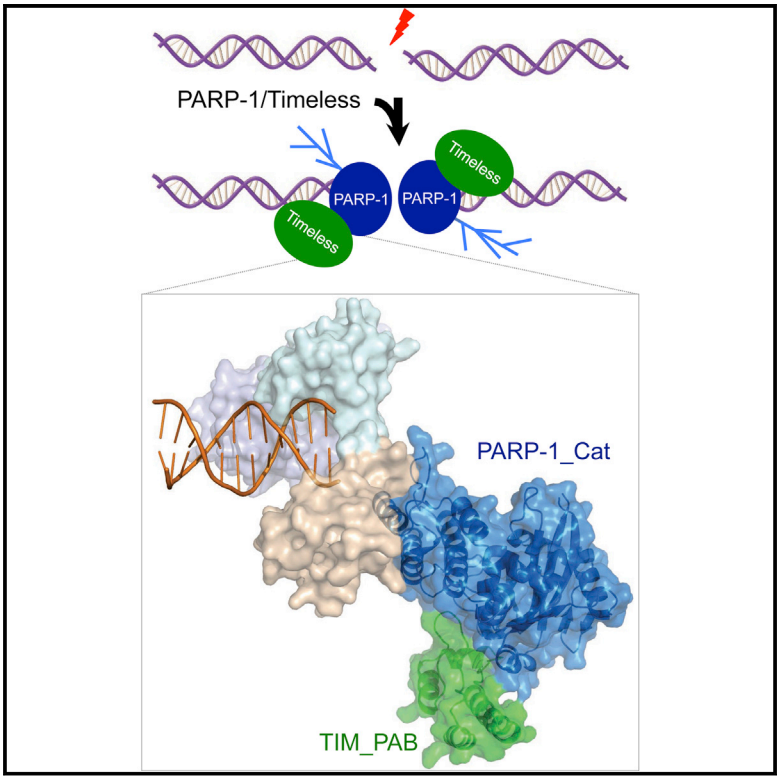
Article

Molecular Cell

Timeless Interacts with PARP-1 to Promote Homologous Recombination Repair

Graphical Abstract



Highlights

- Timeless interacts with PARP-1 independent of poly(ADP-ribosyl)ation
- Crystal structures of Timeless PAB in free form and in complex with PARP-1
- Specific recognition of PARP-1 by Timeless does not affect its enzymatic activity
- PARP-1 is required for Timeless recruitment to DSB sites to promote HR repair

Authors

Si Xie, Oliver Mortusewicz, Hoi Tang Ma, ..., Randy R.Y. Poon, Thomas Helleday, Chengmin Qian

Correspondence

thomas.helleday@scilifelab.se (T.H.), cmqian@hku.hk (C.Q.)

In Brief

Xie et al. present a high-resolution crystal structure of the Timeless-PARP-1 complex and show that this specific interaction is required for PARP-1 mediated, but PAR-independent, Timeless recruitment to damaged DNA and promotes efficient homologous recombination repair.

Xie et al., 2015, Molecular Cell *60*, 1–14
October 1, 2015
http://dx.doi.org/10.1016/j.molcel.2015.07.031

CellPress

Please cite this article in press as: Xie et al., Timeless Interacts with PARP-1 to Promote Homologous Recombination Repair, Molecular Cell (2015), http://dx.doi.org/10.1016/j.molcel.2015.07.031

Molecular Cell
Article

Timeless Interacts with PARP-1 to Promote Homologous Recombination Repair

Si Xie,[1,4] Oliver Mortusewicz,[2,4] Hoi Tang Ma,[3] Patrick Herr,[2] Randy R.Y. Poon,[3] Thomas Helleday,[2,*] and Chengmin Qian[1,*]
[1]School of Biomedical Sciences, The University of Hong Kong, Hong Kong
[2]Science for Life Laboratory, Division of Translational Medicine and Chemical Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, 171 21 Stockholm, Sweden
[3]Division of Life Science, Hong Kong University of Science and Technology, Hong Kong
[4]Co-first author
*Correspondence: thomas.helleday@scilifelab.se (T.H.), cmqian@hku.hk (C.Q.)
http://dx.doi.org/10.1016/j.molcel.2015.07.031

SUMMARY

Human Timeless helps stabilize replication forks during normal DNA replication and plays a critical role in activation of the S phase checkpoint and proper establishment of sister chromatid cohesion. However, it remains elusive whether Timeless is involved in the repair of damaged DNA. Here, we identify that Timeless physically interacts with PARP-1 independent of poly(ADP-ribosyl)ation. We present high-resolution crystal structures of Timeless PAB (PARP-1-binding domain) in free form and in complex with PARP-1 catalytic domain. Interestingly, Timeless PAB domain specifically recognizes PARP-1, but not PARP-2 or PARP-3. Timeless-PARP-1 interaction does not interfere with PARP-1 enzymatic activity. We demonstrate that rapid and transient accumulation of Timeless at laser-induced DNA damage sites requires PARP-1, but not poly(ADP-ribosyl)ation and that Timeless is co-trapped with PARP-1 at DNA lesions upon PARP inhibition. Furthermore, we show that Timeless and PARP-1 interaction is required for efficient homologous recombination repair.

INTRODUCTION

Faithful transmission of genetic information from parent to daughter cells is central for eukaryotic life. However, eukaryotic cells are constantly facing challenges from both environmental and endogenous sources and defects in DNA replication and repair pathways are major causes of cancer and premature aging. Cells have evolved elaborate surveillance mechanisms, or checkpoints, to detect and respond to DNA damage and replication stress. Under conditions of replication stress, components of the DNA replication fork protection complex including Timeless and Tipin act together with a variety of proteins such as RPA, Claspin, ATR/ATRIP, and Chk1 to activate the intra-S phase checkpoint, maintain structural stability of stalled replication forks, and eventually promote restart of the stalled forks (Branzei and Foiani, 2010; Ciccia and Elledge, 2010; Errico and Costanzo, 2012).

Mammalian Timeless was originally identified on the basis of its homology to the *Drosophila* Timeless gene (Tim-1) (Sangoram et al., 1998; Zylka et al., 1998). Later, phylogenetic sequence analysis indicated that mammalian Timeless is more closely related to *Drosophila* Timeout gene (Tim-2) (Benna et al., 2000). *Drosophila* Tim-1 and Tim-2 have been demonstrated to be key players in circadian rhythm (Benna et al., 2010; Hardin, 2005), but it remains debatable if mammalian Timeless functions as a circadian clock factor (Barnes et al., 2003; Gotter, 2003; Gotter et al., 2000). Nonetheless, mammalian Timeless and its paralogs (Tof1 in *Saccharomyces cerevisiae*, Swi1 in *Schizosaccharomyces pombe*, and Tim-1 in *Caenorhabditis elegans*) have been shown to play an essential role in proper progression of DNA replication, activation of cell-cycle checkpoints, and the establishment of sister chromatid cohesion (Chou and Elledge, 2006; Errico et al., 2007; Gotter et al., 2007; Leman et al., 2010; Smith et al., 2009; Unsal-Kaçmaz et al., 2005, 2007; Urtishak et al., 2009; Yoshizawa-Sugata and Masai, 2007). Timeless forms a stable complex with its partner protein Tipin. The Timeless-Tipin complex has been reported to travel along with the replication fork during unperturbed DNA replication. Tipin physically interacts with RPA, while Timeless directly interacts with replisome components including the replicative helicase CDC45-MCM-GINS (CMG) complex and DNA polymerase ε (Aria et al., 2013; Cho et al., 2013). Timeless appears to stimulate Polε polymerase activity, but negatively regulates CMG helicase activity. This unique function of Timeless might be essential to prevent disassembly of the replisome at paused replication forks, and re-assemble a replisome to facilitate fork restart when DNA replication is reactivated. In addition, the Timeless-Tipin complex has also been demonstrated to interact with Claspin and facilitate the accumulation of Claspin at the replication fork in response to replication stress (Yoshizawa-Sugata and Masai, 2007). The Timeless-Tipin-Claspin complex contributes to full activation of the ATR-Chk1 signaling pathway through the recruitment of Chk1 to arrested replication forks for sufficient ATR-mediated phosphorylation. Besides Tipin, Timeless has also been demonstrated to associate with a list of other nuclear proteins including ChIR1, SMC1, Dbf4, and Plk1 and is involved in multiple cellular processes such as sister chromatid cohesion

Please cite this article in press as: Xie et al., Timeless Interacts with PARP-1 to Promote Homologous Recombination Repair, Molecular Cell (2015), http://dx.doi.org/10.1016/j.molcel.2015.07.031

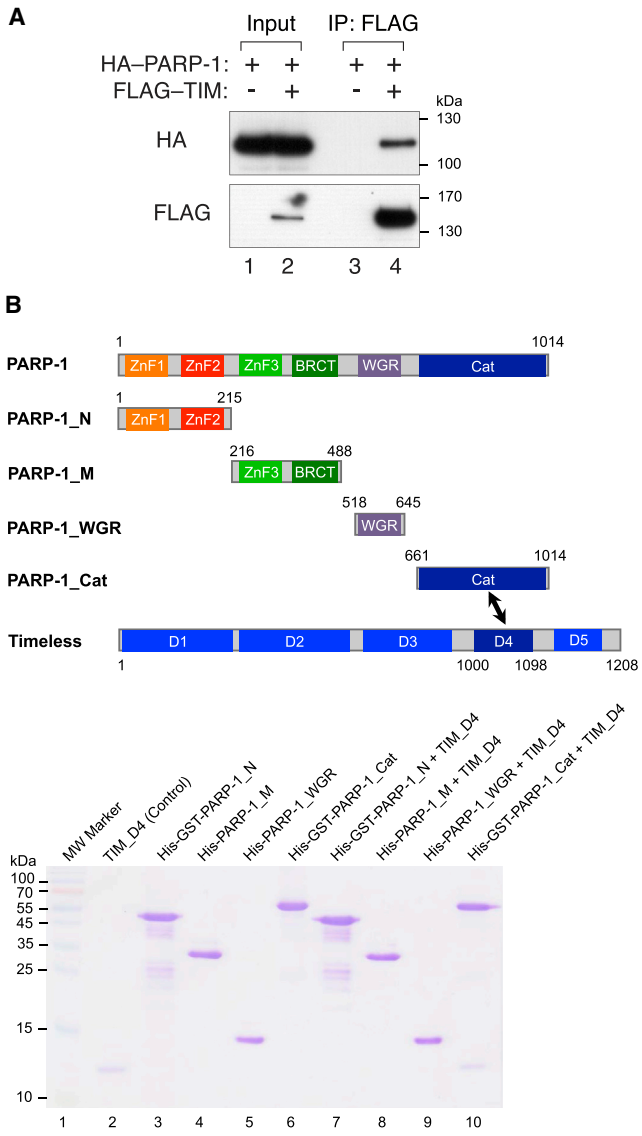


Figure 1. Timeless Interacts with PARP-1

(A) CoIP of fl-Timeless with fl-PARP-1. The HA-PARP-1 was either expressed alone or together with FLAG-Timeless in HeLa cells. The cell-free extracts were prepared and subjected to immunoprecipitation and were afterward analyzed with immunoblotting for FLAG and HA.

(B) Schematic diagram of Timeless and PARP-1 fragments and results from domain mapping experiments indicate that Timeless_D4 (referred to as Timeless PAB) domain binds to the PARP-1 catalytic domain. The His-tagged PARP-1_M, WGR domain, and His-GST-tagged PARP-1_N, PARP-1_Cat were immobilized, washed, and then incubated, respectively, with untagged Timeless_D4. The proteins were subsequently eluted from the beads (lanes 7–10) and visualized in SDS-PAGE gel. The lanes 3–6 are corresponding to different fragments of PARP-1 that are immobilized on Ni-NTA beads after washing away unbound proteins.

(Leman et al., 2012; Murakami and Keeney, 2014; Serçin and Kemp, 2011), temporal control of replication and meiotic double-strand break (DSB) formation, replication termination, and mitotic entry.

In this study, we report that human Timeless forms a tight complex with poly(ADP-ribose) polymerase 1 (PARP-1) that is independent of poly(ADP-ribosyl)ation. In addition, we determined crystal structures of the Timeless PARP-1-Binding domain (PAB) in free form and in complex with the PARP-1 C-terminal catalytic domain. Interestingly, Timeless specifically binds to the catalytic region of PARP-1, but not other PARP family members and this physical interaction does not seem to affect PARP-1 enzymatic activity. Finally, we demonstrate that recruitment of Timeless to DNA damage sites depends on the physical interaction with PARP-1, but is independent of PARP-1 enzymatic activity. We postulate that the interaction of Timeless and PARP-1 could facilitate DNA repair, in particular homologous recombination (HR) repair.

RESULTS

Timeless Directly Interacts with PARP-1

Human Timeless is a multi-domain containing protein involved in various cellular processes. Previous studies have defined four conserved regions in mouse Timeless based on sequence alignment (Yoshizawa-Sugata and Masai, 2007; Zylka et al., 1998); however, the precise molecular functions of these different domains have been elusive. To gain further insights into the structural domain organization of human Timeless, we cloned the individual domains. We first determined the high-resolution crystal structure of the Timeless_D4 domain, encompassing amino acids 1,000 to 1,098. The structural analysis suggests this Timeless_D4 domain may function as a protein-protein interacting modulator. In an effort to unravel the biological function of the Timeless_D4 domain, we performed tandem affinity purification (TAP) using cell lysates prepared from human embryonic kidney (HEK)293T cells stably expressing triple-epitope (S-protein, FLAG, and streptavidin-binding peptide)-tagged Timeless_D4. Mass spectrometry analysis revealed that one of the possible Timeless_D4-associated proteins is PARP-1. The physical interaction between full-length (fl) Timeless and PARP-1 was then confirmed in HeLa cells using co-immunoprecipitation (coIP) followed by western blot analysis (Figure 1A). We also carried out in vitro pull-down assay and confirmed that fl-Timeless associates with PARP-1 (Figure S1A). Furthermore, we demonstrated that the Timeless_D4 domain specifically interacts with the PARP-1 catalytic domain (Figure 1B). Thus, we named the Timeless_D4 domain PARP-1-binding domain (PAB).

Crystal Structure of Timeless PAB Domain

The Timeless PAB domain exists as a dimer in solution, as demonstrated by analytical gel filtration (Figure S1B). Consistently, the Timeless PAB domain forms a head-to-tail homodimer in the crystal structure (Figure 2A and crystallographic statistics are given in Table 1) solved initially with single-wavelength anomalous diffraction. Each PAB subunit shares an almost identical structure that mainly consists of a bundle of five α helices. As shown in cartoon representation, helix α2 connects to helix α3 with a 15-amino acid (aa)-long extended loop, while helices α4 and α5 are linked by a 20-aa-long U-shaped loop. The conformation of the U-shaped loop and the α2/α3 loop is further stabilized by an inter-loop hydrogen bonding network as well as salt

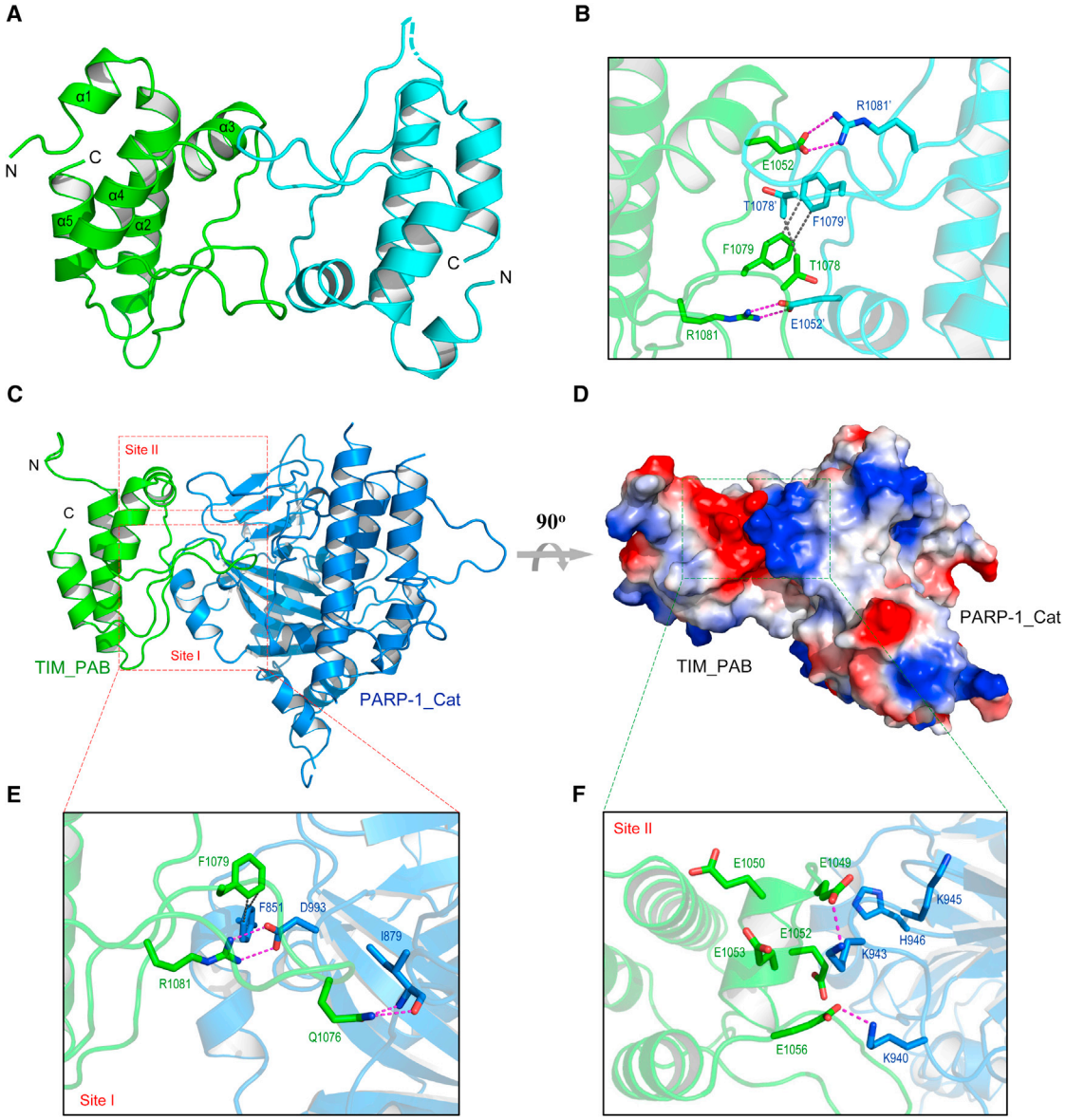


Figure 2. 3D Crystal Structures of the Timeless PAB Domain in Free Form and in Complex with PARP-1 Catalytic Domain

(A) Timeless PAB homodimer is shown as cartoon diagrams. Each PAB subunit is colored in green and cyan, respectively.

(B) Detailed interaction between Timeless PAB subunits. The residues that form the binding interface are depicted as stick models and labeled. The hydrogen bonds and salt bridges are shown in magenta dashed lines and hydrophobic interactions are in black dashes.

(C) Cartoon representation of the structure of Timeless PAB (green) in complex with the PARP-1 catalytic domain (marine). The box areas define two binding interfaces shown in (E) and (F).

(D) Electrostatic surface representation of PARP-1 catalytic domain and the bound Timeless PAB. To highlight the contributions of electrostatic interactions at the site II interface for heterodimer formation, the electrostatic surface potential was generated with the PARP-1 catalytic domain and the Timeless PAB separately. The electrostatic surface potential is contoured at −5 to 5 *kT/e*.

(E) Detailed view of site I binding interface of PARP-1-Timeless complex. The selected side chains are shown as color-coded sticks and are annotated, with the Timeless PAB colored green and the PARP-1 catalytic domain colored marine.

(F) Detailed view of the site II interface, showing the electrostatic interactions between the Timeless PAB helix α3 and the PARP-1 loop region encompassing residues 932–947. The interacting residues are shown as stick models.

bridges formed between side chains of Arg1070 and Glu1077 (Figure S1C). The dimeric interface is mainly composed of residues from the U-shaped loop and helix α3 of each subunit. Both electrostatic and hydrophobic interactions contribute to the formation of the stable homodimer, burying the total solvent accessible surface area of 1,220 Å^2. Notably, residue Glu1052 of helix α3 and Arg1081′ (with the prime indicating the other subunit) located in the U-shaped loop, as well as the counterpart

Table 1. Summary of X-Ray Diffraction Data and Structure Refinement Statistics

Crystal	Timeless_PAB SeMET	Timeless_PAB Native	Timeless/ PARP-1
Data Collection			
Beamline	BL17U	BL17U	BL17U
Protein Data Bank code	4XHW	4XHT	4XHU
Wavelength (Å)	0.97923	1.06997	0.97907
Space group	$C222_1$	$C222_1$	$C121$
Unit cell a, b, and c (Å)	66.4, 100.5, and 149.5	66.6, 100.6, and 149.5	99.4, 98.4, and 116.9
Resolution (Å)	50.0–2.80	50.0–1.65	50.0–2.05
	(2.85–2.80)[a]	(1.68–1.65)	(2.09–2.05)
Observed reflections	181,889	375,510	188,441
Unique reflections	12,777 (613)	59,619 (5,562)	62,689 (3,191)
Completeness (%)	100.0 (100.0)	98.4 (100.0)	99.6 (100.0)
R_{merge}	0.134 (0.486)	0.071 (0.431)	0.070 (0.458)
Average I/σ (I)	27.1 (6.6)	29.65 (4.64)	15.9 (3.0)
Redundancy	14.2 (14.7)	6.3 (6.3)	3.2 (3.2)
Refinement			
Resolution (Å)	24.93–2.80	26.34–1.65	25.1–2.09
	(2.89–2.80)	(1.71–1.65)	(2.16–2.09)
No. reflections	12,751	59,619	60,143
R_{work}/R_{free} [b] (%)	19.3 / 25.3	18.5 / 22.2	18.3 / 21.1
No. atoms	2,854	3,243	6,829
Protein	2,854	2,921	6,548
Ligand	0	0	33
Water	0	317	248
B-factors (Å^2)	46.9	37.5	52
RMSD			
Bond lengths (Å)	0.011	0.007	0.008
Bond angles (°)	1.40	1.09	1.12
Ramachandran Statistics			
Favored (%)	100	99	99
Allowed (%)	0	1	1
Outliners (%)	0	0	0

[a]Numbers in parentheses refer to the highest resolution shell.
[b]R_{free} was calculated using 5% random data omitted from the refinement.

residues Glu1052′ and Arg1081, form two ion pairs (2.9 Å) that contribute significantly to the dimer formation (Figure 2B). Additionally, residues Thr1078 and Phe1079 located in the U-shaped loop have hydrophobic interactions with their equivalent counterparts from the other subunit. It is worth to note that a structural similarity search from the DALI server revealed that the architecture of the Timeless PAB shares certain similarity to that of PUB domains of PNGase and HOIP (Allen et al., 2006; Elliott et al., 2014; Schaeffer et al., 2014), despite the low level of aa conservation at equivalent positions (Figure S1D). The PNGase PUB domain recognizes a conserved tyrosine-based motif in AAA+ ATPase VCP/p97 and modulates ER-associated protein degradation activity (Allen et al., 2006). More recently the PUB domain of HOIP has been demonstrated to bind to OTULIN to control NF-κB signaling (Elliott et al., 2014; Schaeffer et al., 2014). However, the putative pocket in the PUB domain for the partner protein binding is not conserved in the Timeless PAB domain (Figure S1E).

Crystal Structure of Timeless-PARP-1 Complex

We went on to determine the crystal structure of the Timeless PAB domain in complex with the PARP-1 C-terminal catalytic domain, including the helical subdomain (HD) and the ADP-ribose transferase (ART) domain at the resolution of 2.09Å (Figure 2C). The Timeless PAB domain forms a 1:1 heterodimer with the PARP-1 catalytic domain, with a total buried surface area of 1,470 Å^2. Similar to the PAB homodimeric interface, the binding surface of Timeless PAB in the Timeless-PARP-1 heterodimer also comprises the U-shaped loop and helix α3 that constitute the "site I" and "site II" interface of the Timeless-PARP-1 heterodimer as highlighted in Figure 2C. On the other hand, the binding interface of the PARP-1 catalytic domain is on the opposite side of its enzymatic active site. The structure arrangement of the Timeless PAB domain is highly similar to that in free form, with 0.43Å root-mean-square deviation (RMSD) for the aligned Cα atoms. Interestingly, at the site I interface, those residues in the U-shaped loop critical for PAB homodimer formation also participate in Timeless-PARP-1 complex formation (Figure 2E). For example, Timeless Arg1081 forms an ion pair (2.7 and 2.9Å) with PARP-1 Asp993. The side chain of Timeless Phe1079 is in hydrophobic contact with PARP-1 Phe851. Additionally, the Timeless Gln1076 side chain carboxamide group forms hydrogen bonds with PARP-1 Ile879 backbone amine and carbonyl groups. Another salient feature of the Timeless-PARP-1 complex is that the Timeless PAB helix α3, with a strong negative electrostatic potential surface (site II interface), tightly associates with the PARP-1 loop segment encompassing residues 932–947, that has a highly positive charged surface (Figures 2D and 2F). Specifically, side chains of Timeless Glu1049 and Glu1056 form ion pairs with PARP-1 Lys943 and Lys940, respectively. It is likely these massive networks of electrostatic interactions provide the driving force to disrupt the Timeless PAB homodimer and promote the Timeless-PARP-1 heterodimer formation when adding the PARP-1 catalytic domain sample into the Timeless PAB solution. To corroborate the interaction observed in the crystal structure, we applied site-directed mutagenesis and quantitatively measured the effect of mutation on the binding of Timeless and PARP-1 using surface plasmon resonance (SPR). The Timeless PAB domain binds to the PARP-1 C-terminal catalytic domain with a K_d of 26.0 ± 1.5 nM (Figure 3A), whereas mutation on either Timeless R1081G or PARP-1 D993G totally abolishes the binding (Figures S2A and S2B), indicating that the specific interaction of Timeless R1081 with PARP-1 D993 contributes substantially to the complex formation. CoIP and in vitro glutathione S-transferase (GST) pull-down assay further support this conclusion (Figures 3B and 3E).

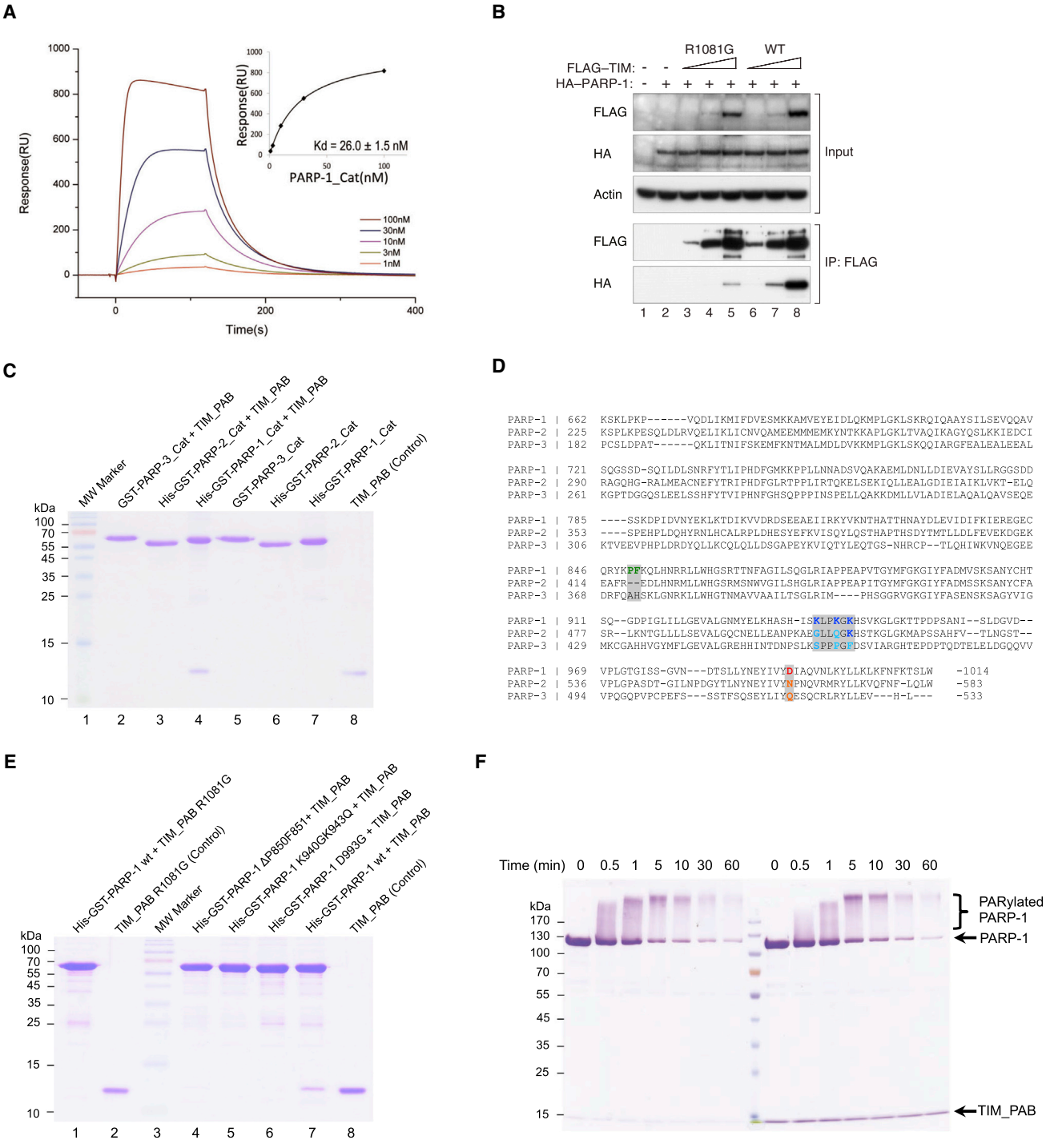


Figure 3. Molecular Analysis of the Binding Specificity between Timeless and PARP-1

(A) SPR measurement of the PARP-1 catalytic domain binding to the immobilized Timeless PAB. Each color in the binding sensorgrams represents the analyte concentration in a separate cycle.

(B) Immunoprecipitation confirms that Timeless R1081G mutation impairs Timeless-PARP-1 interaction in vivo. The different levels of FLAG-Timeless or FLAG-Timeless R1081G were co-expressed with HA-PARP-1 in the HeLa cells as indicated.

(C) In vitro GST pull-down assay of the Timeless PAB domain with the catalytic domains of PARP-1, PARP-2, and PARP-3 indicates the Timeless PAB domain binds specifically to PARP-1, but not PARP-2 or PARP-3.

(D) Structural-based sequence alignment of the catalytic domains of PARP-1, PARP-2, and PARP-3. The residues that are important for the Timeless binding are highlighted with the light gray background.

(legend continued on next page)

Timeless PAB Binds Specifically to PARP-1, but Not PARP-2 or PARP-3

PARP-1 is one of the most abundant nuclear enzymes, and it is estimated to mediate over 80% of cellular poly-ADP-ribose synthesis (Shieh et al., 1998). PARP-1 is also the best characterized member in the poly(ADP-ribose) polymerase family, which currently comprises 17 members (Schreiber et al., 2006). So far PARP-1, PARP-2, and PARP-3 have been implicated in the DNA damage response. PARP-1 contains three zinc fingers at the N terminus that are critical for sensing DNA damage, these DNA binding domains are not present in PARP-2 or PARP-3. However, all of three PARP members share a structurally similar catalytic domain. The result from our domain mapping experiment indicated only the C-terminal catalytic domain of PARP-1 is required for Timeless PAB binding, which prompted us to investigate whether Timeless binds to the catalytic domain of PARP-2 or PARP-3. The GST pull-down assay indicated Timeless PAB binds specifically to the catalytic domain of PARP-1, but not PARP-2 or PARP-3 (Figure 3C). In fact, the structural basis of this binding specificity can be explained by the crystal structure of PARP-1 and the Timeless heterodimer. Structure-based sequence alignment of the catalytic domains of human PARP-1, PARP-2, and PARP-3 is shown in Figure 3D. The residues of the PARP-1 catalytic domain directly involved in the Timeless-PARP-1 complex formation are highlighted by the light gray background. Interestingly, most of the residues in PARP-1 critical for the complex formation are not conserved in PARP-2 or PARP-3. For example, PARP-1 Asp993 forms salt bridges with Timeless Arg1081 and the corresponding residues of PARP-1 Asp993 in PARP-2 and PARP-3 are Asn563 and Gln519, respectively, neither of which could form an ion pair with Timeless Arg1081. Furthermore, PARP-1 possesses positively charged Lys940 and Lys943 that are involved in strong electrostatic interactions with the Timeless PAB domain, but neither PARP-2 nor PARP-3 has positively charged residues at equivalent positions. To further verify the importance of these residues in recognition of Timeless by PARP-1, we generated a number of mutations in PARP-1 to examine the interactions with their wild-type counterparts using both in vitro pull-down assay and SPR. In complete agreement with our structural findings, single mutation of PARP-1 Asp993 to Gly, or double mutation of Lys940 and Lys943 to Gly and Gln that mimics the corresponding region of PARP-2, or double deletion of Pro850 and Phe851, led to the abrogation of interactions with Timeless, as demonstrated by in vitro GST pull-down assay (Figure 3E). Consistently, SPR measurement indicates that PARP-1 Pro850 and Phe851 double deletion or D993G mutation disrupts the binding of the PARP-1 catalytic domain to the Timeless PAB domain, while PARP-1 K940G/K943Q double mutation resulted in an almost 20-fold reduction in Timeless PAB binding (Figures S2B–S2D).

Timeless-PARP-1 Interaction Does Not Interfere with PARP-1 Enzymatic Activity

Considering that the PARP-1 catalytic domain is required for Timeless binding, we wondered whether Timeless-PARP-1 interaction affects PARP-1 enzymatic activity. To analyze this, we performed in vitro PARP-1 enzymatic assays. In presence of DNA, PARP-1 can efficiently poly(ADP-ribosyl)ate itself when adding NAD^+, as demonstrated by SDS-PAGE, the auto-ADP-ribosylated PARP-1 gradually migrated as the reaction proceeded (Figure 3F, left side). Adding the Timeless PAB domain into the reaction did not block PARP-1 auto-ribosylation (Figure 3F, right side). This actually is consistent with the structural observation that Timeless PAB binding does not cause obvious conformational changes in the PARP-1 catalytic domain. A recent structure-based study showed that conformational changes of HD preceding the ART could modulate PARP-1 catalytic activity (Langelier et al., 2012). Structural superimposition of the PARP-1-Timeless complex and PARP-1 in free form revealed that PARP-1 catalytic domains in the two structures are almost identical (Figure S3), no obvious structural distortion was observed in the HD domain, indicating Timeless binding does not induce conformational changes in PARP-1 and thus does not affect its enzymatic activity.

Timeless Accumulates at DNA Damage Sites and Its Recruitment Is Increased upon Inhibition of PARP-1

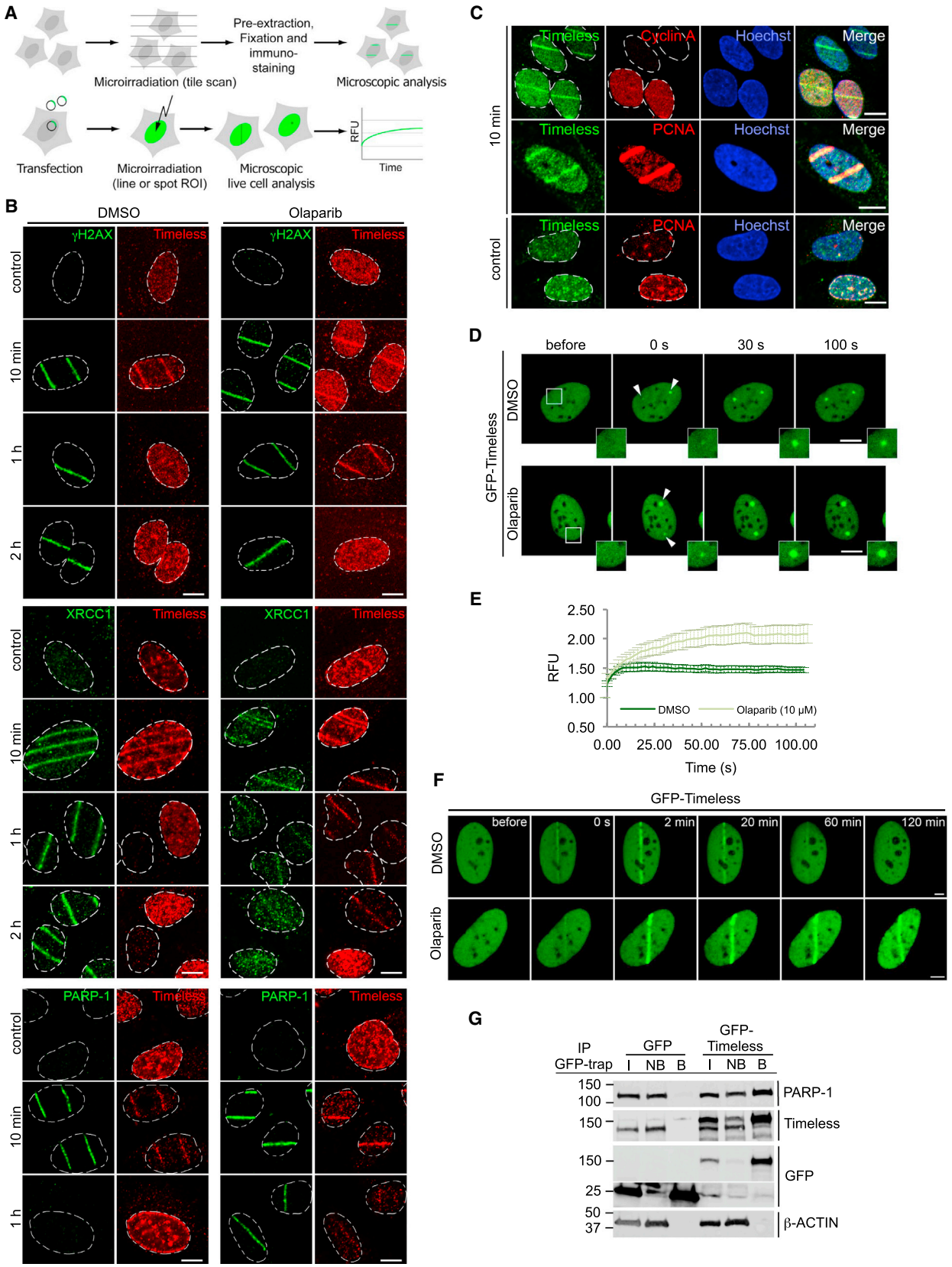
PARP-1 functions as a sensor protein that directly recognizes DNA DSBs or SSBs and promotes the rapid recruitment of a number of proteins to DNA damage sites in a PAR-dependent manner (Ali et al., 2012; Langelier et al., 2012; Gibson and Kraus, 2012). Since we have demonstrated that Timeless interacts with PARP-1, we tested if PARP-1 targets Timeless to DNA damage sites. Endogenous Timeless could be detected at DNA damage tracks as early as 10 min after microirradiation in both S and non-S phase cells (Figures 4B, 4C, and S4A). No Timeless accumulation could be observed at later time points, indicating a rather transient recruitment. To analyze whether PARP-1 mediated poly(ADP-ribosyl)ation at DNA damage sites is required for recruitment of Timeless, we microirradiated U2OS cells in the presence or absence of the PARP-1 inhibitor Olaparib. Interestingly, we observed a prolonged retention of Timeless and PARP-1 at laser tracks, while recruitment of XRCC1 was abolished in the presence of Olaparib (Figures 4B and S4B). The prolonged retention of Timeless at damaged chromatin is likely due to trapping of PARP-1 at DNA damage sites.

To monitor the spatio-temporal accumulation of Timeless and its trapping at DNA damage sites upon PARP-1 inhibition in real time, we performed live-cell analysis in cells expressing GFP-tagged Timeless. GFP-Timeless readily accumulated at laser-induced DNA damage sites and reached the maximum level after 10–15 s (Figures 4D and 4E), suggesting an early role for Timeless in the DNA damage response (DDR). Interestingly, PARP-1 inhibition led to stronger and prolonged recruitment of

(E) In vitro GST pull-down assay of the Timeless PAB domain with the catalytic domain of PARP-1 and its mutants (lanes 4–8), as well as the PARP-1 catalytic domain with Timeless R1081G mutant (lanes 1 and 2).

(F) In vitro PARP-1 auto-modification assay. The time course of PARP-1 auto-poly(ADP-ribosyl)ation in the absence (left side) and presence (right side) of the Timeless PAB domain is displayed in each lane.

Please cite this article in press as: Xie et al., Timeless Interacts with PARP-1 to Promote Homologous Recombination Repair, Molecular Cell (2015), http://dx.doi.org/10.1016/j.molcel.2015.07.031



(legend on next page)

GFP-Timeless, as indicated by the Timeless accumulation reaching its plateau much later at around 75 s. This suggests increased retention of GFP-Timeless at damage sites similar to what has been observed for endogenous Timeless. In contrast, inhibition of PARP-1 dramatically reduced GFP-XRCC1 recruitment (Figure S4C), which depends on poly(ADP-ribosyl)ation. We also performed long-term live cell experiments and found complete dissociation of GFP-Timeless from damage sites within 1 hr, which is in accordance with the results obtained from immunofluorescence (IF) studies. However, in PARP-1 inhibited cells, GFP-Timeless could still be detected at laser-tracks up to 2 hr after irradiation (Figure 4F). In vivo pull-down assays confirmed the interaction of GFP-Timeless with endogenous PARP-1 (Figure 4G). Taken together, we demonstrate that trapping of catalytically inactivated PARP-1 at DNA damage sites leads to pronounced recruitment and longer retention of its interacting partner Timeless.

Recruitment of Timeless to DNA Damage Sites Depends on Its Interaction with PARP-1

PARP-1 mediated poly(ADP-ribosyl)ation is believed to be the main prerequisite for the recruitment of PARP-1-interacting proteins to repair sites. To our knowledge, Timeless is the first PARP-1-interacting protein that is not dependent on PARP-1 enzymatic activity for its accumulation at DNA lesions. To further test whether the presence of PARP-1 at DNA damage sites is required for Timeless recruitment, we used a set of small interfering (si)RNAs specifically targeting PARP-1. PARP-1 depletion led to a dramatic reduction of endogenous and GFP-tagged Timeless recruited to laser-induced DNA damage sites (Figures 5A–5D). Interestingly, depletion of Timeless had no effect on PARP-1 recruitment (Figure S5D). The residual very weak recruitment of Timeless observed in some PARP-1 knockdown cells may be due to an incomplete PARP-1 depletion. We therefore analyzed recruitment of GFP-Timeless in wild-type and PARP-1 knockout mouse embryonic fibroblast (MEFs). Interestingly, in the absence of PARP-1, neither GFP-Timeless nor GFP-XRCC1 could be detected at damage sites (Figures 5A, 5C, and 5E). Co-expression of both GFP-Timeless and DsRed-PARP-1 in PARP-1 knockout MEFs not only restored recruitment of Timeless, but even enhanced its accumulation (Figures S5B and S5E). In contrast, co-expression of GFP-Timeless and FLAG-PARP-1 D993G mutant in PARP-1 knockout cells failed to recruit Timeless to laser tracks (Figures 5E, 5F, S5G, and S5H). This is consistent with the structural and quantitative binding analyses highlighting the ion pair formed by PARP-1 D993 and Timeless R1081 has a critical role in complex formation. Indeed, we observed the GFP-tagged Timeless R1081G mutant failed to accumulate at laser-induced DNA damage sites (Figures 5G, 5H, and S5F). Altogether, our data show that the physical interaction with PARP-1 targets Timeless to DNA damage sites.

The interaction of Timeless with Tipin has been shown to be required for S phase checkpoint activation and replication restart after induction of replication stress. We therefore tested whether Tipin is co-recruited to DNA damage sites along with Timeless by PARP-1. Interestingly, we found that Tipin recruitment not only displays the same kinetics as Timeless, but also depends on the interaction of Timeless with PARP-1, as the PARP-1 D993 mutant could not restore Tipin recruitment in PARP-1 deficient cells (Figures 6A, 6B, and S5I).

Previous studies have also demonstrated that PARP-1 is important for S phase checkpoint activation and restart of stalled replication forks (Bryant et al., 2009; Min et al., 2013; Sugimura et al., 2008). It remains elusive how Timeless and PARP-1 cooperate in response to replication stress. We attempted to study the potential implication of the Timeless-PARP-1 interaction in maintaining replication fork stability by treating GFP-Timeless, GFP-Timeless R1081G, and GFP-PARP-1 expressing cells with hydroxyurea (HU). As unbound protein likely masks the binding of GFP-tagged Timeless and PARP-1 to stalled or collapsed replication forks, we decided to perform fluorescence recovery after photobleaching (FRAP) analysis to determine the mobility of these factors in control and HU-treated cells. We found reduced mobility for GFP-Timeless and GFP-PARP-1 upon induction of replication stress, indicating increased binding at stalled or collapsed forks (Figure 6F). In line with this observation, we could also detect increased Timeless foci formation in response to HU treatment (Figures 6G and 6H), which could be partially dependent on PARP-1, as adding Olaparib slightly increased Timeless recruitment and γH2AX induction, but not RPA recruitment (Figures 6I–6K). Interestingly, the GFP-Timeless R1081G mutant that lost the capacity to bind to PARP-1 showed an increase in mobility in both untreated and HU-treated cells (Figure 6F). In addition, we could detect a minor increase in mobility of GFP-Timeless upon PARP-1 depletion (Figure 6E). HU did not trigger any interaction of Timeless R1081G with PARP-1, nor did it strengthen the interaction of wild-type Timeless with PARP-1 (Figure 6C). Thus PARP-1 might play a

Figure 4. Increased Timeless Recruitment to DNA Damage Sites upon PARP-1 Inhibition

(A) Outline of experimental procedure.

(B) Time course of Timeless recruitment to DNA damage sites in presence or absence of the PARP-1 inhibitor Olaparib. The recruitment of endogenous Timeless could be detected as early as 10 min after induction of DNA damage and co-localized with γH2AX, XRCC1, and PARP-1. The PARP-1 inhibitor treatment results in the prolonged retention of Timeless and PARP-1 at damage sites, while XRCC1 recruitment is lost.

(C) Timeless localizes to replication sites and is recruited to laser tracks in S and non-S phase cells. The cyclin A and PCNA antibodies were used to mark S phase cells.

(D) GFP-Timeless recruitment to DNA damage sites is increased upon PARP-1 inhibition.

(E) Quantification of GFP-Timeless recruitment in control and PARP-1 inhibitor treated cells. Note that GFP-Timeless recruitment is significantly increased in the cells treated with the PARP-1 inhibitor.

(F) PARP-1 inhibition results in prolonged retention of GFP-Timeless at laser-induced DNA damage sites.

(G) Interaction of GFP-Timeless with PARP-1. The 293T cells expressing either GFP or GFP-Timeless were harvested and GFP-tagged proteins pulled down with a GFP nanotrap (loading: Input and Non-Bound = 1% and Bound = 20%). The scale bar represents 10 μM. The data are represented as mean ± SEM. See also Figure S4 and Movie S1.

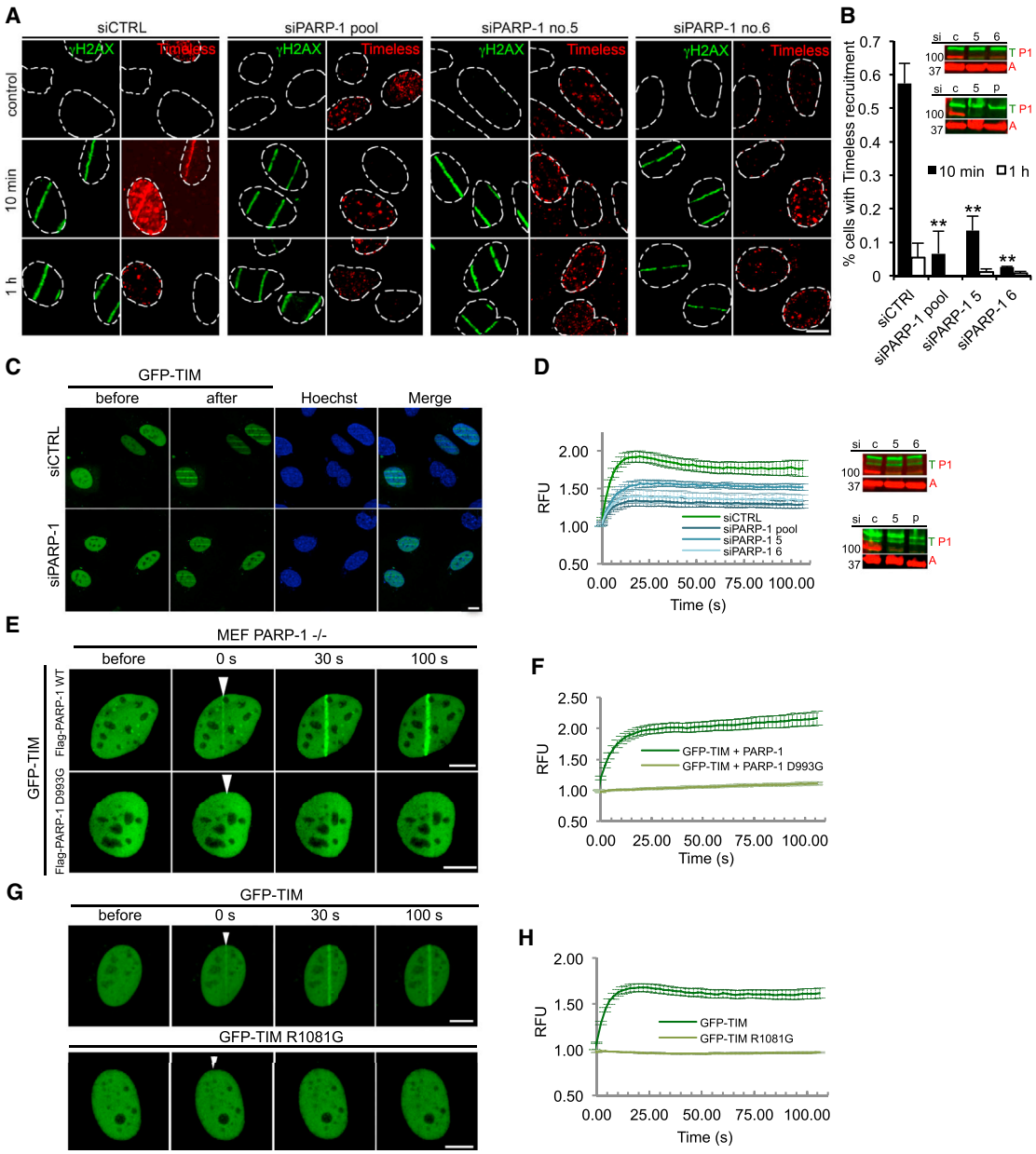


Figure 5. Recruitment of Timeless to DNA Damage Sites Depends on Its Interaction with PARP-1

(A) siRNA mediated knock down of PARP-1 abolishes recruitment of endogenous Timeless to DNA damage sites. The U2OS cells transfected with either control or PARP-1 siRNA were microirradiated, pre-extracted, fixed, and probed for endogenous Timeless. The depletion of PARP-1 via two different siRNAs or a pool of both abolishes the recruitment of endogenous Timeless to the DNA damage sites.

(B) Quantification of Timeless recruitment to laser-induced DNA damage sites co-localizing with γH2AX in control and PARP-1 knockdown cells. The inset shows efficient depletion of PARP-1 (Timeless: T and PARP-1: P1).

(C) Depletion of PARP-1 leads to a reduction of GFP-Timeless recruitment in living cells. The U2OS cells expressing GFP-tagged Timeless were transfected with control or PARP-1 siRNA and microirradiated.

(D) Quantification of recruitment kinetics. The inset shows efficient depletion of PARP-1 (Timeless: T and PARP-1: P1).

(E) Expression of PARP-1 wild-type, but not PARP-1 D993G mutant, restores recruitment of GFP-Timeless to laser tracks in PARP-1 knockout MEFs.

(F) Quantification of recruitment kinetics.

(G) U2OS cells either expressing wild-type GFP-tagged Timeless or GFP-Timeless R1081G mutant were microirradiated and the recruitment of GFP-tagged proteins was followed over time.

(H) Quantification of recruitment kinetics. The scale bar represents 10 μM. The data are represented as mean ± SEM. See also Figure S5.

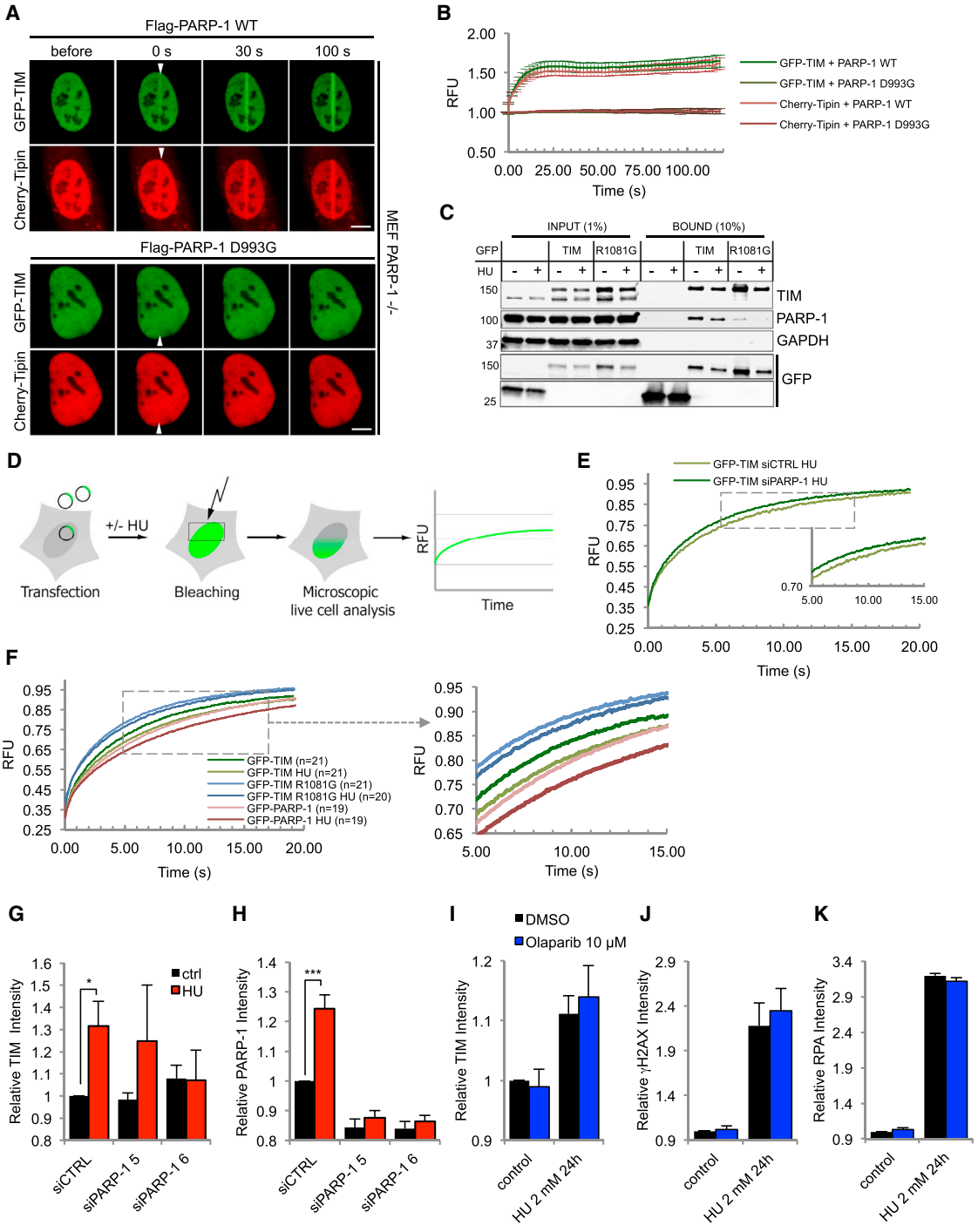


Figure 6. Interaction of Timeless with PARP-1 Is Required for Recruitment of Timeless and Tipin to Laser-Induced DNA Damage Sites

(A) Both GFP-Timeless and Cherry-Tipin readily accumulate at laser induced DNA damage sites in PARP-1 knockout MEFs expressing the wild-type PARP-1, but not the PARP-1 D993G mutant.

(B) Quantification of recruitment kinetics.

(C) HU does not significantly alter the interaction of Timeless and PARP-1. The 293T cells expressing GFP, GFP-Timeless (TIM), or GFP-Timeless R1081G (R1081G) were incubated in medium with or without HU for 24 hr before harvesting and pull down of GFP-tagged proteins with a GFP nanotrap (Input = 1% and Bound = 10%).

(D) Outline of FRAP experiments.

(E) Depletion of PARP-1 leads to minor increase of GFP-Timeless mobility after induction of replication stress. The U2OS cells transfected with control or PARP-1 siRNA were treated with HU for 24 hr before performing the FRAP experiments.

(legend continued on next page)

minor role in localizing Timeless to sites of replication stress, but likely is more important for Timeless recruitment to DSBs and SSBs.

Timeless Is Required for HR Repair

It was previously reported that knock down of Timeless leads to elevated levels of γH2AX foci formation even without genotoxic agents (Chou and Elledge, 2006), which we could confirm in U2OS cells with or without HU treatment (Figures S6A–S6C). Additionally, several studies have suggested that Timeless is also required ATM-dependent Chk2-mediated signaling of DNA DSBs (Urtishak et al., 2009; Yang et al., 2010) and Timeless depletion was found to cause defects in sister chromatid cohesion and chromosome segregation. However, the molecular mechanism of HR repair regulated by Timeless is largely unexplored. We speculated that PARP-1 mediated localization of Timeless to DNA damage sites might promote efficient repair. To determine a potential role of Timeless in HR repair, we performed a direct repeats (DR)-GFP recombination reporter assay in control and Timeless-depleted cells. Knock down of either Timeless or PARP-1 had no significant effect on cell-cycle distribution measured by FACS, EdU incorporation, or staining for the S phase marker PCNA (Figures S6D–S6H). We observed that Timeless knock down by different siRNAs resulted in a marked reduction in the number of GFP-positive cells (Figures 7A, 7B, S7A, and S7B), indicating a significant reduction of HR repair. In addition, PARP-1 depletion also impaired HR activity. Interestingly, double knock down of Timeless and PARP-1 did not have a synergistic effect, but showed an epistatic reduction of HR. Strikingly, expression of siRNA resistant wild-type Timeless rescued the HR defect in siRNA treated cells, while expression of the Timeless R1081G mutant failed to do so (Figures 7B, 7C, S7C, and S7D). In summary, depletion of Timeless or PARP-1 compromises HR-mediated DSB repair and both proteins likely function in the same DNA damage repair pathway.

DISCUSSION

In this study, we demonstrate a different mechanism for PARP-1 mediated protein recruitment to DNA damage sites, which is independent of its enzymatic activity or poly(ADP-ribosyl)ation. The structure of Timeless-PARP-1 complex indicates that the PARP-1 catalytic domain has dual functions: it functions as a catalytic domain to synthesize and attach poly(ADP-ribose) to acceptor proteins, but also directly modulates the interaction of PARP-1 with its protein partners. These two distinct functions seem not to interfere with each other, as the enzymatic assay shows PARP-1 can efficiently auto-poly(ADP-ribosyl)ate itself in the presence or absence of the Timeless PAB domain. It is thus reasonable to deduce that PARP-1 depletion and inhibition should generate different biological outcomes.

Several PARP inhibitors have been developed to efficiently target PARP-1 and are widely used in the clinic to target BRCA1 or BRCA2 mutated cancers (Bryant et al., 2005; Farmer et al., 2005). Here, we demonstrate that PARP inhibitors do not disrupt the PARP-1-Timeless complex in cells. Instead, Timeless is co-trapped with PARP-1 at DNA lesions after PARP inhibition, which could have implications for understanding the mechanism of action of clinically active PARP inhibitors.

Timeless has a phylogenetically conserved role in maintaining and monitoring the integrity of the replication fork (Errico and Costanzo, 2012), while accumulating evidence has shown that PARP-1 is also an important mediator of replication fork stability (Bryant et al., 2009; Min et al., 2013; Sugimura et al., 2008). PARP-1 and Timeless have overlapping roles when replication fork is pausing: both have been shown to modulate the progression of replication in response to DNA damage; both have protective roles to prevent the collapse of stalled forks, and both proteins are involved in S phase checkpoint activation through Chk1. As Timeless and PARP-1 are present in a complex, it is likely that they together participate in damage surveillance against potential replication stress and also in DNA repair, although the role of Timeless in DNA repair is so far poorly studied. Interestingly, Timeless defective cells have a similar increase in spontaneous sister chromatid exchange as reported for PARP-1 defective cells (Urtishak et al., 2009; Wang et al., 1997). In relation to PARP-1, this increase in HR has been suggested to be a consequence of lacking DNA SSB repair (Bryant et al., 2005), indicating there can be several ways in which Timeless and PARP-1 are collaboratively involved in DNA repair.

In this report, we demonstrate that Timeless is rapidly recruited to DNA damage sites, and this recruitment is dependent on its interaction with PARP-1, but independent of poly(ADP-ribosyl)ation. Knock down of Timeless compromised HR-mediated DSB repair, suggesting a previous uncharacterized role of Timeless in the repair of DNA DSBs. Future efforts are required to elucidate how exactly Timeless promotes DSBs repair. Since mounting evidence suggests that HR is the major mechanism to restart stalled replication forks in higher eukaryotes (Mehta and Haber, 2014; Petermann and Helleday, 2010), it is likely that the Timeless-PARP-1 complex has a direct role in the repair of fork-associated damage. We also show here that Tipin is co-recruited to DNA damage sites along with Timeless and PARP-1, with similar recruitment kinetics compared to Timeless. Likely, the recruitment of Tipin to damaged sites relies on the interaction of PARP-1 with Timeless.

(F) The interaction of Timeless with PARP-1 is required for chromatin binding. The U2OS cells expressing GFP-Timeless, GFP-Timeless R1081G, or GFP-PARP-1 were mock treated or treated with HU (2 mM 24 hr) before performing FRAP analysis to determine protein mobility. For clarity, an inset of the FRAP curve was magnified.

(G and H) U2OS cells were transfected with different siRNAs targeting PARP-1 and after 48 hr mock treated or treated with 2 mM HU for 24 hr before fixation and immunostaining.

(I–K) U2OS cells were treated with 2 mM HU and where indicated 10 μM Olaparib for 24 hr before fixation and immunostaining with the indicated antibodies. The images were taken with a high throughput microscope. The relative fluorescence intensities are displayed. The scale bar represents 10 μM. The data are represented as mean ± SEM. See also Figure S5.

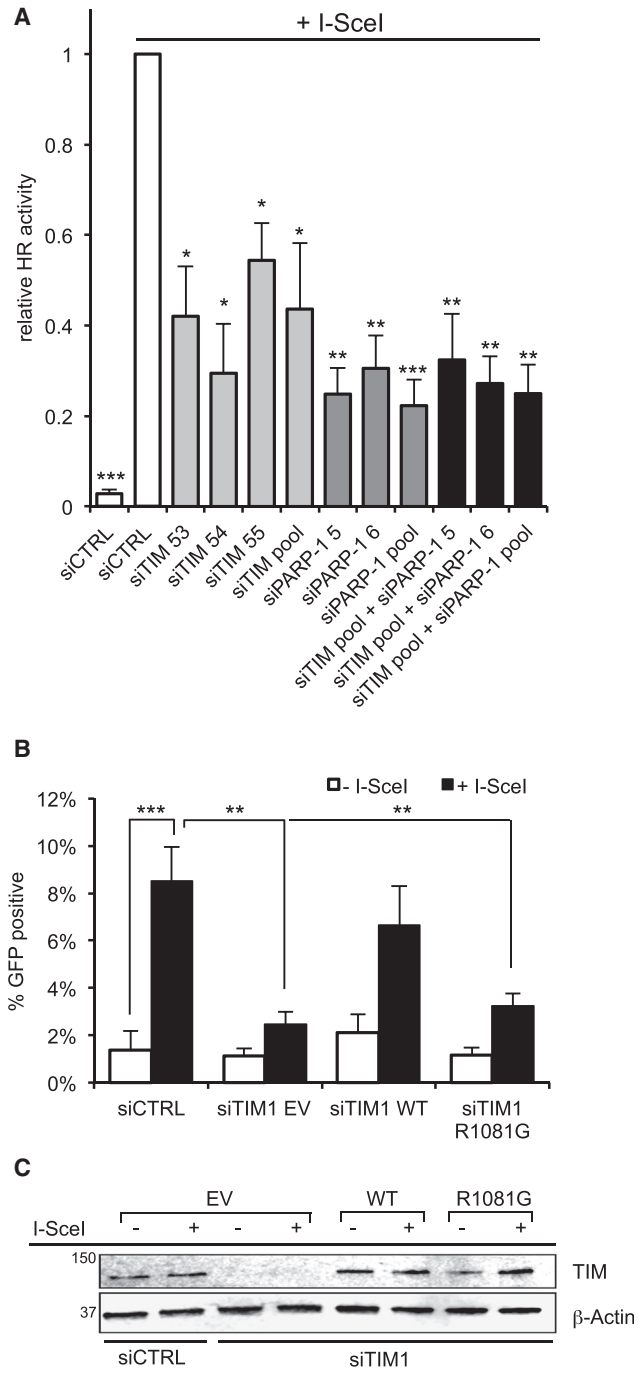


Figure 7. HR Is Impaired in Timeless Depleted Cells

(A) Relative HR activity in control, Timeless, PARP-1, and double knockdown cells. The DR-GFP U2OS cells were transfected with the indicated siRNAs.

(B) Expression of siRNA resistant wild-type Timeless, but not Timeless R1081G, can restore HR activity. The DR-GFP U2OS cells treated with control or Timeless siRNA were co-transfected with indicated expression constructs and pcDNA3.1(+)-Cherry to be able to follow transfection efficiency by red fluorescence. At 48 hr after I-SceI transfection, the cells were fixed and the percentage of GFP positive cells relative to the number of Cherry positive cells was analyzed using high throughput microscopy.

(C) Confirmation of Timeless knockdown and transient overexpression of wild-type and R1081G Timeless in siTIM1 treated U2OS cells were analyzed by western blot. The data are represented as mean ± SEM. See also Figures S6 and S7.

In conclusion, we report the crystal structure of human Timeless-PARP-1 complex and provide evidence that Timeless is recruited to sites of DNA damage through PARP-1 to mediate HR repair of DNA DSBs.

EXPERIMENTAL PROCEDURES

Please refer to the Supplemental Information for additional experimental details.

Plasmid Construction, Protein Expression, and Purification

For the structural and biochemical analyses, the human Timeless, Timeless PAB (also referred to as Timeless_D4) domain, PARP-1 and various PARP-1 fragments and related mutants, PARP-2, and PARP-3 catalytic domains were cloned into pET28a, pGEX-6P-1, pCold-GST, or home-engineered pRSF-SUMO plasmids (Hayashi and Kojima, 2008). For the cell-based analyses, fl-Timeless, fl-PARP-1, and mutants were cloned into pEGFP-C vector and pcDNA3 containing either HA- or FLAG-tag, respectively. Fl-Tipin was cloned into pcDNA3 with a N-terminal cherry tag. GFP-XRCC1 and DsRed-PARP-1 were prepared as previously described (Maeda et al., 2006; Mortusewicz et al., 2007). The details of plasmid construction, mutagenesis, protein expression, and purification can be found in the Supplemental Information.

Interaction Assays

TAP using triple-epitope S protein, FLAG, and streptavidin-binding peptide-tagged Timeless_D4 to identify possible interacting proteins was performed essentially as described previously (Sy et al., 2009). CoIP was performed to verify the Timeless and PARP-1 interaction. In vitro pull-down assay and SPR measurement were performed to map out the interaction of the Timeless PAB domain with various PARP-1 fragments and also used to examine the interactions of the Timeless PAB domain with PARP-1, PARP-2, and PARP-3. CoIP with a GFP-Trap (Chromotek) was performed according to the manufactures protocol. Further details are provided in the Supplemental Information.

Crystallization and Structure Determination

Timeless, PAB domain, and its complex with the PARP-1 catalytic domain were crystallized using sitting drop vapor diffusion method. All crystallographic data for the SeMet-labeled, native Timeless PAB domain and Timeless-PARP-1 complex were collected at SSRF beamline BL17U. Data were indexed, integrated, and scaled using the HKL2000 program suites (Otwinowski and Minor, 1997). The SeMet-labeled Timeless PAB structure was solved using single-wavelength anomalous diffraction (SAD) method at SeMet-peak wavelength. The complex structure was determined by molecular replacement and refined with Phenix and COOT (Adams et al., 2010; Emsley and Cowtan, 2004). X-ray data collection and refinement statistics are listed in Table 1.

Cell Lines

Human U2OS osteosarcoma, HEK293T cells, and wild-type, as well as PARP-1 knockout MEFs cells, were cultured in DMEM (Invitrogen) containing Pen/Strep supplemented with 10% fetal bovine serum (FBS) in a humidified CO_2 atmosphere at 37°C. Details on cell treatments are provided in the Supplemental Information.

Live Cell and High-Throughput Microscopy, FRAP, and Microirradiation

Live cell imaging, photobleaching, and microirradiation experiments were carried out with a Zeiss LSM780 confocal laser scanning microscope equipped with a UV-transmitting Plan-Apochromat 40×/1.30 Oil DIC M27 objective

and a heated environmental chamber. For microirradiation, cells were presensitized in medium containing 10 μg/ml Hoechst for 10 min. High throughput microscopy was performed with an Operetta (Perkin Elmer) or ImageXpress (Molecular Devices) microscope using a 20× lens. Further details on live cell imaging, photobleaching, laser mircoirradiation, FRAP, and IF are provided in the Supplemental Information.

Western Blot

Western blot was carried out as described in the Supplemental Information.

GFP-HR Assay

Details for the GFP-HR assay are provided in the Supplemental Information. In brief, DR-GFP U2OS cells were transfected with corresponding siRNAs and 48 hr later transfected with I-SceI and indicated vectors. GFP-positive cells were either analyzed by FACS or high throughput microscopy (Pierce et al., 1999).

Statistical Analysis

Data from at least three independent experiments were subjected to a two-tailed Student's t test to determine statistical significance and presented ± SEM.

ACCESSION NUMBERS

The coordinates and structure factors for the reported crystal structures have been deposited in the Protein Data Bank with the following accession codes: 4XHT and 4XHW for the Timeless PAB domain in free form (native, SeMet) and 4XHU for the Timeless-PARP-1 complex.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.07.031.

AUTHOR CONTRIBUTIONS

C.Q. conceived the project. T.H., R.P., and C.Q. supervised this study. S.X. did structural analyses, SPR, and enzymatic assay. O.M. performed live cell imaging, FRAP, and DR-GFP assay. P.H. prepared Tipin constructs. H.M. prepared siRNA resistant Timeless constructs and did coIP in HeLa cells. All authors contributed to the manuscript preparation.

ACKNOWLEDGMENTS

We wish to acknowledge the use of the Shanghai Synchrotron Radiation Facility (beamline BL17U) for X-ray data collection. We thank Dr. Michael SY Huen for helping collect TAP data, Dr. Valérie Schreiber and Françoise Dantzer for providing GFP-tagged PARP-1 expression vectors as well as wild-type and knockout MEFs, and Dr. Jean Jankonic for the useful discussion. This study was supported by the Swedish Research Council (T.H.), the Swedish Cancer Society (T.H.), the AXA foundation (T.H.), the Göran Gustafsson Foundation (T.H.), the Helleday Foundation (O.M.), the Wenner-Gren Foundation (P.H.), the Torsten and Ragnar Söderberg Foundation (T.H.), and the Hong Kong Research Grants Council, General Research Fund 776313 and 775712 (C.Q.).

Received: January 14, 2015
Revised: May 1, 2015
Accepted: July 29, 2015
Published: September 3, 2015

REFERENCES

Adams, P.D., Afonine, P.V., Bunkoczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, L.W., Kapral, G.J., Grosse-Kunstleve, R.W., et al. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Cryst. *66*, 213–221.

Ali, A.A., Timinszky, G., Arribas-Bosacoma, R., Kozlowski, M., Hassa, P.O., Hassler, M., Ladurner, A.G., Pearl, L.H., and Oliver, A.W. (2012). The zinc-finger domains of PARP1 cooperate to recognize DNA strand breaks. Nat. Struct. Mol. Biol. *19*, 685–692.

Allen, M.D., Buchberger, A., and Bycroft, M. (2006). The PUB domain functions as a p97 binding module in human peptide N-glycanase. J. Biol. Chem. *281*, 25502–25508.

Aria, V., De Felice, M., Di Perna, R., Uno, S., Masai, H., Syväoja, J.E., van Loon, B., Hübscher, U., and Pisani, F.M. (2013). The human Tim-Tipin complex interacts directly with DNA polymerase epsilon and stimulates its synthetic activity. J. Biol. Chem. *288*, 12742–12752.

Barnes, J.W., Tischkau, S.A., Barnes, J.A., Mitchell, J.W., Burgoon, P.W., Hickok, J.R., and Gillette, M.U. (2003). Requirement of mammalian Timeless for circadian rhythmicity. Science *302*, 439–442.

Benna, C., Scannapieco, P., Piccin, A., Sandrelli, F., Zordan, M., Rosato, E., Kyriacou, C.P., Valle, G., and Costa, R. (2000). A second timeless gene in Drosophila shares greater sequence similarity with mammalian tim. Curr. Biol. *10*, R512–R513.

Benna, C., Bonaccorsi, S., Wülbeck, C., Helfrich-Förster, C., Gatti, M., Kyriacou, C.P., Costa, R., and Sandrelli, F. (2010). Drosophila timeless2 is required for chromosome stability and circadian photoreception. Curr. Biol. *20*, 346–352.

Branzei, D., and Foiani, M. (2010). Maintaining genome stability at the replication fork. Nat. Rev. Mol. Cell Biol. *11*, 208–219.

Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J., and Helleday, T. (2005). Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature *434*, 913–917.

Bryant, H.E., Petermann, E., Schultz, N., Jemth, A.S., Loseva, O., Issaeva, N., Johansson, F., Fernandez, S., McGlynn, P., and Helleday, T. (2009). PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. EMBO J. *28*, 2601–2615.

Cho, W.H., Kang, Y.H., An, Y.Y., Tappin, I., Hurwitz, J., and Lee, J.K. (2013). Human Tim-Tipin complex affects the biochemical properties of the replicative DNA helicase and DNA polymerases. Proc. Natl. Acad. Sci. USA *110*, 2523–2527.

Chou, D.M., and Elledge, S.J. (2006). Tipin and Timeless form a mutually protective complex required for genotoxic stress resistance and checkpoint function. Proc. Natl. Acad. Sci. USA *103*, 18143–18147.

Ciccia, A., and Elledge, S.J. (2010). The DNA damage response: making it safe to play with knives. Mol. Cell *40*, 179–204.

Elliott, P.R., Nielsen, S.V., Marco-Casanova, P., Fiil, B.K., Keusekotten, K., Mailand, N., Freund, S.M., Gyrd-Hansen, M., and Komander, D. (2014). Molecular basis and regulation of OTULIN-LUBAC interaction. Mol. Cell *54*, 335–348.

Emsley, P., and Cowtan, K. (2004). Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. *60*, 2126–2132.

Errico, A., and Costanzo, V. (2012). Mechanisms of replication fork protection: a safeguard for genome stability. Crit. Rev. Biochem. Mol. Biol. *47*, 222–235.

Errico, A., Costanzo, V., and Hunt, T. (2007). Tipin is required for stalled replication forks to resume DNA replication after removal of aphidicolin in Xenopus egg extracts. Proc. Natl. Acad. Sci. USA *104*, 14929–14934.

Farmer, H., McCabe, N., Lord, C.J., Tutt, A.N., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., et al. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature *434*, 917–921.

Gibson, B.A., and Kraus, W.L. (2012). New insights into the molecular and cellular functions of poly(ADP-ribose) and PARPs. Nat. Rev. Mol. Cell Biol. *13*, 411–424.

Gotter, A.L. (2003). Tipin, a novel timeless-interacting protein, is developmentally co-expressed with timeless and disrupts its self-association. J. Mol. Biol. *331*, 167–176.

Gotter, A.L., Manganaro, T., Weaver, D.R., Kolakowski, L.F., Jr., Possidente, B., Sriram, S., MacLaughlin, D.T., and Reppert, S.M. (2000). A time-less function for mouse timeless. Nat. Neurosci. *3*, 755–756.

Gotter, A.L., Suppa, C., and Emanuel, B.S. (2007). Mammalian TIMELESS and Tipin are evolutionarily conserved replication fork-associated factors. J. Mol. Biol. *366*, 36–52.

Hardin, P.E. (2005). The circadian timekeeping system of Drosophila. Curr. Biol. *15*, R714–R722.

Hayashi, K., and Kojima, C. (2008). pCold-GST vector: a novel cold-shock vector containing GST tag for soluble protein production. Protein Expr. Purif. *62*, 120–127.

Langelier, M.F., Planck, J.L., Roy, S., and Pascal, J.M. (2012). Structural basis for DNA damage-dependent poly(ADP-ribosyl)ation by human PARP-1. Science *336*, 728–732.

Leman, A.R., Noguchi, C., Lee, C.Y., and Noguchi, E. (2010). Human Timeless and Tipin stabilize replication forks and facilitate sister-chromatid cohesion. J. Cell Sci. *123*, 660–670.

Leman, A.R., Dheekollu, J., Deng, Z., Lee, S.W., Das, M.M., Lieberman, P.M., and Noguchi, E. (2012). Timeless preserves telomere length by promoting efficient DNA replication through human telomeres. Cell Cycle *11*, 2337–2347.

Maeda, Y., Hunter, T.C., Loudy, D.E., Davé, V., Schreiber, V., and Whitsett, J.A. (2006). PARP-2 interacts with TTF-1 and regulates expression of surfactant protein-B. J. Biol. Chem. *281*, 9600–9606.

Mehta, A., and Haber, J.E. (2014). Sources of DNA double-strand breaks and models of recombinational DNA repair. Cold Spring Harb. Perspect. Biol. *6*, a016428.

Min, W., Bruhn, C., Grigaravicius, P., Zhou, Z.W., Li, F., Krüger, A., Siddeek, B., Greulich, K.O., Popp, O., Meisezahl, C., et al. (2013). Poly(ADP-ribose) binding to Chk1 at stalled replication forks is required for S-phase checkpoint activation. Nat. Commun. *4*, 2993.

Mortusewicz, O., Amé, J.C., Schreiber, V., and Leonhardt, H. (2007). Feedback-regulated poly(ADP-ribosyl)ation by PARP-1 is required for rapid response to DNA damage in living cells. Nucleic Acids Res. *35*, 7665–7675.

Murakami, H., and Keeney, S. (2014). Temporospatial coordination of meiotic DNA replication and recombination via DDK recruitment to replisomes. Cell *158*, 861–873.

Otwinowski, T., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. *276*, 307–326.

Petermann, E., and Helleday, T. (2010). Pathways of mammalian replication fork restart. Nat. Rev. Mol. Cell Biol. *11*, 683–687.

Pierce, A.J., Johnson, R.D., Thompson, L.H., and Jasin, M. (1999). XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. Genes Dev. *13*, 2633–2638.

Sangoram, A.M., Saez, L., Antoch, M.P., Gekakis, N., Staknis, D., Whiteley, A., Fruechte, E.M., Vitaterna, M.H., Shimomura, K., King, D.P., et al. (1998). Mammalian circadian autoregulatory loop: a timeless ortholog and mPer1 interact and negatively regulate CLOCK-BMAL1-induced transcription. Neuron *21*, 1101–1113.

Schaeffer, V., Akutsu, M., Olma, M.H., Gomes, L.C., Kawasaki, M., and Dikic, I. (2014). Binding of OTULIN to the PUB domain of HOIP controls NF-κB signaling. Mol. Cell *54*, 349–361.

Schreiber, V., Dantzer, F., Ame, J.C., and de Murcia, G. (2006). Poly(ADP-ribose): novel functions for an old molecule. Nat. Rev. Mol. Cell Biol. *7*, 517–528.

Serçin, O., and Kemp, M.G. (2011). Characterization of functional domains in human Claspin. Cell Cycle *10*, 1599–1606.

Shieh, W.M., Amé, J.C., Wilson, M.V., Wang, Z.Q., Koh, D.W., Jacobson, M.K., and Jacobson, E.L. (1998). Poly(ADP-ribose) polymerase null mouse cells synthesize ADP-ribose polymers. J. Biol. Chem. *273*, 30069–30072.

Smith, K.D., Fu, M.A., and Brown, E.J. (2009). Tim-Tipin dysfunction creates an indispensible reliance on the ATR-Chk1 pathway for continued DNA synthesis. J. Cell Biol. *187*, 15–23.

Sugimura, K., Takebayashi, S., Taguchi, H., Takeda, S., and Okumura, K. (2008). PARP-1 ensures regulation of replication fork progression by homologous recombination on damaged DNA. J. Cell Biol. *183*, 1203–1212.

Sy, S.M., Huen, M.S., and Chen, J. (2009). PALB2 is an integral component of the BRCA complex required for homologous recombination repair. Proc. Natl. Acad. Sci. USA *106*, 7155–7160.

Unsal-Kaçmaz, K., Mullen, T.E., Kaufmann, W.K., and Sancar, A. (2005). Coupling of human circadian and cell cycles by the timeless protein. Mol. Cell. Biol. *25*, 3109–3116.

Unsal-Kaçmaz, K., Chastain, P.D., Qu, P.P., Minoo, P., Cordeiro-Stone, M., Sancar, A., and Kaufmann, W.K. (2007). The human Tim/Tipin complex coordinates an Intra-S checkpoint response to UV that slows replication fork displacement. Mol. Cell. Biol. *27*, 3131–3142.

Urtishak, K.A., Smith, K.D., Chanoux, R.A., Greenberg, R.A., Johnson, F.B., and Brown, E.J. (2009). Timeless maintains genomic stability and suppresses sister chromatid exchange during unperturbed DNA replication. J. Biol. Chem. *284*, 8777–8785.

Wang, Z.Q., Stingl, L., Morrison, C., Jantsch, M., Los, M., Schulze-Osthoff, K., and Wagner, E.F. (1997). PARP is important for genomic stability but dispensable in apoptosis. Genes Dev. *11*, 2347–2358.

Yang, X., Wood, P.A., and Hrushesky, W.J. (2010). Mammalian TIMELESS is required for ATM-dependent CHK2 activation and G2/M checkpoint control. J. Biol. Chem. *285*, 3030–3034.

Yoshizawa-Sugata, N., and Masai, H. (2007). Human Tim/Timeless-interacting protein, Tipin, is required for efficient progression of S phase and DNA replication checkpoint. J. Biol. Chem. *282*, 2729–2740.

Zylka, M.J., Shearman, L.P., Levine, J.D., Jin, X., Weaver, D.R., and Reppert, S.M. (1998). Molecular analysis of mammalian timeless. Neuron *21*, 1115–1122.