129]. *S. cerevisiae* Pif1, *S. pombe* Pfh1, and several of the *Trypanasoma* Pif1 helicases are critical for maintenance of mitochondrial DNA. Clear telomere functions have been demonstrated for the two *S. cerevisiae* proteins and suggested for the *S. pombe*, human, and mouse proteins.

4.1. S. cerevisiae Pif1: general biology and telomere roles

Pif1 is the only family member that is relatively easy to purify and thus is the Pif1 family helicase for which there is the most extensive biochemical analyses [130]. On conventional linear DNA substrates, Pif1 is poorly processive, although its activity is higher on forked DNA substrates [3]. Although Pif1 cannot load onto RNA, it has higher activity removing RNA from an RNA/DNA hybrid than DNA from an equivalent DNA/DNA hybrid [3]. Moreover, Pif1 is even more active on forked RNA/DNA molecules than on linear RNA/DNA hybrids or forked DNA/DNA substrates. In fact, Pif1 can unwind these structures in a processive manner, that is, under single cycle conditions. Like many of the RecQ helicases, Pif1 also unwinds G-quadruplex structures [36]. In fact, in side by side comparisons, Pif1 unwinds G-quadruplex structures ~100-times faster than the human WRN protein and again is able to do so under single cycle conditions (KP and VAZ, in preparation).

Pif1 was first identified because of its role in promoting recombination in mitochondrial DNA [131]. It was rediscovered in a mutant screen for genes affecting telomeres [125]. Depletion of Pif1 results in long telomeres, and this lengthening is telomerase-dependent [125,132]. Over-expression of Pif1 leads to modest telomere shortening [132]. Thus, telomere length is inversely proportional to Pif1 levels. The effects of Pif1 on telomere length require its ATPase/helicase activity, and because Pif1 is telomere-associated in vivo, it likely acts directly to affect telomerase [132].

The most dramatic effect of Pif1 on telomerase is its inhibition of telomere addition to DSBs, which is increased ~600-fold in *pif1*Δ cells [125,133]. Pif1 is phosphorylated in response to DSB formation [134], and this phosphorylation activates Pif1 activity at DSBs. In contrast, Pif1 function at telomeres does not require phosphorylation [134]. Gross chromosomal rearrangements (GCRs), which are complex chromosome changes similar to those observed in human tumor cells, increase ~1000-fold in *pif1*Δ cells. Most of the GCR events recovered in *pif1* cells are a result of telomerase dependent telomere addition to DSBs [135]. Thus, Pif1 inhibits both telomere lengthening and telomere addition by negatively regulating telomerase.

Pif1 also affects the specificity of telomere addition. Unlike wild type cells where the rare telomere addition events almost always occur near telomere-like sequences, telomere addition in *pif1* cells occurs at many sites which often have virtually no telomere-like DNA near the break site [125,133,136]. By inhibiting telomere addition to DSBs, Pif1 promotes genome integrity by preventing the generation of terminally deleted chromosomes.

In vivo and in vitro experiments indicate that Pif1 inhibits telomerase not by altering telomerase activity but rather by removing it from DNA. In vivo, Pif1 over-expression reduces telomerase levels at telomeres, while Pif1 depletion increases these levels at both telomeres [137] and DSBs (J. Phillips, KP and VAZ, in preparation). The ability of Pif1 to

inhibit telomerase depends on its interaction with Est2, the catalytic subunit of the telomerase holoenzyme [138].

Pif1 inhibits telomerase directly by removing it from its DNA substrate, as shown by in vitro studies using purified Pif1 and partially purified telomerase. Surprisingly, in this in vitro system, total synthesis of telomeric DNA is actually increased in the presence of Pif1. However, telomerase processivity is reduced and Est2, is released from DNA in the presence of catalytically active Pif1. As shown by competition experiments, the displaced telomerase is still active and therefore able to lengthen another DNA oligonucleotide, explaining why total synthesis is higher even though telomerase processivity is reduced. Presumably, when Pif1 releases telomerase from telomeres in vivo, the concentration of telomeres is not high enough for the released enzyme to find another telomere in the narrow window of the cell cycle in which yeast telomerase is active. Given its facility at unwinding forked RNA/DNA hybrids in vitro [3], Pif1 might displace telomerase from DNA ends by unwinding the hybrid between telomerase RNA and single strand telomeric DNA. Since there is no Est2 detectable at telomeres in the absence of telomerase RNA [139,140], dissociating this hybrid is likely to release the protein subunits of telomerase. Alternatively, like certain other helicases that can remove proteins from DNA, Pif1 could displace the holoenzyme directly [138]. Another possibility is that the effects of Pif1 on telomerase are linked to its ability to unwind G-quadruplexes [36]. However, in vitro assays show that telomerase activity at telomeres is blocked by intramolecular G-quadruplexes [37,38,141]. Thus, it is difficult to imagine how Pif1's ability to unwind G-quadruplexes could explain its inhibitory effects on telomerase.

In addition to its telomeric and mitochondrial functions, Pif1 has more general roles in chromosomal DNA replication. Genetic [142] and biochemical [143] studies indicate that Pif1 has roles in Okazaki fragment maturation. Together with DNA polymerase *δ*, Pif1 contributes to the generation of long 5′ flaps on Okazaki fragments that are then cleaved by Dna2 [142–144]. Pif1 might also remove the last RNA primer during Okazaki fragment maturation at telomeres [142].

4.2. S. cerevisiae Rrm3: telomere functions

The second *S. cerevisiae* Pif1 family member (Fig. 1), *RRM3* (rDNA recombination mutant), was identified by two groups, one finding that mutation in Rrm3 results in increased rDNA recombination [145] and the other noting its sequence similarity to Pif1 [146]. Like Pif1, Rrm3 is found in both nuclei and mitochondria. However unlike Pif1, Rrm3 is not important for the maintenance of mitochondrial DNA [147,148]. Furthermore unlike Pif1, which is recruited to its sites of action (KP and VAZ, unpublished results), Rrm3 travels with the replication fork [149]. 2D gel analyses and genome-wide microarray studies demonstrate that efficient replication fork progression at specific sites is facilitated by Rrm3 helicase activity [56,146,149,150]. Replication forks slow at over 1000 genomic loci in *rrm3*Δ cells including tRNA genes, inactive replication origins, centromeres, multiple sites within each rDNA repeat, and, as discussed below, telomeres. All Rrm3 sensitive sites are incorporated into stable protein complexes. Disruption of these complexes eliminates Rrm3 dependence [150,151]. These findings suggest that Rrm3 uses its helicase activity to dissociate proteins that are bound to these sites.

At first glance, Rrm3 seems to have only modest effects on telomeres as telomeres are only slightly longer, and telomeric silencing is only modestly reduced in its absence [56]. However, 2D gel analysis of replication intermediates [56] and genome-wide studies of replication fork movement by DNA polymerase 2 occupancy [57] demonstrate that in wild type cells replication forks slow as they move through telomeric DNA. Pausing within telomeric DNA is exacerbated about 10-fold in *rrm3*Δ cells. Slow replication through

telomeres is not due to the terminal position of these sequences as replication through internal tracts of telomeric sequence is also slow in wild type cells, and this slowing is heightened in the absence of Rrm3. These studies were the first to demonstrate that semi-conservative replication of telomeric DNA is a problem for the conventional replication apparatus.

4.3. S. pombe Pfh1: telomere functions

Unlike *S. cerevisiae* and certain other fungi, *S. pombe* encodes only one PIF1 family helicase (Pfh1) (Fig. 1). Like its *S. cerevisiae* counterparts, Pfh1 is a 5′–3′ DNA helicase [132] that can unwind RNA/DNA as well as DNA/DNA duplexes and is more active on forked substrates [152]. Although neither of the *S. cerevisiae* Pif1 proteins is essential and even a *pif1*Δ *rrm3*Δ strain is viable [146], Pfh1 is essential [132,153]. Like the two *S. cerevisiae* Pif1 family proteins, Pfh1 is found in both nuclei and mitochondria, and its ATPase/helicase activity is essential in both compartments [126]. Although nuclear Pfh1 has roles in both repair and replication, only its replication function is essential. The best clue as to the nature of the essential nuclear function of Pfh1 is that it can be supplied by *S. cerevisiae* Rrm3, suggesting that Pfh1 might promote fork progression through hard to replicate sites [126]. However, Pfh1 may have a more general role in DNA replication as genetic assays suggest that Pfh1, like the *S. cerevisiae* Pif1, functions during Okazaki fragment maturation [152–154].

So far, the only evidence that Pfh1 functions at telomeres comes from telomere length analyses in Pfh1-depleted cells. When heterozygous *pfh*1+/*pfh*1Δ cells go through meiosis, the resulting *pfh1*Δ spore clones divide zero to four times before arresting, and telomeres in these *pfh1*Δ spore clones are modestly shorter than in wild type cells [128]. In a second study where Pfh1 expression was reduced to very low levels using a repressible promoter, there was no change in telomere length [126]. However, in more recent analyses, a more complete repression of Pfh1 results in telomere shortening (K.M., N. Sabouri, and VAZ, unpublished results). The question of whether or not Pfh1 affects telomeres is still not resolved, but given that its depletion does not result in telomere lengthening, it does not appear to be an inhibitor of telomerase like the *S. cerevisiae* Pif1.

4.4. Mammalian Pif1 family proteins

Like *S. pombe*, mouse (mPif) and humans (hPif) encode a single Pif1 family helicase (Fig. 1) [155]. An N-terminally truncated version as well as full length hPif1 protein have been purified and shown to unwind both DNA/DNA and DNA/RNA substrates with 5′–3′ directionality. However, hPIF is more active on forked structures that resemble replication intermediates [156–158]. In vitro hPif reduces telomerase processivity and binds preferentially to telomeric TTAGGG repeats [158]. mPIF is not essential because mice that are homozygously deleted for mPif1 are viable and show no differences in telomere length, DNA damage response, cell cycle progression, or chromosome integrity [159]. Thus, if mPIF is important in vivo, its activity is likely redundant with that of other helicases/translocases.

In cultured human cells, hPIF is a low abundance protein that shows dramatic cell cycle regulation with significant expression limited to late in the cell cycle [155]. This expression pattern is due to ubiquitin-mediated degradation via the anaphase promoting complex, a pattern of regulation that hPIF shares with the *S. cerevisiae* Pif1 [155,160]. However, not all Pif1 family proteins are cell cycle regulated, as levels of *S. cerevisiae* Rrm3 are constant throughout the cell cycle [149].

There are conflicting in vivo data for hPif function at telomeres as in one study [158] but not in another [155], over-expression of hPIF in tissue culture cells results in telomere shortening. An additional indication of mammalian PIF function at telomeres comes from co-immunoprecipitation experiments in which both mouse [159] and human [155] PIF proteins are associated with the catalytic subunit of telomerase.

5. FANCJ helicases

The SFI FANCJ family of 5′–3′ DNA helicases is the most recently identified helicase family with telomere effects. The human FANCJ helicase, which was originally called BACH1/BRIP1, was first identified as a DNA helicase that interacts with BRCA1, the product of a human gene whose mutation is associated with a high incidence of early onset breast cancer [161]. Like BRCA1 mutations, FANCJ mutations can also lead to an inherited predisposition to early onset breast cancer [162]. However, FANCJ is probably best known for being one of 13 genes whose mutation leads to the human genetic disorder Fanconi anemia (FA) [163–165]. FA is characterized by genome instability, especially hyper-sensitivity to inter-strand DNA cross-linking agents, such as mitomycin C. FA patients suffer bone marrow failure and increased cancer rates.

BLAST analysis and alignment to FANCJ and RTEL family members show that FANCJ family helicases are also found in some prokaryotes and single-celled eukaryotes. Widespread in metazoans, they are best studied in humans, but also in mouse, chicken and *C. elegans*. The family includes the FANCJ proteins themselves as well as the more distantly related Dog1 protein (*C. elegans*) and the RTEL helicases (Fig. 1). *C. elegans*, as well as some vertebrates, encodes several FANCJ family proteins, FANCJ itself (*dog-1* in *C. elegans*) and RTEL. In general, FANCJ-like helicases function in response to DNA damage, DNA repair, and maintenance of genomic stability (review [166,167]).

In almost all studied FANCJ helicases, a Fe–S cluster is present in the ATP binding domain that is essential for helicase activity and recognition of damaged DNA [168,169]. The BRCA1 binding site, which is important for DNA repair, is found at the C-terminus of the human and mouse FANCJ proteins (reviewed in [167]).

5.1. C. elegans DOG-1 helicase

Although the *C. elegans* Dog-1 protein is 32% identical to human FANCJ over the helicase domain, they differ in size with Dog-1 being 983 amino acids and FANCJ being 1249 amino acids in length. This size difference is due to the lack of the BRCA1 interaction domain at the C-terminus of Dog-1 [170,171]. The *Dog-1* gene (deletions of guanine-rich DNA) was studied initially because of its sequence similarity to mammalian RTEL (see Section 5.3.2). Its mutation yields a mutator phenotype and reduced brood size [172]. Closer examination of the *dog-1* associated mutations led to the realization that they are largely due to deletions that initiate throughout the genome in tracts of G-rich sequences with the potential to form G-quadruplex structures [172,173]. In some loci, mutation frequencies are as high as 4% per animal generation [173], and the mutation frequency correlates positively with the length of the G-rich tract that initiates the deletion [172]. In addition, large chromosomal rearrangements are detected genome-wide in *dog-1* mutants [174]. Similar to what is seen in humans defective for FANCJ, *dog-1* mutants are hypersensitive to DNA cross linking agents [170]. *Dog-1* mutations do not have dramatic effects on telomere length, perhaps because the *C. elegans* telomeric repeat (TTAGGC) has low G-quadruplex forming potential [172].

5.2. Human FANCJ

In vitro, human FANCJ preferentially binds and unwinds forked duplex substrates. Given its anticipated roles in recombination and repair, it is perhaps surprising that it cannot unwind Holliday junctions. However, it does unwind D-loop structures, another potential recombination intermediate [175]. Purified FANCJ also unwinds G-quadruplexes [176–178]. Cell lines from human FA patients with mutations in FANCJ show accumulation of genomic deletions that overlap predicted G-quadruplex motifs [178]. Moreover, telomestatin, a compound that stabilizes G-quadruplexes in vitro [179–181], causes impaired proliferation and elevated levels of apoptosis and DNA damage in FANCJ-deficient cells [177]. Although these data are consistent with a telomeric role for FANCJ, there are as yet no experiments that link the helicase directly to telomeres.

5.3. RTEL helicases

Mouse RTEL (regulator of telomere length) is the founding member of this helicase family [182]. This gene was first identified as a locus that affects telomere length in crosses between mouse species with different starting telomere lengths [183]. As described below, human RTEL is the only family member that has been subjected to biochemical analysis. Human RTEL has ATPase activity [184], but no helicase assays have been reported.

5.3.1. C. elegans RTEL—Like mammals, *C. elegans* encodes a second FANCJ family protein called RTEL [184]. This protein is 32% identical (62% similar) over the entire ORF to the *C. elegans* FANCJ homolog *Dog-1* and 31% identical to the human RTEL1 ORF. It was identified in a genetic strategy to find *C. elegans* helicases with an anti-recombination activity similar to that of the *S. cerevisiae* Srs2 helicase. Since *S. cerevisiae srs2 sgs1* double mutants are not viable, the authors searched for helicases whose mutation confers a synthetic lethal phenotype on *him-6* deficient worms (*him-6* encodes the *C. elegans* BLM, a RecQ helicase). This analysis identified *rtel-1* as a gene that is essential in a *him-6* deficient background [184].

Although the RTEL1 protein has no sequence similarity to the *S. cerevisiae* Srs2 helicase, genetic and biochemical studies indicate that it too inhibits homologous recombination. For example, in meiosis, *rtel-1* deficient worms have large numbers of *RAD51* foci, which are diagnostic for recombination events as well as elevated rates of recombination. Despite its similarity to *Dog-1*, the functions of the two FANCJ family helicases are different as G-rich tracts are not unstable in *rtel-1* deficient cells. So far, the *C. elegans rtel-1* has not been linked to telomeres.

5.3.2. Mammalian RTEL—Telomeres in laboratory mice (*M. musculus*) are much longer than in wild type *Mus spretus* mice. Because the two species are inter-fertile, they were crossed to identify genes involved in telomere length regulation. From linkage analysis, a region on chromosome 2 was identified that when derived from the *M. musculus* parent acts in a dominant manner to elongate telomeres. Although the interval had no previously identified telomere factor, it encodes a helicase-like gene that is highly conserved among mammals, which was named RTEL (regulator of telomere length) [182,183]. In humans, the RTEL locus encodes a protein that is 27% identical (55.5% similar) over its entire ORF to human FANCJ. The amino terminal 750 amino acids of the predicted ~1200 amino acid protein contain the seven helicase motifs.

Subsequent genetic analyses showed that the mouse RTEL is an essential gene, with homozygous null animals dying early in embryogenesis with abnormalities in multiple organs [182]. Homozygous null ES cells are viable and have variable length telomeres that are ~70% shorter than in wild type ES cells. If the null ES cells are allowed to differentiate,

the differentiating cells display massive genome instability, including telomere signal free ends, chromosome fusions, broken chromosomes, and multi-chromosome fusions. Some of the chromosomal abnormalities suggest that RTEL functions at non-telomere sites as well as at telomeres. Because mammalian RTEL shares sequence similarity to *C. elegans Dog-1*, which is needed to maintain G-rich DNA (see Section 5.1), the authors asked if non-telomeric G-rich internal tracts are unstable in RTEL null ES cells. However, none of the more than 30 G-rich sites examined in this study had deletions or insertions [182].

One suggestion to explain the telomeric role of RTEL is that it resolves G-quadruplex structures formed during DNA replication. In support of this idea, mouse cells depleted for RTEL using siRNA have a fragile telomere phenotype similar to that seen in TRF1 or BLM depleted cells [60]. Another possibility is that RTEL regulates telomere length by inhibiting telomere recombination. This possibility is supported by biochemical studies using purified human RTEL that were inspired by genetic studies that suggested that the *C. elegans rtel-1* inhibits recombination in vivo [184]. Depletion of human RTEL1 with siRNA in cultured cells results in a four-fold increase in DSB repair via homologous recombination. In vitro, purified human RTEL1 has ATPase activity and can prevent D-loop formation as well as disrupting preformed D-loops. However, unlike the *S. cerevisiae* Srs2, human RTEL1 does not disrupt Rad51-DNA filaments vitro. Human *Rtel* is amplified in certain gastric tumors [185], but RTEL function during tumor development is not known.

6. Dna2 helicases

The SFI Dna2 family of 5′–3′ DNA helicases was first discovered in *S. cerevisiae* as an essential gene required for complete synthesis of chromosomal DNA due its role in Okazaki fragment processing (reviewed in [186]). Dna2 is conserved throughout eukaryotes, and it has been well studied in *S. cerevisiae*, *S. pombe*, *C. elegans*, and humans [154,186–191]. The Dna2 helicase motifs are located at the C-terminus, while the N-terminal region is not conserved in size or sequence [192,193]. Although Dna2 helicase motifs are well defined, the detection of helicase activity in human Dna2 is disputed, and it has not been possible to demonstrate helicase activity for purified *S. pombe* or *Xenopus* Dna2 [153,186,194,195]. Moreover, the helicase activity of *S. cerevisiae* Dna2 is not essential in some growth conditions, suggesting it may not be necessary for Okazaki fragment maturation [196]. A single stranded DNA specific endonuclease activity is an essential feature of Dna2 in *S. cerevisiae* and *S. pombe* [197,198].

Genetic and biochemical studies show that in *S. cerevisiae* and *S. pombe*, the Dna2 endonuclease has a genome-wide role in processing the 5′ ends of “long flap” Okazaki fragments. These flaps are generated by DNA pol δ extending the 3′ end of an Okazaki fragment and thereby displacing the 5′ end of the adjacent Okazaki fragment. Genetic and biochemical data indicate that the *S. cerevisiae* Pif1 helicase, described in Section 4.1, participates in generating these long flaps [142], and its deletion can suppress the lethality of *dna2*Δ cells [142]. Human Dna2 is found in both nuclei and mitochondria and has key roles in replication of both genomes [199,200].

6.1. Telomeric roles of S. cerevisiae and S. pombe Dna2 proteins

So far, telomere effects of Dna2 proteins have been reported only in *S. cerevisiae* and *S. pombe*. The N-terminus of *S. cerevisiae* Dna2 was identified as one of ten genes whose over-expression reduces telomeric silencing (DOT gene, disruptor of telomeric silencing) [201]. Over-expression of Dna2 also results in telomere shortening and increased levels of single stranded G-overhangs [196,202]. Levels of telomere silencing correlate positively with telomere length in wild type cells [5]. Thus, the telomere shortening that results from over-expressing Dna2 may explain why overexpression decreases silencing. Multiple

different mutations in *dna2* result in telomere lengthening [196]. Reduced Dna2 levels also result in faster senescence of telomerase deficient cells and faster appearance of type II survivors [203].

Although more complicated models are also possible, it is not unreasonable that all of the effects of Dna2 on telomere structure are due to its role in Okazaki fragment processing [204], as impaired lagging strand replication is expected to inhibit C-strand resynthesis and thereby affect G-tails. Changes in G-tails could in turn affect telomerase access. Dna2 likely affects telomeres directly as it localizes to telomeres by both one-hybrid and ChIP analyses, and this association is cell cycle regulated [203]. Finally, in vitro *S. cerevisiae* and human Dna2 can unwind G-quadruplex DNA structures [205], providing the only suggestion that the helicase activity, rather than the Dna2 endonuclease activity, has a role in telomere biology.

In *S. pombe*, like *S. cerevisiae*, Dna2 is a flap endonuclease that is important for Okazaki fragment maturation and hence viability [198]. That this activity is important for telomeric G-tails comes from their loss in *dna2-C2* mutant cells growing at semi-permissive temperatures [206]. Since wild type Dna2 binds telomeres as shown by ChIP, and this binding is reduced in *dna2-2* mutants at high temperatures, *S. pombe* Dna2 likely affects telomeres directly. Telomeres progressively shorten, and telomerase telomere binding is reduced in *dna2-2* cells growing at semi-permissive temperatures. Thus, the data from both *S. cerevisiae* and *S. pombe* indicate that Dna2 is important for maintaining G-tails and telomerase-mediated telomere replication. It is possible in both organisms that the effects of *dna2* mutants on telomeres are due to faulty Okazaki fragment maturation.

7. Future directions

Helicases are critically involved in all processes involving nucleic acids. This dependence is true throughout the genome but is likely to be particularly important for G-rich and shelterin bound telomeric DNAs. To date, members of four helicase families – RecQ, Pif1, FANCJ, and DNA2 – are known to have roles in telomere biology. Mutations in RecQ and FANCJ helicases cause inherited human diseases, characterized by genome instability, increased cancer susceptibility, and premature aging. Given the relatively young age of telomere-helicase research and the large number of eukaryotic helicases, there are likely to be additional helicases with telomeric roles that are important for human health and longevity.

One critical area of helicase-telomere research over the next years is to elucidate the role of G-quadruplex structures in telomere biology. Do G-quadruplex structures form during semi-conservative telomere replication? If so, does their formation affect replication fork progression? Does the demonstrated ability of certain helicases to unwind G-quadruplex structures in vitro explain their in vivo effects on telomeres? Another interesting question is whether the unusual chromatin structure of telomeres impacts their replication in organisms other than *S. cerevisiae*, and if so does their replication involve helicases, such as the *S. cerevisiae* Rrm3, with the specialized ability to bypass stable protein–DNA structures. Identifying helicases that regulate telomerase is another important area. So far only the *S. cerevisiae* Pif1 is known to have a direct effect on telomerase. Do other Pif1 family helicases affect telomerase? The case is not yet made, but there are intriguing links between mammalian Pif proteins and telomerase. The exact roles of human RecQ helicases in ALT may clarify the roles of these proteins in cancer. Another intriguing question is to determine if (and if so, how) the *S. cerevisiae* and *S. pombe* subtelomeric helicases contribute to the recombination that maintains telomeres when telomerase is not active as well as to determine if subtelomeric helicases are a general feature of telomeric DNA. The involvement of helicases in TERRA is totally unexplored and a likely area for exciting

findings. Future experiments will continue to elucidate the diverse and important roles that helicases perform in telomere metabolism and will lead to the identification of additional helicases with telomere functions.

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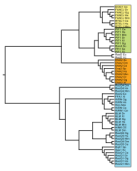

Fig. 1.

Sequence alignments of the RecQ, Pif1, FANC-J, and DNA2 family helicases. Sequences were aligned using ClustalX, and the phylogenetic relationship among them was drawn as a rooted tree using the unrelated human beta actin protein (NP_001092) as an outgroup (not shown) with TreeView v. 1.6.6. software (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). Tree lines are not scaled. Helicase family members from the following organisms were aligned: *Caenorhabditis elegans* (Ce), *Danio rerio* (Dr), *Drosophila melanogaster* (Dm), *Gallus gallus* (Gg), *Escherichia coli* (Ec), *Homo sapiens* (Hs), *Mus musculus* (Mm), *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Vibrio cholera* (Vc), *Xenopus laevis* (Xl). *Drosophila melanogaster* has four different annotated PIF1A isoforms (designated A–D; PIF1B is an unrelated protein), but none of the isoforms closely align to any known PIF1 helicase member. It is unclear if this is due to the misannotation of PIF1A or some other problem. Due to this discrepancy, PIF1 Dm is not shown in the tree. For each organism all related proteins are listed, if annotated as such in the database. The GenBank accession numbers are as follows: BLM Dm, AAD41441; BLM Dr, XP_701357; BLM Gg, NP_001007088; BLM Hs, NP_000048; BLM Mm, NP_001035992; BLM Xl, NP_001079095; DNA2 Ce, NP_496515; DNA2 Dm, ACS78060; DNA2 Dr, CAX13876; DNA2 Gg, NP_001006497; DNA2 Hs, AAI11741; DNA2 Mm, AAI15717; DNA2 Sc, AAB68010; DNA2 Sp, CAB38508; DNA2 Xl, NP_001079231; DOG1 Ce, NP_493618; FANCJ Dr, ABO27623; FANCJ Gg, Q3YK19; FANCJ Hs, NP_114432; FANCJ Mm, Q5SXJ3; HIM6 Ce, AAM26298; PIF1 Hs, NP_079325; PIF1 Ce, BAA28677; PIF1 Dr, NP_942102; PIF1 Gg, XP_426648; PIF1 Mm, AAH46611; PIF1 Sc, CAA86260; Rrm3 Sc, NP_011896; PIF1 Sp, NP_596488; PIF1 Xl, AAZ41379; RecD Bc, YP_085716; RecD Ec, CAQ33145; RecD Vc, NP_231950; RecQ Ec, YP_002331581; RecQ1 Ce, AAK21428; RecQ1 Dr, NP_001038561; RecQ1 Gg, NP_989724; RecQ1 Hs, NP_002898; RecQ1 Mm, NP_075529; RecQ4 Dm, AAF42939; RecQ4 Hs, NP_004251; RecQ4 Mm, BAD11131; RecQ5 ce, CAA86232; RecQ5 Dm, AAD43051; RecQ5 Gg, BAI79325; RecQ5 Hs, NP_004250; RecQ5 Mm, BAD11130; Rqh Sp, CAA91177.1; RTEL1 Hs, NP_116575; RTEL1 Ce, NP_492769; RTEL1 Mm, AAI44979; Sgs1 Sc, AAB60289.1; WRN Gg, BAI79323; WRN Hs, AAC63361; WRN Mm, AAH60700; WRN Xl, NP_001081838; WRN1 Ce, NP_495324.

Table 1

Summary of helicase functions at telomeres.

Function	RecQ	Pif1	FancJ	Dog-l	RTEL	Dna2
Telomere binding	(✓) hWRN	✓ (ScPifl, ScRrm3, hPif)				✓ (SpDna2)
Telomerase regulation/interaction		✓ (scPifl, hPif, mPif)				
Homologous recombination at telomeres	✓ (hBLM, hWRN, mWRN, SpTlh, Rqhl?, Sgsl)		✓ (hFANCJ?)		✓ (all)	
Telomere length regulation by ALT	✓ (hBLM, hWRN, mWRN, SpTlh, Rqhl?, Sgsl)					✓ (scDNA)
Telomere semi-conservative replication	✓ (WRN?, hBLM?, Rqhl?)	✓ (scRrm3)		(Dog-l?)	✓ (mRTEL?)	✓ (scDna2)
G-quadruplex unwinding/regulation	✓ (hWRN, hBLM, Sgsl)	✓ (scPifl)	✓ (hFANCJ)	✓ (Dogl)		✓ (hDNA2, scDna2)
Telomere length regulation	✓ (hBLM, hWRN)	✓ (scPifl, ScRrm3, Pfhl?, hPifl?)		(Dog-l?)	✓ (mRTEL)	✓ (scDNA2)
Telomeric overhang	✓ (hWRN, Sgsl)	(scPifl?)				✓ (hDna2, SpDna2, ScDna2)
Preventing telomere fusion	✓ (Rqhl?)				✓ (mRTEL)	

Columns indicate helicase families. Rows indicate predicted function at telomeres. Checkmark (✓) indicates a direct role for this helicase family at this telomere event. In parenthesis are the names of the specific helicase shown to have this functional activity. ? indicates a modest or indirectly demonstrated effect on this telomere event.