PrimPol is required for replication reinitiation after mtDNA damage

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Eukaryotic PrimPol is a recently discovered DNA-dependent DNA primase and translesion synthesis DNA polymerase found in the nucleus and mitochondria. Although PrimPol has been shown to be required for repriming of stalled replication forks in the nucleus, its role in mitochondria has remained unresolved. Here we demonstrate in vivo and in vitro that PrimPol can reinitiate stalled mtDNA replication and can prime mtDNA replication from nonconventional origins. Our results not only help in the understanding of how mitochondria cope with replicative stress but can also explain some controversial features of the lagging-strand replication.

DNA repair | fork rescue | mtDNA damage | mtDNA replication

PrimPol is an unusual mammalian primase-polymerase belonging to the archaeo-eukaryotic primase superfamily of primases (1, 2). The superfamily includes all known replicative primases in Archaea and Eukaryotes and is evolutionarily unrelated to the bacterial topoisomerase-primases (TOPRIMs) (3, 4). Similarly to the related archaeal PriS/L replicative primases (5, 6), PrimPol has a clear preference for dNTPs over NTPs, allowing it to synthesize DNA primers and function as a DNA-dependent DNA polymerase (2). It has been suggested that the priming, as well as primer extension activities, are required for DNA damage tolerance, such as translesion synthesis (TLS) across lesions such as 8-oxo-7-hydrodeoxyguanosine (2, 7), abasic sites, and UV lesions (8–10). Concordantly, PrimPol-KO cells are viable (1, 2, 9), but have an increased sensitivity to DNA-damaging agents such as UV and hydroxyurea (11). In addition, PrimPol contributes to the repriming of replication forks that are arrested at G-quadruplex structures in the template (12).

Like many DNA repair proteins (13), PrimPol is known to be localized in the nucleus and mitochondria (2), suggesting that it may play similar roles in the maintenance of mtDNA as it does with nuclear DNA. Although PrimPol has been proposed to be involved in multipriming events on mtDNA (2), no specific role for PrimPol in mtDNA maintenance has been experimentally demonstrated to our knowledge. In contrast to the nucleus, mitochondria are thought to have a limited set of DNA repair pathways; for example, they are unable to repair cyclobutane pyrimidine dimers caused by UV damage (14). Repair of DNA lesions represents only a subset of genome maintenance mechanisms, and the most dangerous types of DNA damage can result from complications during DNA replication. This seems to also be the case in mitochondria, as replication fork stalling has been implicated as the main cause of pathological mtDNA rearrangements (15, 16). mtDNA replication can stall as a result of mutations in TWNK helicase and the catalytic subunit of DNA polymerase γ (Pol γA) (16–18), chain-terminating nucleoside analogs such as 2′-3′-dideoxycytidine (ddC) (19), and DNA template damage (20). Unlike some catalytic mutations or ddC interference, oxidative or UV damage-induced stalling does not result in mtDNA copy number depletion, indicating that mitochondria have effective mechanisms to cope with such damage (21).

To our knowledge, nothing is known about the fate of stalled replication forks in mitochondria, and evidence suggests that the outcomes might be different in different tissues (15). Mitotic cells, like those used in tissue culture, mainly employ a highly strand-asymmetric replication mechanism, whereby the lagging-strand DNA is synthesized with a considerable delay (22). This replication mechanism results in typical patterns on 2D agarose gels used in DNA replications studies (23, 24). Although there is still debate about the details, the two proposed models for strand-asymmetric replication mechanism are very alike with the exception of the displaced strand being coated with preformed RNA (23) or the mitochondrial single-strand binding protein mtSSB (25). As there is evidence for (24) and against (26) RNA covering the displaced mtDNA strand in vivo, we use "strand-asymmetric mechanism" as a general term without differentiating between the two models. The main origin of leading-strand replication in the strand-asymmetric mechanism is the origin of heavy-strand replication (O_H) in the noncoding region (NCR) of mtDNA (27). Replication from O_H is assumedly primed by mitochondrial RNA polymerase (MTRPOL) transcribing from one of the two light-strand promoters (28). A major origin for the lagging-strand synthesis is at the origin of light-strand replication (O_L), two thirds of the genome downstream of O_H, and is also initiated by MTRPOL (16, 29), although there is evidence for other light-strand origins (25).

In the present work, we examine the role of PrimPol in the restart of stalled mtDNA replication forks and its significance for mtDNA maintenance after damage. We find that PrimPol is not only

Significance

Failure to maintain mtDNA integrity can lead to a wide variety of neuromuscular disorders. Despite its central role in the development of these disorders, many mechanistic details of mtDNA maintenance are still unclear. In the present work, we have studied the role of PrimPol, an unusual primase-polymerase, in mammalian mtDNA maintenance. We report here that PrimPol is specifically required for replication reinitiation after DNA damage. PrimPol synthesizes DNA primers on an ssDNA template, which can be elongated by the mitochondrial replicative polymerase γ, a solution to reprime replication beyond DNA lesions and to facilitate lagging-strand replication. Our findings show that PrimPol has biological relevance for mtDNA maintenance.

responsible for the replication reinitiation downstream of DNA lesions, but that it is also involved in the completion of partially ss mtDNA molecules. We conclude that, even though PrimPol is not essential for mtDNA maintenance, it provides an adaptational mechanism against genotoxic stress in mitochondria and might also enable origin-independent initiation of lagging-strand synthesis.

Results

PrimPol Is Required for Repriming of Stalled mtDNA Replication.
Chain-terminating nucleoside analogs (CTNAs) represent a very specific type of problem for DNA replication that can be mainly resolved via repriming and not, for example, by a TLS-type of activity. As PrimPol was recently shown to be important in maintaining nuclear DNA replication in the presence of CTNAs (11), we wanted to investigate whether this is also the case for mtDNA replication. In fact, Pol γ is the only mammalian replicative polymerase capable of incorporating ddC during DNA synthesis (30), resulting in specific blockage of mtDNA replication that can be detected by using 2D agarose gel electrophoresis (AGE; Fig. 1 and Fig. S1; further details are provided in *SI Results and Discussion*). Although *Primpol*-KO MEFs do not show any obvious replication phenotype under normal conditions (Fig. 1C), a 48-h treatment with 175 μM ddC results in complete replication stalling and depletion of mtDNA copy number (Fig. 1D and Fig. S2), revealing major differences in the replication responses between the WT and KO cells (Fig. 1E). WT MEFs showed a strong increase in replication bubbles, indicating increased replication initiation within the O_H-containing region of mtDNA (explained in Fig. 1B and Fig. S1). In contrast, *Primpol*$^{-/-}$ cells seem to lose their replication intermediates upon ddC treatment (Fig. 1E), demonstrating that the compensatory increase in replication initiation is fully dependent on the presence of PrimPol. As replication cannot be completed in either cell type as a result of the recurrent ddC incorporation, the WT MEFs show a similar depletion of mtDNA copy number under ddC as *Primpol*$^{-/-}$ (Fig. 1D). However, when ddC is removed from the growth medium, mtDNA copy number recovery is significantly delayed in *Primpol*$^{-/-}$ cells compared with WT MEFs (Fig. 1D). No difference in mtDNA integrity was observed between the two cell lines (Fig. S2C).

To confirm that PrimPol can reinitiate replication when strand elongation is blocked at the mtDNA replication fork, we simulated the situation in vitro by using M13mp18 ssDNA template, purified recombinant Pol γAB$_2$ (for simplicity, hereafter referred to as Pol γ), and PrimPol. When provided with a normal oligonucleotide primer, Pol γ was able to efficiently synthesize [α-^{32}P]dGTP-labeled full-length (7.3-kb) product from M13mp18 template (Fig. 2A, lane 2, black arrowhead). The shorter DNA bands observed in this reaction are the result of Pol γ pausing in front of secondary DNA structures, which form on this ssDNA template in the absence of single-strand binding proteins. The addition of PrimPol to the reaction resulted in additional DNA products (Fig. 2A, lane 3, white arrowheads), which differ from the ones caused by Pol γ pausing (Fig. S3). PrimPol alone cannot generate these species because PrimPol is unable to synthesize long DNA fragments as a result of its low processivity (Fig. 2A, lane 1). In agreement with the published data on inefficient removal of chain terminators by Pol γ proofreading activity (30), Pol γ was unable to elongate a primer with a 3′ ddC-monophosphate (ddCMP) (Fig. 2A, lane 5). The observed faint full-length DNA product is likely to result from ddCMP-oligonucleotide impurity. When PrimPol is added to the reaction, it will provide new primers for Pol γ and enables DNA synthesis, with the majority of products arising from specific priming events on the M13mp18 template (Fig. 2A, lane 6, white arrowheads). It should be noted that Pol γ is unable to initiate DNA synthesis in the absence of a primer (Fig. 2A, lane 8), but the addition of PrimPol to the reaction enables the synthesis of full-length DNA products (Fig. 2A,
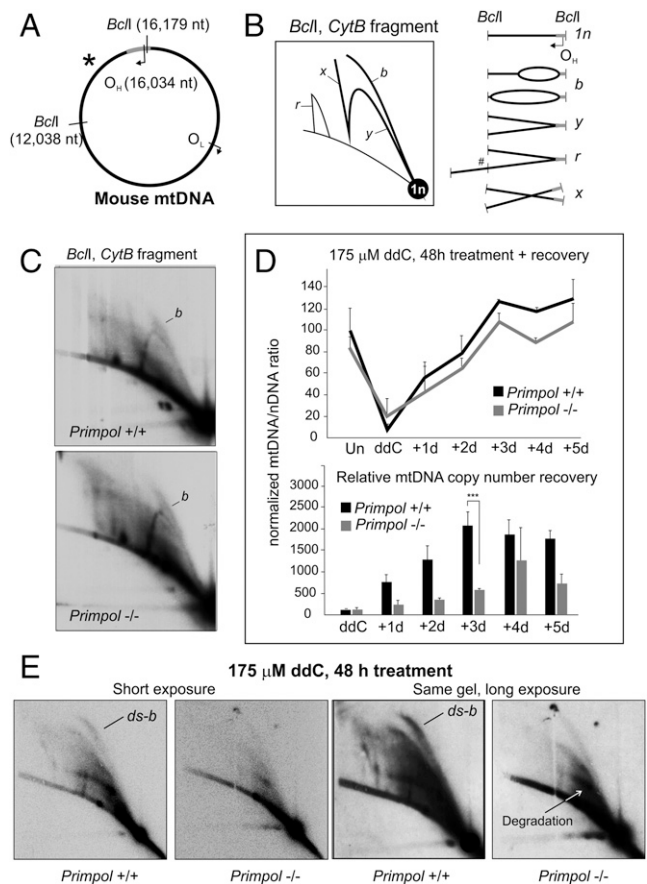

Fig. 1. PrimPol is required for replication restart in mitochondria. (A) A schematic illustration of mouse mtDNA showing the canonical replication origins (O_H, O_L) and BclI restriction sites adjacent to the probed fragment, marked with an asterisk. (B) An interpretation of 2D-AGE patterns from BclI-digested mouse mtDNA with an illustration of the corresponding replication intermediates. Linear fragments corresponding to the restriction fragment size (1n) will constitute the majority of the molecules. Replication that has initiated within the fragment forms replication bubbles, migrating as a bubble arc (marked as "b"), whereas replication that has exited the fragment (or initiated elsewhere) will form the Y-arc ("y"). The bubble arcs can be partially single-stranded or fully double-stranded (*SI Results and Discussion*). Replication intermediates resistant against restriction digestion (#) migrate in high molecular weight arcs ("r") and recombination intermediates on the x-arc ("x"). (C) Two-dimensional AGE of BclI-digested mtDNA from WT (*Primpol*$^{+/+}$) and *Primpol*-KO (*Primpol*$^{-/-}$) MEFs, probed for the O_H-containing fragment as illustrated in A. No differences in the replication intermediates can be observed. Signal intensities were quantified with a PhosphorImager, and autoradiographic films shown here represent comparable exposures. (D) ddC treatment causes a dramatic depletion in the mtDNA copy number in both cell types, with *Primpol*$^{-/-}$ cells still showing delayed recovery at 72 h after the removal of the drug [n = 9; P < 0.01, one-way ANOVA with post hoc Tukey honest significant difference (HSD) test; error bars represent SD]. (*Lower*) Quantification of the copy number recovery, standardized against mtDNA copy number, after 48 h ddC treatment to demonstrate the differences in recovery kinetics. Note that mtDNA depletion in *Primpol*$^{-/-}$ cells is not as strong, and that mtDNA steady-state levels are lower than in WT. (E) A 48-h treatment of cells with 175 μM ddC causes an accumulation of replication intermediates in WT cells, but a notable decrease in all replicative forms in *Primpol*$^{-/-}$ MEFs. Note especially the strong increase in dsDNA replication bubbles (*ds-b*) in the WT MEFs after ddC treatment and the degradation of stalled intermediates. Longer exposure of the same gel is given as a comparison.

lane 9), further corroborating that PrimPol can provide DNA primers for Pol γ (2).

During in vivo experiments, addition of ddC to the cell growth media results, after several phosphorylation steps, in a mixed deoxycytide/di-deoxycytidine triphosphate (dCTP/ddCTP) cellular
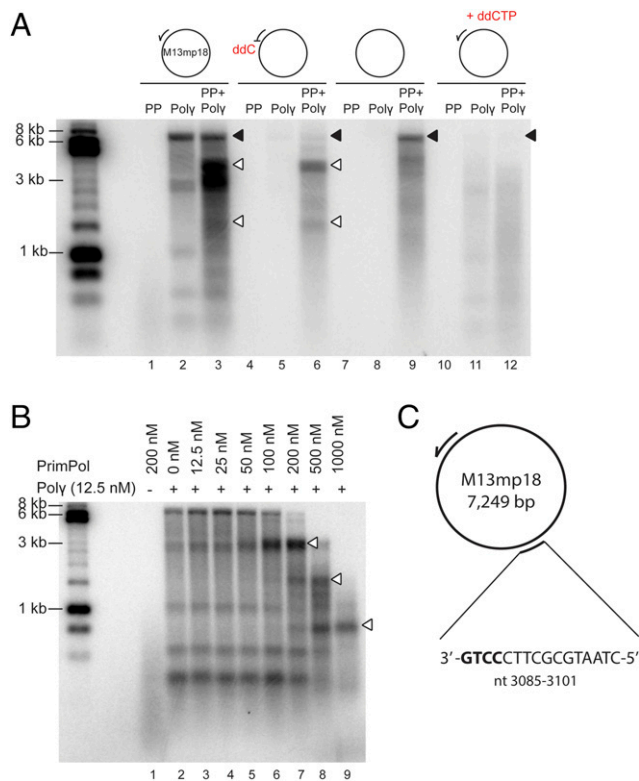
Fig. 2. PrimPol can prime Pol γ-dependent DNA synthesis. (*A*) In vitro DNA synthesis incorporating ^{32}P-radiolabeled dGTP on M13ssDNA. PrimPol alone is unable to synthesize long DNA products in the absence of Mn^{2+}, even when provided with a synthetic annealed DNA primer (lane 1). In contrast, Pol γ (lanes 2 and 3) can effectively utilize the annealed primer and synthesize a full-length product (black arrow). The addition of PrimPol results in additional DNA products (white arrows), assumedly originating from sequence-specific priming downstream of the provided primer. As expected, Pol γ is unable to elongate an annealed primer containing a 3′ ddCMP (lane 5), but can synthesize long DNA products on this template in the presence of PrimPol (lane 6). Similarly, PrimPol can prime DNA synthesis in the absence of additional synthetic annealed primers (lane 9). The addition of ddCTP inhibits Pol γ DNA synthesis, which can partially be rescued by PrimPol (lanes 11 and 12). (*B*) Pol γ extension of 5′ radiolabeled primer in the presence of increasing PrimPol concentrations visualized on a denaturing alkaline gel. Whereas PrimPol can synthesize only products shorter than 500 nt (lane 1), Pol γ can extend the annealed primer to give rise to full-length DNA products (lane 2). Shorter DNA products are also visible as a result of Pol γ stalling at assumed secondary DNA structures. Pol γ-dependent primer extension is unaffected at low PrimPol concentrations (12.5–50 nM; lanes 3–5). At 100–200 nM PrimPol, the synthesis of the 7.3-kb full-length DNA product is reduced, but an abundant DNA product migrating at ~3.7 kb (lanes 6 and 7) is generated. At high PrimPol concentrations (500–1,000 nM), synthesis from the annealed primer decreases and new stalling sites appear (lanes 8 and 9, white arrows). (*C*) Schematic view of the preferred PrimPol priming site on M13ssDNA, positioned relative to the 28-nt primer used in *A*. The preferred PrimPol priming site (3′-GTCC-5′; nucleotides 3,086–3,089) was mapped by using an adapted 5′-RACE method.

pool. To recapitulate the situation in vitro, we next performed replication reactions with a mixed population of free ddCTP and dCTP. Under these conditions, DNA synthesis by Pol γ is weak as a result of the frequent termination of polymerization when ddCTP is incorporated (Fig. 2*A*, lane 11). The addition of PrimPol leads to substantially more DNA synthesis in the presence of ddCTP (Fig. 2*A*, lane 12). Unlike Pol γ, PrimPol's polymerase activity is not significantly inhibited by ddCTP (Fig. S4*A*). More importantly, primer synthesis by PrimPol is only slightly affected by the presence of ddNTPs (Fig. S4*B*). Such a strong discrimination in favor of a 3′-OH group in the incoming nucleotides guarantees that the primers

made by PrimPol do not abort and can be elongated by the Pol γ replicase. The ability of PrimPol to assist Pol γ by reinitiation of efficient DNA polymerization is hampered only when relatively high concentrations of ddCTP are used (Fig. S4*B*). In summary, Pol γ cannot continue DNA synthesis after ddC insertion and requires the priming activity of PrimPol for replication restart (Fig. 2*A*). As long as ddCTP is present, recurrent replication stalling will occur despite the presence of PrimPol, resulting in accumulation of partially replicated molecules in vitro and depletion of mtDNA copy number in vivo (Fig. 1 *D* and *E*).

Sequence-Specific Priming by PrimPol. The concentration of Prim-Pol used (200 nM) equals approximately 40 monomers of the enzyme per M13mp18 template with a potential priming site every 180 nt. As noted earlier, instead of observing a range of elongation products, only a major DNA species was observed, indicating a preferred priming site on the offered template. This priming site became dominant over a ^{32}P-labeled synthetic primer at PrimPol concentrations greater than 50 nM, whereas, at higher concentrations (500–1,000 nM), more and smaller replication products were obtained (Fig. 2*B*, lanes 8 and 9). However, this does not mean that excess of PrimPol is inhibiting DNA synthesis in these reactions. When the same DNA products are separated over a neutral Tris-borate-EDTA (TBE) gel, instead of a denaturing gel, it is possible to visualize all DNA products, regardless of gaps between labeled primer and PrimPol-primed DNA synthesis, demonstrating that high PrimPol levels actually support more DNA synthesis (Fig. S5*A*). The observed main priming site on M13mp18 was highly specific, as the size of the main PrimPol-primed product was dependent on the position of the ddCMP-oligonucleotide primer (Fig. S5*B*). To map this site, we used a modified method for 5′-RACE and discovered that DNA priming started opposite the 3′-GTCC-5′ sequence (Fig. 2*C*), indicating that PrimPol has an identical sequence preference as HSV1 primase (31) and similar to known prokaryotic primases (32).

PrimPol Is Required for UV-Induced mtDNA Replication Initiation. Because UV damage induces similar accumulation of replication intermediates as ddC treatment (21), we sought to identify whether this is also a consequence of PrimPol-dependent replication reinitiation. Whereas UV exposure caused a rapid increase in replication bubbles in normal MEFs, this reaction was completely absent in *Primpol*^{−/−} cells (Fig. 3*C*). Our interpretation is that UV exposure induces a PrimPol-dependent increase of replication initiation in O[H]-containing fragments and must be regulated by some damage-response pathway. In contrast to the ddC treatment, in which replication intermediates were lost (Fig. 1*E*), UV exposure had no effect on the replication intermediates in *Primpol*^{−/−} cells (Fig. 3*C*) or mtDNA copy number (Fig. S6).

PrimPol Overexpression Increases Lagging-Strand Initiation During mtDNA Replication. As PrimPol is able to synthesize primers on ssDNA, which Pol γ can elongate (Fig. 2*A*, lane 9), we decided to next analyze whether PrimPol expression alone could modify ss mtDNA replication intermediates in vivo. We found this plausible, as PrimPol interacts with TWNK and Pol γ in mitochondria (Fig. S7). Furthermore, TWNK is also reported as a PrimPol (as CCDC111) partner in the BioPlex database (33). To get a view of the whole mitochondrial genome, long-range 2D-AGE analysis of full-length mtDNA, cut once downstream or upstream of O[H], was performed (Fig. 4 and Fig. S8). Although PrimPol overexpression notably decreased the levels of the partly single-stranded circles ("ssc" in Fig. 4), it also generally increased all replicative molecules and, more specifically, dsDNA intermediates, such as the double-y forms ("dy" in Fig. 4). These changes in replication intermediates are dependent on mitochondrially localized PrimPol and, more specifically, on its primase activity, as demonstrated by a primase-deficient variant of PrimPol (Fig. S8).
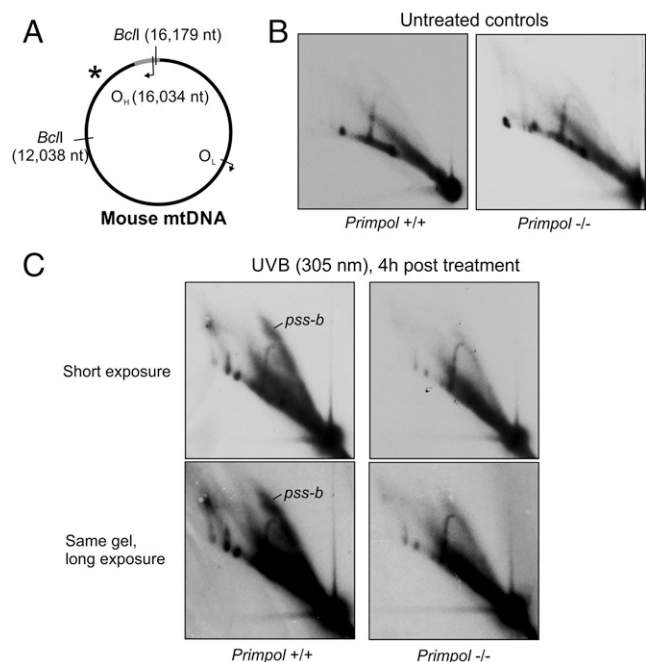
Fig. 3. PrimPol is responsible for increased mtDNA replication initiation after UV exposure. (*A*) Schematic illustration of mouse mtDNA as in Fig. 1. (*B*) Two-dimensional AGE of BclI-digested mtDNA from untreated *Primpol* WT (+/+) and *Primpol*-KO (−/−) MEFs. Comparative exposures of the gels are shown. (*C*) After 4 h recovery from a 30-s exposure to 305 nm UVB, the control WT cells show a significant increase in partially single-stranded replication bubbles (*pps-b*; SI Results and Discussion), whereas *Primpol*-KO MEFs are unaffected. Longer exposure (*Bottom*) was given to illustrate the qualitative and quantitative differences in the replicative forms.

Taken together, PrimPol overexpression increases ds mtDNA forms at the expense of partially single-stranded forms (Fig. S9C), demonstrating that PrimPol can also prime DNA synthesis on a single-stranded template in vivo.

Discussion

Almost all recently discovered mtDNA maintenance proteins are shared with the nucleus (13), making it difficult to dissect their specific importance for the mitochondrial compartment. Furthermore, nuclear DNA damage activates a number of signaling pathways that block cell proliferation or target the cell for apoptosis, making any simultaneous damage in the mitochondrial compartment trivial. The same also applies for the role of PrimPol. Although not essential for life (9), PrimPol is beneficial for nuclear genome maintenance, as its loss influences the mitotic checkpoint responses after damage (34). However, mitochondria also require efficient DNA repair and damage-tolerance mechanisms for long-term survival. Mitochondria have formidable intrinsic sources of DNA damage, most notably reactive oxygen species originating from the electron transport system (35), capable of causing oxidative damage that can block mtDNA replication (20, 21). mtDNA replication can also stall as a result of impaired replisome proteins (17, 18) or sequence-specific replication pause sites (36). Unless the replication is reinitiated, stalling can lead to replication fork collapse and double-strand breaks, resulting in the formation of pathological deletions (16). Our experiments provide direct evidence that PrimPol is required for replication reinitiation in mitochondria, identifying it as a central player in mtDNA replication fork rescue.

PrimPol Is Required for Increased Replication Initiation After DNA Damage.
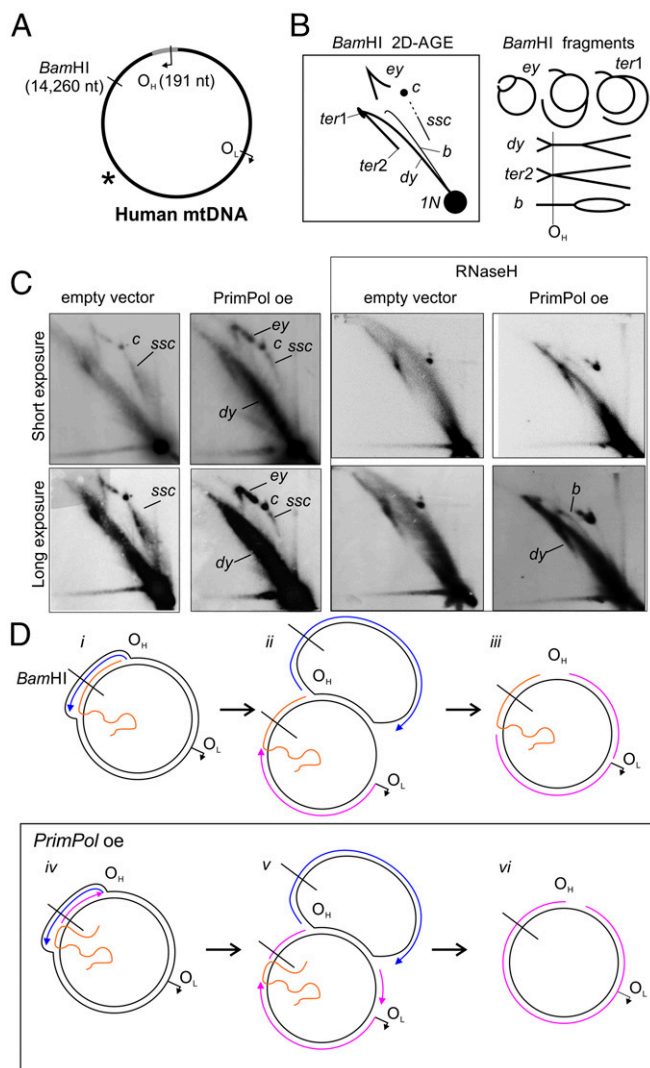The increase in replication intermediates, especially when accompanied by a decrease in mtDNA copy number, has

Fig. 4. PrimPol overexpression in human T-REx 293 cells induces specific changes in the replication intermediates, corresponding to increased lagging-strand priming. (*A*) Schematic illustration of human mtDNA showing the canonical replication origins, BamHI (downstream of O_H) restriction site, and probe location (asterisk). (*B*) Illustration of BamHI-restriction fragments resulting from replicating mtDNA. BamHI cuts directly downstream of O_H, resulting in molecules migrating on the so-called eyebrow arc (*ey*), which represent replication intermediates that are cut only on the leading strand as a result of RNA coverage or single-strandedness of the lagging strand. After the leading-strand replication is completed, the displaced strand template will remain uncut until the lagging strand is entirely replicated (*ter1*). Fully dsDNA intermediates are cut on both strands and result in y forms (*y*) and double-y forms (*dy*), which convert to termination intermediates (*ter2*) after the replication is completed. Note that the distances where the replication bubble is converted to y and then double-y forms are fairly short, resulting in poor separation of the latter. Partially single-stranded circles (*ssc*) streak southeast from the uncut double-stranded circles (*c*, arrows). (*C*) A 48-h overexpression of PrimPol (5 ng/mL Dox) depletes the partially single-stranded circles (*ssc*) while increasing the dsDNA intermediates (*dy*, *y*). Host cell line with an integrated empty expression vector represents the control. (*D*) Under normal conditions (*i–iii*), the majority of mtDNA replication intermediates are partially ssDNA as a result of only a few active lagging-strand origins, such as O_L. When PrimPol is overexpressed (*iv–vi*), lagging-strand synthesis is initiated at multiple loci, resulting in more dsDNA intermediates that can be cut by the restriction enzyme (e.g., BamHI). Note that, despite the general increase in dsDNA, some restriction sites remain uncut.

been treated as a hallmark of replication stalling in a number of studies that used 2D-AGE (17, 18, 37). However, if the stalled replication forks are actively processed or turned over, an increase in replication intermediates can be obtained only through a concomitant increase in replication initiation. In the case of ddC, the accumulation of replication intermediates is caused by recurrent initiation and stalling events (Figs. 1 and 2). In the absence of PrimPol, no reinitiation occurs, resulting in the loss of replication intermediates (Fig. 1C), further demonstrating that replication reinitiation is required for replication maintenance in the presence of ddCTP. As shown here, the primase and polymerase activity of PrimPol are not significantly affected by the presence of ddNTPs, precluding the formation of abortive primers that could compromise fork restart and progression. Despite PrimPol's ability to reprime replication after a CTNA, mtDNA will eventually be lost because of the repeated incorporation of ddCTP by Pol γ (Fig. S10).

Similar to the situation of treatment with ddC, PrimPol is responsible for the increased replication initiation after UV exposure (Fig. 3), although the damage seems to be tolerated in cells lacking PrimPol. It is likely that PrimPol is only one of several players involved in mtDNA damage response and its activities. Despite PrimPol contributing to the defense against a range of genotoxic insults, it is not essential for cell survival after acute damage. This is apparent also from the fact that PrimPol can facilitate recovery after UV damage to nuclear DNA, but does not influence cell survival (34). As stalled replication forks pose a great risk for genome integrity as a result of their tendency to collapse and form double-strand breaks with potentially catastrophic consequences, cells have evolved a number of partially redundant mechanisms to avoid such damage (38).

PrimPol Primes mtDNA Synthesis Independent of the Replication Origin.
Fully dsDNA replication intermediates exist in mitochondria (39, 40) and have been suggested to originate from bidirectional replication that is initiated downstream of O_H (41) (SI Results and Discussion). Under normal conditions, dsDNA replication represents the minority of replicative molecules in mitotic cells, and it remains unsettled to which extent these intermediates represent an independent replication and not just more frequent lagging-strand initiation during strand-asymmetric replication. Interestingly, overexpression of PrimPol results in the generation of fully dsDNA replication intermediates (Fig. 4). As PrimPol can provide primers for Pol γ on single-stranded substrate (Fig. 2A, lanes 7–9), it is also likely to prime the lagging strand during asymmetric replication. Increased lagging-strand priming would be the easiest explanation for the reduction of partially single-stranded circles ("ssc" in Fig. 4C) as well as the increase in fully dsDNA replication intermediates in PrimPol-overexpressing cells (e.g., "dy" in Fig. 4 and Fig. S9).

Although replication stalling is acknowledged as an important culprit behind pathological mtDNA deletions (15, 16), almost nothing is known about the fate of stalled replication intermediates, such as their subsequent processing, and which enzymatic players are involved. Our finding that PrimPol is required for the reinitiation of replication in mitochondria will hopefully pave the way for further understanding of mtDNA replication mechanisms and how the mitochondrial genome is protected against various intrinsic and extrinsic stressors.

Materials and Methods

Immortalization of MEFs and Generation of Flp-In T-REx 293 PrimPol Cell Line.
WT Primpol $(^{+/+})$ and Primpol-KO $(^{-/-})$ cells (Fig. S1A) were generated from primary MEFs (2) that were immortalized by transfection with an SV40T antigen expression vector using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's recommendation. After transfection,

the cells were grown to confluence and passaged onto 10-cm tissue culture dishes, followed by another five 1/10 passages (i.e., a 1/100,000-fold splitting of the original cells), which exposes the cells to a strong negative selection against nontransformed cells. Cells that continue growing after 6–10 passages are considered immortalized. Additionally, an inducible cell line expressing WT PrimPol was established by using Flp-In T-REx 293 cells. The cloning of PrimPol cDNA with a C-terminal flag-tag into the pcDNA5FRTO and the generation of the cell line was performed essentially as in the work of Wanrooij et al. (18). In this system, the transgene is expressed upon addition of doxycycline (Dox) to the growth medium. Dox 5–10 ng/mL was determined to give a stable long-term expression of the transgene (Fig. S7). At these concentrations, Dox is nontoxic for mitochondrial functions (42).

Cell Culture, ddC, and UV Light Treatment.
Flp-In T-REx 293 and MEF cells were cultured in DMEM containing 4.5 g/L glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µg/mL uridine, and 10% FBS at 37 °C in a humidified atmosphere with 8.5% CO_2 atmosphere. No antibiotic agents were added to the growth medium. After the indicated times, cells were pelleted and DNA samples were extracted. To induce mtDNA replication stalling, cells were treated for 48 h with 175 µM of ddC (Sigma-Aldrich) (37). As previously described for UV damage (21), cells were exposed on the tissue culture dish with DMEM to a single 30-s dose of 30 J/m² UVB using a Benchtop 2UV Transilluminator 302 nm instrument (UVP). UV light doses were controlled by using a handheld UV radiometer (UM-25; Minolta).

mtDNA Copy Number Analysis.
Total cellular DNA was isolated by using proteinase K and SDS lysis followed by phenol:chloroform extraction and ethanol precipitation (40). mtDNA levels were analyzed by separating 2 µg HindIII-digested total DNA on a 0.4% agarose gel in 1× TBE 1.2 V/cm for 16 h at room temperature. Southern blotting and DNA hybridization were carried out as previously described (40) by using a Cytb (mouse; nucleotides 14,783–15,333) probe for mtDNA, and an 18S rDNA probe (nucleotides 24–772; National Center for Biotechnology Information accession no. M10098) as loading control. Radioactive signal was captured on Kodak storage phosphor screen SO230, detected by using a Molecular Imager FX (BioRad), and quantified by using the associated QuantityOne software.

mtDNA Isolation and Analysis of mtDNA Replication Intermediates.
mtDNA was isolated by using 1 h 20 µg/mL cytochalasin (Sigma-Aldrich) treatment for MEF cells or 30 min for T-Rex 293 cells before cell breakage, followed by differential centrifugation and sucrose gradient purification (43). The 2D-AGE analysis was performed essentially as in the work of Pohjoismäki et al. (37). Further details are provided in SI Materials and Methods.

In Vitro Replication Assays.
M13mp18 ssDNA (M13ssDNA) was used as a template to assay replication by PrimPol and Pol γ in the presence of dideoxynucleotides. Pol γA, Pol γB (forming the holoenzyme Pol γAB₂), and PrimPol were expressed and purified as described previously (20, 44). Standard reaction mixtures contained 10 mM Bis-Tris propane, 10 mM MgCl₂, 1 mM DDT, 200 µM dNTPs, 1 mM ATP, [α-³²P]dGTP as radioactive tracer, and the indicated amount of ddCTP. When indicated, 12.5 nM Pol γA, 18.75 nM Pol γB (as a dimer), and 200 nM PrimPol was added. The reaction was performed by using 5 nM of singly- or nonprimed M13ssDNA (the 28-mer primer is complementary to nucleotides 6,218–6,245). Reactions were incubated for 60 min at 37 °C, stopped with 0.5% SDS/25 mM EDTA, purified with G-25 columns (GE Healthcare), and loaded on a 1% alkaline (30 mM NaOH, 1 mM EDTA) agarose gel. The gel was run at 20 V for 16 h (4 °C), dried, exposed to a storage phosphor screen, and scanned with a Typhoon 9400 device (Amersham Biosciences).

Mapping of PrimPol Priming Site.
Mapping of PrimPol priming site was performed by an adapted protocol for 5′-RACE (45). Further details are provided in SI Materials and Methods.

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