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MINIREVIEW

To Peep into Pif1 Helicase: Multifaceted All the Way from Genome Stability to Repair-Associated DNA Synthesis

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Pif1 DNA helicase is the prototypical member of a 5′ to 3′ helicase superfamily conserved from bacteria to humans. In *Saccharomyces cerevisiae*, Pif1 and its homologue Rrm3, localize in both mitochondria and nucleus playing multiple roles in the maintenance of genomic homeostasis. They display relatively weak processivities *in vitro*, but have largely non-overlapping functions on common genomic loci such as mitochondrial DNA, telomeric ends, and many replication forks especially at hard-to-replicate regions including ribosomal DNA and G-quadruplex structures. Recently, emerging evidence shows that Pif1, but not Rrm3, has a significant new role in repair-associated DNA synthesis with Polδ during homologous recombination stimulating D-loop migration for conservative DNA replication. Comparative genetic and biochemical studies on the structure and function of Pif1 family helicases across different biological systems are further needed to elucidate both diversity and specificity of their mechanisms of action that contribute to genome stability.

Keywords: Pif1 helicase, *Saccharomyces cerevisiae*, genome stability, replication, recombination

Introduction

Helicases are essential for nearly all nucleic acid metabolic transactions in living organisms that transfer genetic materials to their offspring. They are referred to as molecular motor proteins that translocate along DNA phosphodiester backbone unidirectionally using the energy of NTP hydrolysis to separate stable DNA duplex into single strands (Patel and Donmez, 2006; Lohman *et al.*, 2008). Most helicases have common biochemical features as above and are classified into six major superfamilies according to the conserved signature motifs and consensus sequences they contain (Caruthers and McKay, 2002; Tuteja and Tuteja, 2004; Singleton *et al.*, 2007). A variety of helicases exist even in one single cell type as approximately 1% of the genes of many eukaryotic genomes code for them, suggesting that multiple helicases have different structural requirements of the substrate at various aspects of DNA metabolism including replication, transcription, repair, chromosome segregation, and telomere maintenance (Bernstein *et al.*, 2010; Wu, 2012). Since the first discovery of DNA helicase in *E. coli* in 1976, a growing number of helicases have been isolated from bacteria to higher eukaryotes, and even in viruses (Abdel-Monem *et al.*, 1976; Tuteja and Tuteja, 2004). The human genome encodes for 95 non-redundant helicases and current estimate in budding yeast reaches at least over 120, which corresponds to ~2% of its genome (Shiratori *et al.*, 1999; Ribeyre *et al.*, 2009; Umate *et al.*, 2011). Special attention has been drawn to understanding the functions of diverse helicases with the findings that several inherited human disorders are caused by mutations in genes for helicases (Ellis, 1997; Bessler *et al.*, 2001).

Pif1, as a prototypical member of Pif1 family of 5′ to 3′-directed DNA helicases, belongs to superfamily 1 (SF1) helicases and is widely found in nearly all eukaryotes from yeast to humans as well as in diverse bacteria, and has multiple roles both in nuclear and mitochondrial genome maintenance including Okazaki fragment processing, telomere homeostasis, and resolving G-quadruplex (G4) structures, using its helicase activity that unwinds duplex DNA or disrupts stable nucleoprotein complexes (Lahaye *et al.*, 1991, 1993; Zhou *et al.*, 2000; Budd *et al.*, 2006; Ribeyre *et al.*, 2009; Bochman *et al.*, 2011). Furthermore, many intriguing findings were recently shown that Pif1 acts as a suppressor of DNA damage preventing replication pausing and double-strand breaks (DSBs) at G4 motifs, as well as it inhibits gross chromosomal rearrangement (GCR) by regulating telomerase action at DSBs, suggesting that Pif1 has indispensable roles for maintenance of genome stability (Makovets and Blackburn, 2009; Lopes *et al.*, 2011; Paeschke *et al.*, 2011). Above and beyond the unique functions of Pif1 helicase that are currently understood, this review highlights very recent evidence that Pif1 has wider roles than previously appreciated, especially in DSB repair-associated DNA replication. A subsidiary goal of this review is to compare distinctive activities of several Pif1 family members in different model systems and, accordingly, to extend our understanding of their functional diversity to maintain genomic integrity in the end.

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Maintenance of mitochondrial genome

The *Saccharomyces cerevisiae* Pif1 helicase was originally isolated 30 years ago in a genetic screen for mutations that affects recombination frequency between mitochondrial *rho*$^+$ and *rho*$^-$ genomes having tandemly arrayed repeat units (Foury and Kolodynski, 1983). In Pif1-deficient cells, mitochondrial DNA (mtDNA) recombination is reduced, after which this gene (petite integration frequency) was named, and mtDNA is more frequently lost than in wild-type (WT) cells especially at high temperatures, implying that Pif1 is crucial for mtDNA replication and recombination (Foury and Van Dyck, 1985; Lahaye *et al*., 1991; Van Dyck *et al*., 1992). Mitochondrial genome is much more susceptible to damages than nuclear DNA as reported that the rate of mutations in mtDNA is approximately 10 times greater than that in chromosomal DNA (Linnane *et al*., 1989). This is probably because mtDNA is not protected by histones, unlike nucleosomes in the nucleus, and oxidative damage-causing reactive oxygen species (ROS) are continuously produced in the mitochondria due to the cellular respiration by oxidative phosphorylation. In *pif1* mutant, cells are sensitive to ethidium bromide (EtBr)-induced DNA damage and mtDNA is subject to fragmentation and the ensuing loss (O'Rourke *et al*., 2005; Cheng *et al*., 2007). Pif1 is physically associated with the entire mitochondrial genome and its effects are likely direct, proposing that Pif1 either prevents or repairs DSBs in mtDNA, as mtDNA breaks are observed at specific sites under natural conditions in cells lacking Pif1 (Cheng *et al*., 2007).

While metazoans and most higher eukaryotes contain only one Pif1 helicase, *S. cerevisiae* encodes two Pif1 family members, Pif1 and Rrm3 (Ivessa *et al*., 2000; Bochman *et al*., 2010). *RRM3* gene encodes another 5′ to 3′ directed DNA helicase and was first identified by its mutation causing elevated recombination in the ribosomal DNA (rDNA) in the nucleus, but later also known to localize to mitochondria by mitochondrial proteome analysis (Keil and McWilliams, 1993; Prokisch *et al*., 2004). Although both Pif1 and Rrm3 are structurally similar and contribute to genomic integrity by acting on common DNA targets, they have non-overlapping and sometimes opposing roles in mitochondria. Although *rrm3* strains have no evident mitochondrial defect, additional deletion of *RRM3* partially rescues the accelerated loss of mtDNA in *pif1* mutant cells, suggesting that Pif1 and Rrm3 are both involved in the maintenance of mtDNA, but in distinct ways (Ivessa *et al*., 2000; O'Rourke *et al*., 2005). These paralogs also show discrepancies in other biological functions in replication in the rDNA as well as in telomeres, which will be discussed later in the part of their nuclear functions. These differences are probably due not only to the helicase domain but also to the divergent amino-terminal sequences, as mutant alleles of *RRM3* lacking N-terminus show similar phenotypes to Rrm3-deficient cells (Bessler and Zakian, 2004). It seems, at least, that the sequences with high similarity between Pif1 and Rrm3 (40% identity and 60% similarity) explain why they could work on common DNA substrates (Bessler *et al*., 2001).

The fission yeast *Schizosaccharomyces pombe* has only one Pif1 family helicase, Pfh1, and, unlike *pif1* mutant cells in budding yeast, Pfh1-deficient cells are not viable (Tanaka *et al*., 2002). This result seems not plausible because even *pif1 rrm3* double mutant is not lethal in *S. cerevisiae*, but, in a sense, acceptable from the reports that *S. pombe* is not able to survive without mtDNA unlike *S. cerevisiae*, and Pfh1-deficient cells quickly lose mtDNA (Ivessa *et al*., 2000; Pinter *et al*., 2008). Moreover, the growth of mutant spores depleted for Pfh1 is arrested showing terminal phenotypes of DNA replication defect, suggesting that Pfh1 has essential roles in chromosomal DNA replication (Zhou *et al*., 2002). In the absence of Pfh1, telomeres are modestly shortened (~50 bp), but this does not seem to explain why *S. pombe* cells require Pfh1 for viability because Pfh1 is still essential in cells with circularized chromosomes (Zhou *et al*., 2002).

Inhibition of telomere formation

Pif1 was rediscovered as a negative regulator of *de novo* telomere formation and telomere elongation in yet another screen to detect mutants that frequently lose subtelomeric gene expression (Schulz and Zakian, 1994). In the absence of Pif1, all telomeres increase in length by ~75 base pairs (Schulz and Zakian, 1994). Pif1 strongly inhibits telomerase-mediated telomere addition not only to chromosome ends but also to intrachromosomal DSBs as *de novo* telomere addition to a broken yeast artificial chromosome (YAC) increases ~600 fold in cells lacking Pif1, and this still occurs ~180 fold more frequently at the break of yeast intrinsic chromosomal DNA in the absence of Pif1 in *rad52* mutant background (Schulz and Zakian, 1994; Mangahas *et al*., 2001). Moreover, a *pif1* deletion mutant is also referred to as a mutator mutant as it displays approximately 1,000-fold increase in GCR rate in a telomerase-dependent manner (Myung *et al*., 2001). Overproduction of Pif1 protein in *pif1* mutants restores mtDNA recombination proficiency and reduces telomere length modestly, but is toxic to yeast cells as observed by slower growth (Lahaye *et al*., 1991; Zhou *et al*., 2000; Vega *et al*., 2007). Overexpression of Pif1 results in reduced viability of *cdc13-1* and Ku-deficient strains and this phenotype was suppressed by additional mutations in *EXO1*, which encodes a telomere-degrading exonuclease, suggesting that the removal of telomerase by Pif1 aggravates the reduced telomere end protection (Vega *et al*., 2007). Pif1 reduces the processivity of telomerase and directly dissociates telomerase from telomeric ends, and this inhibition is totally dependent on its helicase activity, as cells expressing the catalytically-inactive Pif1-K264A, in which the Lys residue in the Walker A box is replaced with Ala, have the same phenotype as *pif1* deletion mutants (Boule *et al*., 2005). Pif1 is not able to bind RNA, but is preferentially active on the forked RNA-DNA hybrids rather than DNA-DNA substrates, probably making its role fit in releasing *TLC1*, the RNA component of telomerase, out of telomeric ends using its catalytic helicase activity (Boule *et al*., 2005; Zhang *et al*., 2006). Although it was demonstrated by chromatin immunoprecipitation (ChIP) analysis that Pif1 dissociates telomerase at least through direct removal of Est2, the catalytic subunit of telomerase complex, the mutant analysis of Est2 finger subdomain also showed that Est2 facilitates the inhibitory action of Pif1 at telomeres in conjunction with *TLC1* RNA (Boule *et al*., 2005; Eugster *et al*., 2006; Boule and Zakian, 2007).

It was not clear whether cells can distinguish intrachromosomal DSBs from telomeres in terms of regulating telomerase activity. Since telomerase possibly interrupt correct DSB repair by adding *de novo* telomeres to the break inappropriately, cells likely need to be armed with an inhibitory mechanism of telomerase activity in response to DNA damage for the accurate repair. Recently, it has been reported in *S. cerevisiae* that DNA damage signaling gives rise to phosphorylation of Pif1, which blocks telomerase activity at DSBs but not at chromosome ends (Makovets and Blackburn, 2009). A short amino acid sequence (TLSSAES) in the C-terminus of Pif1 is the target of phosphorylation by Mec1- and Rad53-dependent checkpoint signaling, but Rad53 is not required for the physical recruitment of Pif1 to DSBs, indicating that DNA damage response pathway is crucial for Pif1 phosphorylation but not its localization. In fact, it has already been expected from the results by Myung *et al.* (2001) that *mec1 sml1* mutant shows highly elevated (~200 fold) GCR rate, which is as much rate shown in *pif1-m2* mutant, although it seems not solely dependent on telomerase activity, suggesting that Mec1 kinase-dependent signaling pathway is involved in the regulation of telomere activity at DSBs. Moreover, it is possible that cells have more Mec1-dependent telomerase-inhibiting mechanisms other than just Pif1 phosphorylation, based on the results that *mec1 sml1* mutant in the absence of Pif1 shows even higher level of GCR rate and *de novo* telomere addition (Myung *et al.*, 2001; Makovets and Blackburn, 2009). In addition to searching for novel telomerase-inhibiting mechanisms, it remains to be elucidated whether this pathway is conserved in higher organisms.

S. cerevisiae PIF1 gene encodes two isoforms of enzyme that are expressed from two different in-frame translational start codons. Pif1-m1 isoform with mutation of the first methionine causes unstable mitochondria, but telomere length is normal because it localizes only in the nucleus, whereas the other with mutation of the second methionine produces only a longer mutant form, Pif1-m2, that localizes in mitochondria with mitochondrial targeting signal (MTS) between first and second start codons, causing longer telomeres (Schulz and Zakian, 1994). It was demonstrated by Western blot analysis, however, that mitochondrial form, Pif1-m2, migrates faster than nuclear form after cleavage of N-terminal MTS (Zhou *et al.*, 2000; Vega *et al.*, 2007).

Unlike the telomerase activity-dependent high rate of GCR shown in *pif1* mutant, mutation of *RRM3* gene affects neither the GCR rate nor *de novo* telomere addition (Myung *et al.*, 2001; Ivessa *et al.*, 2002). However, Rrm3 is still associated with telomere *in vivo* and has an important role in timely replication of telomeric DNA affecting telomere length (Ivessa *et al.*, 2002).

The amino acid sequence of human PIF1 shows almost equal homology to both *S. cerevisiae* Pif1 and Rrm3 (Mateyak and Zakian, 2006). Human PIF1 binds and unwinds stalled replication fork-like DNA substrates *in vitro* (George *et al.*, 2009). It also interacts with hTERT, the catalytic subunit of telomerase, *in vitro* and *in vivo*, and reduces telomere length when overexpressed, demonstrating that it is functionally related more to *S. cerevisiae* Pif1 than to Rrm3 at telomere region (Mateyak and Zakian, 2006; Zhang *et al.*, 2006). In mice, however, Pif1 is completely different from *S. cerevisiae* Pif1 in both genetic and biochemical properties. The expression of Pif1 is highly restricted so that it is detected only in highly proliferating embryonic stem cells. Murine Pif1 associates with telomerase but telomere elongation activity is not affected *in vitro* and *mPif1*$^{-/-}$ knockout strain displays neither phenotypic abnormality nor alteration of telomere length even after four generations (Snow *et al.*, 2007).

Okazaki fragment maturation

Because of the antiparallel structure of duplex DNA, cells need to employ two distinct mechanisms for replication. While leading strand is extended continuously toward the replication fork, lagging strand DNA replication is the consecutive processes with creation and joining of a series of segments, called Okazaki fragments (Bambara *et al.*, 1997). These short stretches of DNA begin with RNA-DNA primers, in which synthesis of RNA (~10 nt) by primase is followed by Polα-mediated short extension of DNA (~20 nt), and are further extended by Polδ to form ~150-nt discrete DNA fragments (Bullock *et al.*, 1991; Nethanel *et al.*, 1992; Rossi *et al.*, 2006; Kang *et al.*, 2010). In *S. cerevisiae*, Polδ produces about a hundred thousand Okazaki fragments throughout the genome during each cell cycle, and each of these fragments needs to be joined into a continuous DNA strand with high fidelity to avoid accumulation of unrepaired nicks leading to DSBs and cell lethality (Garg *et al.*, 2004).

The removal of initiator RNA and gap filling during Okazaki fragment maturation occur through sequential action of several different enzymes. Polδ processively extends Okazaki fragments in the 5′ to 3′ direction to their full length. Upon encountering the 5′ end of the downstream Okazaki fragment, Polδ displaces it into a short single-stranded flap, which is then cleaved by Fen1 (flap endonuclease 1 encoded by *RAD27*), forming a ligatable nick to be eventually sealed by DNA ligase I (Garg *et al.*, 2004; Rossi *et al.*, 2006). If the displaced flap escapes cleavage by Fen1 and reaches the size longer than 30 nt, however, it is bound by replication protein A (RPA), the yeast homolog of *E. coli* single-stranded DNA binding protein, SSB (Maga *et al.*, 2001). Once the flap is coated by RPA, it inhibits cleavage by Fen1 but stimulates cleavage by Dna2 (Bae *et al.*, 2001; Kao *et al.*, 2004). Dna2 is a well-conserved essential endonuclease/helicase and its biochemical preference to 5′ single-stranded flap is well-suited to process Okazaki fragments (Bae *et al.*, 1998).

Evidence that Pif1 has a role in Okazaki fragment processing was originally given from genetic studies with *S. pombe*, where temperature-sensitive (ts) allele of Cdc24, a protein that forms a complex with Dna2, is suppressed by mutants within *pfh1*$^{+}$, a homologue of *S. cerevisiae PIF1*, and the ts growth defect of *dna2-C2* mutant is suppressed by *pfh1-R20*, a cold-sensitive (cs) mutant allele of *pfh1* (Tanaka *et al.*, 2002; Ryu *et al.*, 2004). Later, the similar genetic interaction was observed in *S. cerevisiae*, as *pif1* mutant suppresses the lethal phenotype of *dna2* mutant at 30°C, but not at higher temperatures (Budd *et al.*, 2006). Moreover, the *pif1 dna2* double mutant is viable even at 37°C with additional deletion of *POL32*, which encodes nonessential subunit of Polδ, suggesting that Pif1 and Pol32 contributes to the displace-

ment of downstream Okazaki fragment and the production of longer flaps by stimulating the processivity of polδ (Budd *et al*., 2006; Stith *et al*., 2008). This proposes that the activities of Pif1 and Pol32 during the lagging strand synthesis create the intermediate substrates that require Dna2 activity to complete DNA replication.

Resolving G-quadruplex structures

Genomic DNA can adopt a variety of unconventional secondary structures such as Z-DNA, cruciforms, and G-quadruplexes (G4). Among these, G4 structures can be the major obstacles in performing a faithful replication as they are very resistant to thermal denaturation and have potential to impede the progression of replication forks. Especially single-stranded regions between Okazaki fragments during the lagging strand DNA synthesis are prone to form G4 structures, which slow down replication and easily leads to fork collapse and chromosome breaks (Bochman *et al*., 2012). They are named because they can form an unusual four-stranded structure in which four guanine bases associate together through cyclic Hoogsteen hydrogen bonding to form a square planar structure (Sunquist and Klug, 1989; Lipps and Rhodes, 2009). In addition to *in vivo* evidence by electron microscopy that G4 DNA forms cotranscriptionally upon G-rich region, a genome-wide bioinformatic studies showed that G4 structures do not exist randomly in the genome, but rather form a cluster in specific loci such as telomeres, rDNA arrays, DSB hot spots, and promoter regions (Duquette *et al*., 2004; Hershman *et al*., 2008; Capra *et al*., 2010; Huppert, 2010). Although they seem to have protective roles at telomeric ends with 3′ single-stranded G-rich overhang and stimulatory roles in gene transcription, other beneficial functions are largely unknown (Paeschke *et al*., 2005; Du *et al*., 2008).

Until now, more than 20 different helicases are known to bind and unwind G4 structures *in vitro*, and most of their human counterparts are implicated in human diseases that give rise to genome instability and cancer development (Huber *et al*., 2002; Ribeyre *et al*., 2009; Bochman *et al*., 2012). Recently, ChIP analysis revealed that Pif1 is a very strong G4 unwinder *in vivo* and replication fork progression slows down and becomes fragile with increased recombination in the vicinity of G4 motifs in Pif1-deficient cells (Paeschke *et al*., 2011). Pif1 associates with G4 motifs maximally in late S/G2 phase, indicating the possibility that Pif1 works to make sure hard-to-replicate conformations are completely resolved before mitotic division begins, and it is also consistent with the observations that both Pif1 abundance and telomerase activity are cell cycle regulated and peaking at late S/G2 phase (Mateyak and Zakian, 2006; Vega *et al*., 2007). RPA binds and stabilizes single-stranded DNA during various DNA transactions by suppressing the formation of unusual secondary structures that might leads to genome instability. However, it has distinct preference for pyrimidine-rich strand such as yeast autonomous replicating sequences (ARS), but shows very low affinity to polypurine tracts that frequently appear in telomeres and G4 structures, the stability in which cases seems to be offered by Pif1 instead (Kim *et al*., 1992; Wold, 1997; Paeschke *et al*., 2011). It is consistent with the recent findings that the affinity of Pif1 to G4 DNA is about 500-fold higher than to Y-structure mimicking replication fork, indicating that G4 structure is one of the most favorite substrates of Pif1 (Paeschke *et al*., 2013).

More interestingly, the same group found that heterologous Pif1 helicases such as bacterial, human, or even viral Pif1 can successfully suppress the phenotype of high GCR rates shown in Pif1-deficient yeast cells. However, the lengthened telomere was restored only by human PIF1, implying that *in vivo* function of Pif1 is largely conserved throughout evolution, but their substrate specificities or preferences might be slightly different. It remains in question pending more evidence which activity is the major contribution of Pif1 for telomere protection, antagonizing the formation of G4 structure in telomere or directly removing telomerase out of it. Although Pif1 is mainly responsible for rescuing genomes from harmful effect of G4 structures, Rrm3 also can suppress damage at G4 motifs when Pif1 is not available, suggesting that they both suppress G4-induced genome instability despite their largely non-overlapping functions (Paeschke *et al*., 2013). It remains to be further deciphered whether mitochondrial function of Pif1 and/or Rrm3 is also linked to G4 unwinding activity owing to the observation that *S. cerevisiae* mitochondrial genome contains numerous G-rich stretches, which might also work as structural targets for recombination (Ribeyre *et al*., 2009).

Replisome pausing at rDNA region

A tightly bound DNA-protein complex is another circumstance that potentially stall replication forks. The rDNA locus is one of hard-to-replicate sites in the genome owing to frequent transcriptions from highly repetitive region. It consists of about 150 tandem repeats of 9.1 kb sequence, occupying over 1 Mb in size in the genome and more than 60% of transcriptions occurring in a yeast cell is attributed to the production of rRNAs (Kobayashi *et al*., 1998; Warner, 1999). It makes the progression of replication fork through this region almost impossible especially when the direction of replication is opposed to that of transcription. However, a replication fork barrier (RFB) bound with Fob1 is located near the 3′ end of rDNA and blocks replication forks in the direction opposite to rDNA transcription, preventing the collision of two large machineries for replication and transcription (Kobayashi, 2003).

In *rrm3* mutant, there is an increase in replisome pausing at RFB in the rDNA and this leads to frequent rDNA breakage and accumulation of rDNA circles (Ivessa *et al*., 2000). Rrm3 is required for promoting fork movement past stable protein-DNA complexes at an estimated 1,400 loci in the yeast genome including the RFB, repressing recombination in rDNA locus and tRNA-rich region as well (Ivessa *et al*., 2003; Torres *et al*., 2004). In contrast, Pif1 is required for efficient pausing at the RFB in the rDNA and maintaining it, working as an inhibitor of fork movement, suggesting that both Rrm3 and Pif1 are involved in rDNA replication but have opposing effects on the fork progression (Ivessa *et al*., 2000).

Why is it that Pif1 promotes replication for progression at G4 structure but, in contrast, pause it at the RFB? Fork stabilizer protein Tof1 that associates with MCM helicase and

regulates the progression of normal replication forks is also required for pausing of replication fork in the rDNA to make sure that replication proceeds only in the same direction as highly active transcription (Hodgson *et al*., 2007). In the absence of Tof1, however, the fork stalling increases at hairpin-forming CGG repeat barriers that cause chromosomal fragility (Voineagu *et al*., 2009). While both Srs2 and Sgs1 helicases are significant in preventing instability and fragility of expanded CAG repeat sequences, only Srs2 helicase is required for unwinding of and fork progression through CGG repeats, where Sgs1 or Pif1 is dispensable (Kerrest *et al*., 2009; Anand *et al*., 2012). However, Srs2 has no effect on replication through G4 structure or protein-bound repeat sequences (Anand *et al*., 2012). Intriguingly, all of Pif1, Sgs1, and Srs2 can unwind G4 structures although Pif1activity is more vigorous than the others (Paeschke *et al*., 2013). Based on all the above observations, it is likely that cells employ differential helicases to exquisitely deal with a variety of replication barriers. Each and every helicase prefers differential location or interaction at fork with differential substrate specificity.

It is also possible that differential oligomeric states of helicases could account for their limited activities with high substrate specificity. Pif1 belongs to SF1 family of helicases that include a variety of monomeric and dimeric helicases. Barranco-Medina and Galletto (2010) applied quantitative approaches to determine the mode of Pif1 binding to DNA and showed that while Pif1 is a monomer in solution, dimerization of Pif1 is induced upon binding to single-stranded DNA (ssDNA). Pif1 dimerizes on DNA unwinding substrates even in the presence of non-hydrolysable ATP analogues, suggesting that dimerization of Pif1 occurs prior to and independently of its unwinding action. Recently, however, Pif1 monomer is shown to be able to translocate along with ssDNA to remove proteins from DNA, but its activity as monomer is not sufficient for duplex unwinding (Galletto and Tomko, 2013). These results are reminiscent of other SF1 helicases such as *E. coli* UvrD and *S. cerevisiae* Srs2, which function to displace RecA and Rad51, respectively, from ssDNA during DNA recombination, probably using their ssDNA translocase activity as monomer. UvrD monomer functions as rapid and processive ssDNA translocase, but not as helicase *in vitro* (Fischer *et al*., 2004). Oligomerization of helicases and/or interactions with accessory proteins may regulate their activities on structurally and metabolically diverse nucleic acid substrates (Lohman *et al*., 2008). Whether the dimeric form of Pif1 works more efficiently as translocase on ssDNA is currently not known. Taken together with these observations, which oligomeric state of Pif1 prefers to be involved in which type of DNA transactions remains to be explored. It should also include the study on telomerase displacement activity of Pif1, for instance, since it is still not clear yet whether Pif1 operates its helicase activity for duplex unwinding, another activity to translocate on ssDNA, or both to inhibit telomere elongation.

Repair-associated DNA synthesis

In *S. pombe*, Pfh1 is required not only for the completion of DNA replication but also for the appropriate responses to DNA-damaging agents. The cs mutant *pfh1-R20* cells are highly sensitive to methyl methanesulfonate (MMS), the DNA alkylating agent, and hydroxyurea (HU) at their permissive temperature, implying a role in the repair of DNA damage (Tanaka *et al*., 2002). When treated with camptothecin, a cytotoxic reagent that collapses replication forks with activity of DNA topoisomerase I, Pfh1 is recruited to form DNA damage foci and posttranslationally-modified isoform of Pfh1 increases dramatically (Pinter *et al*., 2008).

In *S. cerevisiae*, *pif1* mutant is just mildly sensitive to MMS and HU compared to *S. pombe pfh1* mutant. However, nuclear Pif1, but not Rrm3, colocalizes to repair foci with Rad52, the homologous recombination protein, in the nucleus after gamma irradiation, suggesting that Pif1 has a role specific for DSB repair and recombination (Wagner *et al*., 2006). Moreover, multiple defective phenotypes including high sensitivity to MMS and HU shown in *top3* mutant cells are suppressed by high-copy Pif1 expression in a Sgs1-dependent manner (Wagner *et al*., 2006). The accumulated observations that Pif1 helicase genetically interacts with Sgs1/Top3 pathway, Dna2 nuclease, and a non-essential subunit of DNA polymerase δ, Pol32, offer an appealing possibility that Pif1 is directly implicated in the DSB repair pathway by homologous recombination (Budd *et al*., 2006; Wagner *et al*., 2006; Zhu *et al*., 2008). In fact, although it was revealed that Sgs1 helicase and Dna2 nuclease activities in yeast are crucial for the extensive DSB end resection, the earliest step of homologous recombination that degrades 5′ strand to expose 3′ ssDNA, it seemed that Pif1 has no role for the step because the resection occurs normally in the absence of nuclear Pif1 (Zhu *et al*., 2008).

Chung *et al*. (2010) found a clue that Pif1 has a significant role in generating DSB repair product through break-induced replication (BIR) pathway. BIR is a repair pathway of homologous recombination where only one end of DSB is homologous to the template DNA so that, after strand invasion, the 3′ end is extended to the end of chromosome mimicking normal DNA replication (Malkova *et al*., 1996; Morrow *et al*., 1997). Yeast disomic experimental system used to study BIR in that study is also useful to screen novel factors potentially involved in damage-induced DNA synthesis step during homologous recombination because more than 100 kb-long chromosome has to be duplicated to complete BIR and the final repair products are to be determined with ease by marker detection or pulsed-field gel electrophoresis (PFGE) (Deem *et al*, 2008; Chung *et al*., 2010).

Very recently, the same group demonstrated that Pif1 strongly promotes recombination-specific DNA synthesis primed from Rad51 nucleofilament-invaded strand in D-loop and this stimulation occurs exclusively in a Polδ-dependent manner (Wilson *et al*., 2013). In *pif1* mutant cells, the recruitment of Polδ to the break site is impaired and DSB repair by BIR is severely reduced generating aborted half-crossover products instead. Purified Pif1 dramatically stimulates Polδ-mediated DNA synthesis in a migrating D-loop, relieving topological constraint properly by unwinding newly-synthesized DNA strand. Electron microscopy (EM) analysis elegantly confirmed that Pif1 supports extensive DNA synthesis generating free, long ssDNA, reminiscent of replicating bubble migration model catalyzed by UvsX protein and Dda helicase of bacteriophage T4 during the re-

combination-dependent DNA replication (Formosa and Alberts, 1986). This mode of replication is distinct from semi-conservative synthesis as in conventional replication fork and consistent with the recent observation that the newly synthesized strands segregate with the broken chromosome, not with the template DNA, suggesting that BIR occurs by conservative DNA synthesis (Donnianni and Symington, 2013; Saini *et al*., 2013). Extensive D-loop migration mediated by Pif1 and Polδ and the ensuing conservative DNA synthesis in BIR could be highly mutagenic because rapid dissociation of newly-synthesized DNA strand by Pif1 would prevent timely proofreading of misincorporated bases and potential mismatch correction afterwards, explaining the extremely high mutation frequency observed in BIR (up to ~2,800-fold increase) compared to normal DNA replication in S phase (Deem *et al*., 2011; Saini *et al*., 2013). Pif1 also promotes crossover recombination during the gene conversion together with Polδ, and the stimulatory effects of Pif1 on DNA synthesis are not substituted by Rrm3 (Wilson *et al*., 2013). While Rrm3 moves along the replication fork as a component of the replisome, it was thought that Pif1 does not travel with the replication fork but rather is recruited to certain genomic loci (Azvolinsky *et al*., 2006; Paeschke *et al*., 2011). However, it seems not the case in recombination-associated DNA synthesis because ChIP analysis showed Pif1 moves along with damage-induced replication machinery during the D-loop migration albeit direct physical interactions between Pif1 and Polδ is not known yet (Wilson *et al*., 2013). If they interact directly, it might be worth enunciating the binding motif of each protein. Even if they do not, however, it is still arousing our curiosity what else can be responsible for the difference in genetic requirements between recombination-mediated DNA replication and canonical replication in S phase.

Conclusion

Pif1, an evolutionarily conserved helicase, has been implicated in various nucleic acid transactions such as maintenance of mtDNA, negative regulation of telomere length, Okazaki fragment maturation, and pausing or unwinding at many hard-to-replicate regions (Fig. 1). Notably, stimulation of repair-specific DNA synthesis coupled with Polδ during the homologous recombination is recently identified as another valuable nuclear function of Pif1 in *S. cerevisiae* (Fig. 1). Most intriguingly with all the above roles, however, the contribution of Pif1 helicase to genome instability looks somewhat controversial. The absence of Pif1 provokes GCR via elevated *de novo* telomere addition and accumulation of G-quadruplex structures, but the presence of Pif1, on the other hand, induces fast D-loop migration and the ensuing highly mutagenic conservative replication as well as high level of crossover formation. Overproduction of Pif1 also shows toxic effects including the retarded cell growth. Even when the catalytically inactive allele is overexpressed, the doubling time of yeast strain is comparable to the case of WT Pif1 overexpression, demonstrating a negative effect even without helicase activity (Wagner *et al*., 2006). It is likely that the intracellular amount of Pif1 is quite small and needs to be tightly regulated as its peak expression is shown only in late S/G2 phase of cell cycle, which might be

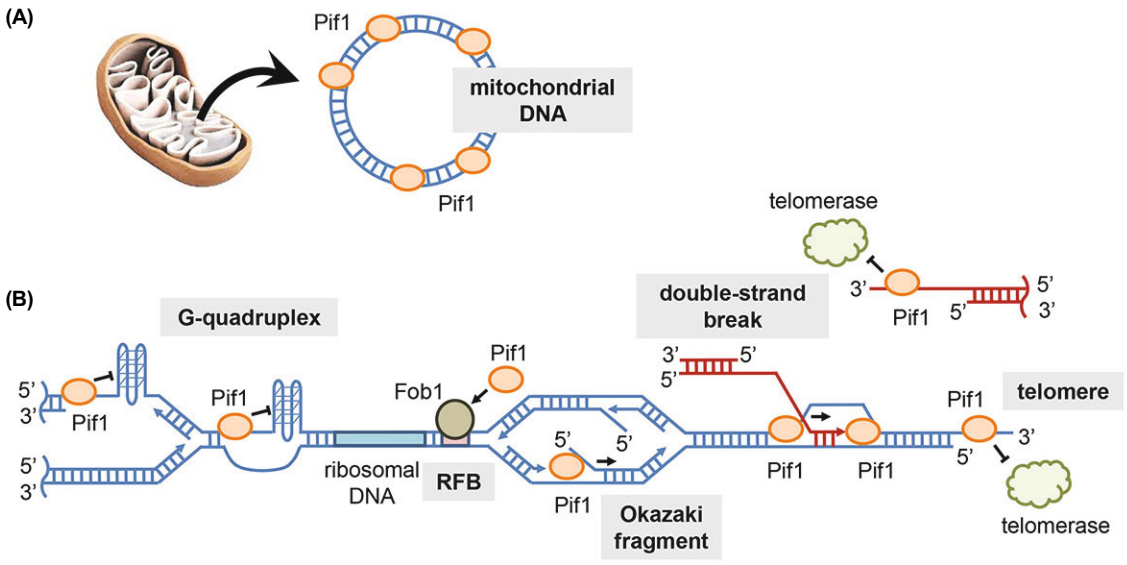

Fig. 1. Models for action mechanism of *S. cerevisiae* Pif1 helicase. (A) Pif1 is important for mtDNA replication and recombination. Pif1 seems to be directly associated with the entire mitochondrial genome. Pif1-deficient cells show frequent breakage of mtDNA at specific sites that are otherwise used for the locus of Pif1-dependent recombination. (B) Pif1 is involved in multiple DNA transactions at a variety of loci in the nucleus. As a negative regulator, Pif1 inhibits *de novo* telomere addition and telomere elongation at DSB sites and telomeric ends, respectively. At Okazaki fragments during lagging strand DNA synthesis, Pif1 unwinds RNA-DNA primer creating long 5′-flap that requires Dna2 activity. Pif1 binds and unwinds G4 structures that form at various G-rich regions and perform faithful replication before mitosis. Pif1 is crucial for pausing and maintaining of replication fork at the RFB in the rDNA region. Recently, it was found that Pif1 stimulates recombination-associated DNA synthesis with Polδ by displacing newly-synthesized strand and promoting extensive D-loop migration during homologous recombination.

Table 1. A variety of functions and substrate specificities of Pif1 family helicases across different model systems

	S. cerevisiae Pif1	*S. cerevisiae* Rrm3	*S. pombe* Pfh1	Mouse mPif1	Human PIF1
Mitochondrial genome stability	↑ (Foury and Van Dyck, 1985)	↓ (O'Rourke *et al.*, 2005)	↑ (Pinter *et al.*, 2008)	?	?
Inhibition of telomerase	↑ (Schulz and Zakian, 1994)	– (Ivessa *et al.*, 2002)	– (Pinter *et al.*, 2008)	– (Snow *et al.*, 2007)	↑ (Zhang *et al.*, 2006; Mateyak and Zakian, 2006)
Okazaki fragment maturation	↑ (Budd *et al.*, 2006)	–	↑ (Ryu *et al.*, 2004)	?	?
Unwinding G4 structure	↑ (Ribeyre *et al.*, 2009)	↑ (Paeschke *et al.*, 2013)	?	?	↑ (Sanders, 2010)
rDNA pausing	↑ (Ivessa *et al.*, 2000)	↓ (Ivessa *et al.*, 2000)	↓ (Sabouri *et al.*, 2012)	?	?
Repair-associated DNA synthesis	↑ (Wilson *et al.*, 2013)	– (Wilson *et al.*, 2013)	?	?	?
Lethality of deletion mutant	–	–	Lethal (Tanaka *et al.*, 2002)	–	–

" ↑ " : 'promotion' or 'stimulation', " ↓ " : 'inhibition', "–" : 'no effect', and "?" designates 'not known'.

the most plausible period for inhibition of telomerase activity, completion of difficult replication, and homologous recombination.

Despite its versatility as an all-round DNA metabolic player in the budding yeast cells, the roles of Pif1 do not seem consistent with those of its orthologs in the fission yeast cells or higher eukaryotes (Table 1). Pif1 null mutation in mice displays almost no visible phenotype including unaltered telomere length. Although the functions of human PIF1 are largely unknown, mutation of a conserved PIF1 residue is associated with increased breast cancer predisposition, suggesting that human PIF1 may function as a tumor suppressor (Chisholm *et al.*, 2012). The highlight of this puzzling enigma lies on Pfh1 in *S. pombe*. The reason why only Pfh1 is essential is still largely undescribed. Even when *S. cerevisiae* Pif1 and Rrm3, and human PIF1 are expressed to be localized to both mitochondria and nucleus in *S. pombe*, none of these homologs could supply all essential functions of Pfh1. Only *S. cerevisiae* Rrm3 could suppress the accumulation of DNA damage foci but not the HU sensitivity of Pfh1-deficient cells in *S. pombe* (Pinter *et al.*, 2008).

It remains in question pending corroborate evidence that each member of Pif1 helicase family has its own unique activity owing to some decisive differences in their structures, oligomeric status, substrate preferences, or specific binding partners to play appropriate roles together *in vivo*. In order to link the structure-function relationships to their own operational specificity, well-defined genetic approaches and *in vitro* studies need to be further developed at the mechanistic level. By the same token, the characterization of global protein interaction networks of Pif1 family helicases and mapping their interaction sites would disclose more details of Pif1 action mechanisms, considering their broad range of distinct functions in the cell.

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