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MINI-REVIEW

Dynamic regulation of Pif1 acetylation is crucial to the maintenance of genome stability

Onyekachi E. Ononye[1] · Christopher W. Sausen[2] · Matthew L. Bochman[2] · Lata Balakrishnan[1]

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Abstract

PIF1 family helicases are evolutionarily conserved among prokaryotes and eukaryotes. These enzymes function to support genome integrity by participating in multiple DNA transactions that can be broadly grouped into DNA replication, DNA repair, and telomere maintenance roles. However, the levels of PIF1 activity in cells must be carefully controlled, as Pif1 over-expression in *Saccharomyces cerevisiae* is toxic, and knockdown or over-expression of human PIF1 (hPIF1) supports cancer cell growth. This suggests that PIF1 family helicases must be subject to tight regulation in vivo to direct their activities to where and when they are needed, as well as to maintain those activities at proper homeostatic levels. Previous work shows that C-terminal phosphorylation of *S. cerevisiae* Pif1 regulates its telomere maintenance activity, and we recently identified that Pif1 is also regulated by lysine acetylation. The over-expression toxicity of Pif1 was exacerbated in cells lacking the Rpd3 lysine deacetylase, but mutation of the NuA4 lysine acetyltransferase subunit Esa1 ameliorated this toxicity. Using recombinant proteins, we found that acetylation stimulated the DNA binding affinity, ATPase activity, and DNA unwinding activities of Pif1. All three domains of the helicase were targets of acetylation in vitro, and multiple lines of evidence suggest that acetylation drives a conformational change in the N-terminal domain of Pif1 that impacts this stimulation. It is currently unclear what triggers lysine acetylation of Pif1 and how this modification impacts the many in vivo functions of the helicase, but future work promises to shed light on how this protein is tightly regulated within the cell.

Keywords Pif1 helicase · Lysine acetylation · DNA replication · DNA repair · G4 resolvase · NuA4 (Esa1) · Rpd3

Introduction

The *Petite Integration Frequency 1* (*PIF1*) gene was identified in 1983 during a screen for mutants that disrupt mitochondrial DNA recombination in the budding yeast *Saccharomyces cerevisiae* (Foury and Kolodynski 1983). A decade later, the Pif1 gene product was purified and shown to have single-stranded DNA (ssDNA)-dependent ATPase and 5′-3′ DNA helicase activities (Lahaye, et al. 1993, 1991). Little did the field know that this yeast enzyme was the founding member of the PIF1 family of multifunctional DNA helicases found in both prokaryotes and eukaryotes (Bochman et al. 2010, 2011) whose metazoan members would eventually be linked to cancer (Chen et al. 2020; Chisholm et al. 2012; Gagou et al. 2011, 2014; Luo et al. 2009), aging (Zhang et al. 2006), and obesity (Bannwarth et al. 2016; Belmonte et al. 2019).

In many respects, the second wave of PIF1 research began with the "rediscovery" of *S. cerevisiae PIF1* as a gene whose mutation affects telomere length (Schulz and Zakian 1994). Since that time, Pif1 has been shown to have roles in multiple DNA transactions in vivo, and insights into the function(s) of *S. cerevisiae* Pif1 in these processes are included below. In vitro, biochemical and biophysical approaches have demonstrated that Pif1 preferentially unwinds DNA fork substrates compared to substrates with a single ssDNA tail (Lahaye et al. 1993; Ononye et al. 2020; Ramanagoudr-Bhojappa et al. 2013), suggesting that Pif1

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✉ Matthew L. Bochman
bochman@indiana.edu

✉ Lata Balakrishnan
latabala@iupui.edu

[1] Department of Biology, School of Science, Indiana University Purdue University Indianapolis, Indianapolis, USA

[2] Molecular and Cellular Biochemistry Department, Indiana University, Bloomington, USA

undergoes secondary interactions with the strand of DNA complementary to the one upon which it translocates. In support of this, RNA:DNA hybrid forks stimulate Pif1 activity above that displayed on DNA forks, when the DNA strand supplies the 5′ ssDNA tail (translocating) and the RNA supplies the 3′ ssDNA tail (non-translocating) of the fork structure (Boule and Zakian 2007; Chib et al. 2016; Ononye et al. 2020; Zhou et al. 2014). Notably, RNA–DNA hybrids adopt an A-form conformation instead of the canonical B-form of duplex DNA (Wong and Shao 2017), and this is not the only non-canonical structure that is preferentially unwound by PIF1 family helicases. For PIF1 enzymes from multiple species, it has been shown that G-quadruplex structures also stimulate helicase activity (Mohammad et al. 2018; Paeschke et al. 2013; Sanders 2010).

Lesser-studied biochemical activities of PIF1 helicases include ssDNA annealing and nuclease activity. Strand annealing by *S. cerevisiae* Pif1 was first demonstrated by the Raney group, and this activity occurs in vitro under unwinding conditions (*i.e.*, in the presence of ATP), even in the presence of single-stranded binding proteins (Ramanagoudr-Bhojappa, et al. 2014). This strand annealing activity is evolutionarily conserved with hPIF1 and intrinsic to its helicase domain (George et al. 2009), which is the most conserved portion of all PIF1 proteins (Bochman et al. 2010, 2011). Thus, strand annealing may be a general feature of all PIF1 helicases. Another putative conserved PIF1 biochemical function is 3′-5′ exonuclease activity, which has been reported for recombinant *Candida albicans* Pif1 and other fungal PIF1 proteins, including *S. cerevisiae* Pif1 (Wei et al. 2017). However, these findings are controversial because high concentrations of *S. cerevisiae* Pif1 were necessary to observe the nuclease activity, and the absence of a contaminating nuclease from the recombinant protein expression host was not rigorously demonstrated.

Crystal structures of the *S. cerevisiae* helicase domain have been solved (Lu et al. 2018), as well as structures of PIF1 family helicases from *Bacteroides* species (Chen et al. 2016; Zhou et al. 2016) and the hPIF1 helicase domain (Dehghani-Tafti et al. 2019). Although these structures have revealed some commonalities and species-specific differences between PIF1 family helicases, it is unclear how informative they will be to the genome integrity field at large. This is because research has shown that in certain instances, PIF1 helicase domains are generic motor modules whose activity is either regulated by accessory domains or complemented by flanking domains necessary for proper in vivo function (Andis et al. 2018). The N- and C-terminal domains that flank the central helicase domain are also likely why a full-length eukaryotic PIF1 structure has eluded the field. These domains vary in length and sequence between species (Bochman et al. 2010, 2011), but a common characteristic among them is that they are predicted to be natively disordered (Nickens et al. 2019). Thus, modern structural biology approaches such as cryo-electron microscopy will be needed to solve the structures of these enzymes, which may exist in an ensemble of conformations in solution. The utility of such natively disordered regions in proteins has gained recent attention as they are often sites of protein–protein interaction (Fong et al. 2009), post-translational modifications (Bah and Forman-Kay 2016), and/or involved in liquid–liquid phase separation (Kato et al. 2012).

Multifunctional activities of nuclear Pif1

Based on its ability to unwind DNA and RNA/DNA hybrid duplexes, nuclear Pif1 has been implicated in a multitude of nucleic acid transactions. For instance, during Okazaki fragment maturation, Pif1stimulates the strand displacement activity of DNA polymerase delta (pol δ), creating long 5′ primer flaps (Pike et al. 2009). Lengthening of this flap gives rise to the accumulation of the ssDNA binding protein, replication protein A (RPA), which when bound, prevents illegitimate recombination, formation of secondary structures, and inappropriate degradation of genetic material (Bartos et al. 2008). RPA-bound flaps prevent primer cleavage by flap endonuclease 1 (FEN1), pushing Okazaki fragment processing toward an alternative pathway, known as the long-flap pathway. This necessitates the activity of the helicase/nuclease Dna2 mediates RPA displacement and flap removal (Pike et al. 2010; Stewart et al. 2008). This proposed mechanism succeeded initial genetic data by Budd et al. (2006), which revealed that the presence of Pif1 necessitates the endonuclease activity of Dna2, presumably by functioning together in the lagging strand maturation process (Budd et al. 2006). Pif1 also plays a role in repetitive ribosomal DNA (rDNA) replication. Movement of the replication fork proceeds in a manner such that the right moving fork is unhindered, while the left moving fork experiences a stop at the replication fork barrier (RFB) (Ivessa et al. 2000). The presence of the RFB is essential so that replication proceeds in the same direction as transcription, preventing macromolecular collisions (Kobayashi 2003). The protein Fob1 binds to these RFB sequences and serves as a unidirectional block to fork progression (Kobayashi and Horiuchi 1996). Pif1 directly interacts with Fob1 to maintain this barrier, though the exact mechanism by which this happens has yet to be fully explored (Muellner and Schmidt 2020). Interestingly, the second PIF1 family helicase in *S. cerevisiae*, Rrm3, has the opposite function during rDNA replication, mitigating fork stalling at the RFB (Ivessa et al. 2000). Additionally, ScPif1 also plays a backup role to Rrm3 in tRNA gene replication (Tran et al. 2017).

While majority of the studies investigating the role of PIF1 helicases during DNA replication focus on the

elongation phase, more recently, Pif1 and Rrm3 have been associated with replication termination. Their unwinding activity is proposed to relieve torsional strain in the final section of the parental DNA in budding yeast (Deegan et al. 2019; Su et al. 2019). Failure to unwind this region prevents the convergence of replication forks, thereby giving rise to late termination intermediates (LTIs) that stall replication termination (Steinacher et al. 2012). Compared to other *S. cerevisiae* helicases that are known to resolve structured intermediates, such as Sgs1 and Chl1, only Pif1 and Rrm3 allow for proper replication termination of preformed LTIs in vitro (Deegan et al. 2019).

Pif1 also acts as a G-quadruplex (G4) resolvase on both strands of the replication fork. In its absence, the lagging strand containing these stable non-canonical DNA secondary structures experience stalling (Dahan et al. 2018; Sparks et al. 2019). Therefore, although other helicases have displayed the capability to resolve G4 structures in vitro, such as Sgs1 and Dna2, it is now clear that in comparison to the activity of Pif1, such helicases function much less efficiently on intramolecular G4s, which are more commonly formed during lagging strand replication (Lerner and Sale 2019; Liu et al. 2010; Paeschke et al. 2013). Similarly, PIF1 family helicases can also efficiently unwind other non-B form DNA structures such as R-loops. This activity was initially implicated by the stimulation of *S. cerevisiae* Pif1 helicase activity by RNA–DNA hybrids (Boule and Zakian 2007) and the finding that Pif1 binding is enriched at highly transcribed genes genome wide (Paeschke et al. 2011). More recently, it has been demonstrated that both Pif1 and Rrm3 promote genome stability at tRNA genes by removing R-loops (Tran et al. 2017). In addition to its role in structure resolution, Pif1 can also remove protein barriers from dsDNA (Koc et al. 2016). Furthermore, along with the various roles of Pif1 during replication, the helicase is also involved in the repair of single-stranded nicks via break-induced replication (BIR) (Malkova and Ira 2013). During this process, Pif1 recruits pol δ to D-loops that are formed following strand invasion and stimulates pol δ synthesis via bubble migration as the helicase interacts with PCNA (Buzovetsky et al. 2017).

Finally, Pif1′s activities also modulate—and are modulated by—protein–protein interactions such as is the case with telomerase. Biochemical studies reveal that Pif1 inhibits Est2, the reverse transcriptase subunit of telomerase, using its ATPase activity to unwind the telomerase RNA and the telomeric DNA hybrid (Boule et al. 2005; Phillips et al. 2015). Overexpression and deletion studies of Pif1 in vivo reveal a decrease and increase in telomere length, respectively, showing that telomere length is inversely proportional to the amount of Pif1 activity in *S. cerevisiae* (Boule et al. 2005). Although Pif1 is a known regulator of telomerase at telomeres, it is equally capable of regulating telomerase at sites of DNA double-strand breaks (DSBs). Indeed, to specifically prevent the de novo addition of telomeres at DSBs instead of telomeres, Pif1 is phosphorylated in a Rad53-dependent manner (Makovets and Blackburn 2009). This regulation of Pif1 telomere maintenance activity also occurs via an interaction with the Hrq1 helicase (Bochman et al. 2014), and the combined activities of these two helicases has been shown to synergistically tune telomerase activity up and down in vitro (Nickens et al. 2018).

The known central functions of Pif1 in various cellular pathways are featured in Fig. 1. Given the myriad of pathways mediated by Pif1, the cell must have multiple mechanisms in place to regulate the activity of this low copy number protein within the cell.

Lysine acetylation of nuclear Pif1

Dynamically reversible protein post-translational modifications (PTMs) function to enhance the diversity of the proteome without much energetic cost to the cell. Following identification of Pif1 phosphorylation in *S. cerevisiae* (Makovets and Blackburn 2009), quantitative proteomic analyses further revealed hPIF1 to also be modified by phosphorylation and by ubiquitination (Kettenbach et al. 2011; Sharma et al. 2014; Udeshi et al. 2013). In our recent work, we show that *S. cerevisiae* Pif1 is acetylated in vivo and can also be modified in vitro (Ononye et al. 2020). The acetylation signature of Pif1 spans the entire protein, with lysine residues being modified on the N-terminus (Lys 118, Lys 129), within the helicase domain (Lys 525, Lys 639, Lys 725), and the C-terminus (Lys 800). Using lysine acetyltransferase (KAT) and lysine deacetylase (KDAC) mutant strains, we assessed the impact of altering the acetylation status of Pif1 on cell viability. While overexpression of Pif1 in itself is highly toxic to the cell, specific KDAC knockouts accentuated this toxicity phenotype. In contrast, a specific KAT knockout ameliorated the overexpression toxicity. Based on the viability of Pif1-overexpressing cells, we were able to assign the NuA4 (Esa1) KAT and its counteracting KDAC Rpd3 as responsible for dynamically altering the acetylation status of Pif1 within the cell. Because Pif1 acetylation had a profound impact on cell viability, we characterized the biochemical changes to Pif1 function upon lysine acetylation. Helicase assays using preferred substrates of Pif1 showed that the acetylated form of the helicase was much better at unwinding duplex strands (either DNA/DNA or RNA/DNA hybrid) compared to unmodified Pif1. Analyzing the reason for this increased helicase activity revealed that while acetylated Pif1 did not display an increased rate of unwinding, it was simply able to remain bound to the substrate longer, allowing for improved unwinding function. This observation directly correlates with our binding assays, wherein Pif1 displayed

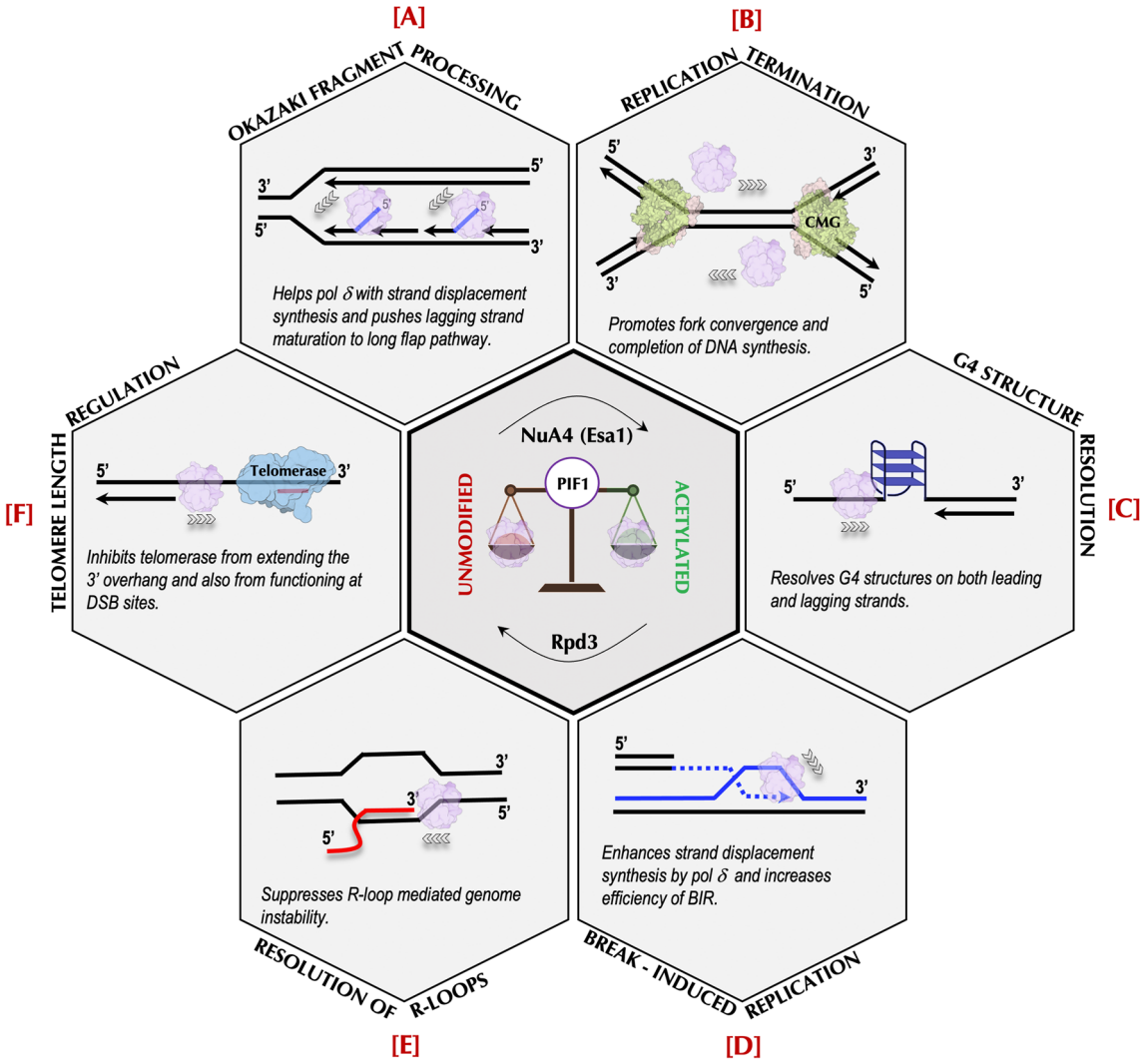

Fig. 1 Role of nuclear Pif1 in different biological DNA transactions. Pif1 functions in a flap lengthening during Okazaki fragment processing; b modulating fork convergence during replication termination; c aiding G4 structure resolution; d promoting break-induced replication; e unwinding R-loops; and f regulating telomere length. Acetylation of Pif1 mediated by the lysine acetyltransferase NuA4 (Esa1) and lysine deacetylase (Rpd3) may play a regulatory role in how Pif1′s functions are altered in all of these outlined pathways. Triple arrows indicate directionality of Pif1 activity

higher binding affinity to its cognate substrates compared to unmodified Pif1. Similar to the stimulated helicase activity and increased DNA binding affinity, we also detected higher ATPase efficiency by acetylated Pif1.

Due to being natively disordered, the functional role of the N-terminus of Pif1 has remained somewhat of an enigma. Though acetylation marks were identified across multiple domains of Pif1, we were particularly interested in understanding the role of this modification on the N-terminus of the helicase. Over-expression of a Pif1 mutant lacking its N-terminus (Pif1ΔN) resulted in decreased cellular toxicity compared to the full-length helicase. The toxicity phenotype of Pif1ΔN was further rescued in strains lacking *RPD3*. This observation clarified the role of the N-terminus in contributing to the acetylation-based regulation of Pif1′s enzymatic activities. Biochemical studies showed that similar to the full-length protein, the helicase and ATPase functions of Pif1ΔN were stimulated by acetylation. However, the mutant helicase did not exhibit increased DNA binding affinity when modified.

We were curious about the role of lysine acetylation in driving conformational changes in the Pif1 structure, which could impact its function. Limited proteolysis assays revealed that acetylation afforded Pif1 protection from cleavage, suggesting a change in conformation compared to unmodified Pif1. The unmodified Pif1ΔN was more resistant to proteolysis, suggesting that the N-terminus directly impacts overall protein structure, and perhaps acetylation

of this domain modifies the three-dimensional architecture of Pif1 to stimulate its biochemical activities. Future structural analyses will be required to delineate the role of the acetylated N-terminus in altering the Pif1 structure, thereby regulating the binding, unwinding, and ATPase functions of the protein.

Current perspectives and future research

The activity of Pif1 in the cell needs to be finely tuned to ensure the maintenance of genome stability and cell viability. Too much or too little Pif1 activity creates havoc in the cell, leading to checkpoint activation and ultimately cell death. Our observation that lysine acetylation leads to increased Pif1 enzymatic activities suggests that PTM of the helicase could be another mode by which the cell can dynamically regulate the functions of this protein. Modification of Pif1 could also change the outcome of a specific biological pathway. For example, increased helicase function of acetylated Pif1 could lead to rampant unwinding of Okazaki fragments, causing the accumulation of checkpoint-triggering ssDNA in the cell. Alternatively, this could also push Okazaki fragment maturation (OFM) to the long-flap pathway of processing, wherein the pol synthesized primers would be completely displaced, promoting resynthesis by the higher fidelity polymerase, pol. Because deletion of *PIF1* rescues the lethal phenotype of *dna2* in *S. cerevisiae*, it has been proposed that both of these helicases work in conjunction during OFM (Budd et al. 2006), and multiple biochemical studies lend credence to this proposal (Ayyagari et al. 2003; Pike et al. 2009, 2010; Rossi et al. 2008). However, recent genetic studies also suggest that formation of long flaps is not widespread in the cell (Kahli et al. 2019). A very recent study suggests that it is not their activity during OFM but the combined activity of these helicases in replication fork restart that make them vital to cell viability (Appanah et al. 2020; Falquet et al. 2020). Human Dna2 was previously shown to be acetylated in vivo, and biochemical analysis revealed that its nuclease, helicase and binding activities were stimulated in vitro (Balakrishnan et al. 2010), similar to our report of increased activity by acetylated yeast Pif1 (Ononye et al. 2020). Combined modification of Pif1 and Dna2 may directly impact their role in both flap processing and/or replication fork restart. A multitude of proteins functioning in replication and repair are also known to be modified by lysine acetylation, including the hBLM (Wang 2017) and hWRN helicases (Lozada et al. 2014; Muftuoglu et al. 2008). While the alterations to enzymatic activities of these acetylated replication proteins have not all been characterized, we are now starting to gain a better understanding of this mode of protein functional regulation.

Our recent study serves as good first step in defining the role of lysine acetylation in regulating the biochemical functions of the Pif1 helicase, but many questions remain about this mode of protein regulation. Specifically, it will be important to know what fraction of Pif1 is constitutively acetylated within the cell or if there are specific triggers for lysine acetylation of the helicase, such as cell cycle phases or the DNA damage response. Alterations in the acetylation signature of the helicase upon different triggers would help us to identify how specific lysine modifications could impact Pif1′s interactions with other proteins in a biological pathway. Further, it would be interesting to know if the same enzyme modifiers that we identified in our study (*i.e.*, the KAT, NuA4 [Esa1], and KDAC, Rpd3) are exclusively responsible for altering Pif1′s acetylation status or if other redundant acetylation-specific modifiers also play a role in the PTM of this enzyme. Further, we need to characterize the impact of Pif1 acetylation on the myriad of biochemical processes that the helicase has been implicated in (outlined in Fig. 1) and the consequences of this modification on these biological pathways. Finally, while we have preliminary evidence showing that hPIF1 is acetylated in vivo (unpublished observation), we are currently unaware of the biochemical consequences of this modification and its impact on human genome integrity. Multiple avenues exist to further understand this regulatory modification on PIF1 family helicases, and future research on this topic will help to shed light on the significance of this protein in genome maintenance and cell viability.

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