Redox-sensitive alteration of replisome architecture safeguards genome integrity

Kumar Somyajit, Rajat Gupta, Hana Sedlackova, Kai John Neelsen, Fena Ochs, Maj-Britt Rask, Chunaram Choudhary, Jiri Lukas

DNA replication requires coordination between replication fork progression and deoxynucleotide triphosphate (dNTPs)—generating metabolic pathways. We find that perturbation of ribonucleotide reductase (RNR) in humans elevates reactive oxygen species (ROS) that are detected by peroxiredoxin 2 (PRDX2). In the oligomeric state, PRDX2 forms a replisome-associated ROS sensor, which binds the fork accelerator TIMELESS when exposed to low levels of ROS. Elevated ROS levels generated by RNR attenuation disrupt oligomerized PRDX2 to smaller subunits, whose dissociation from chromatin enforces the displacement of TIMELESS from the replisome. This process instantly slows replication fork progression, which mitigates pathological consequences of replication stress. Thus, redox signaling couples fluctuations of dNTP biogenesis with replisome activity to reduce stress during genome duplication. We propose that cancer cells exploit this pathway to increase their adaptability to adverse metabolic conditions.

Replication dynamics must be tightly aligned with metabolic pathways that generate deoxyribonucleotide triphosphates (dNTPs), the essential building blocks for replicating DNA. In the absence of such regulation, intrinsic oscillations or stochastic fluctuations of metabolic pathways might compromise replication fork integrity and thereby undermine the fidelity of genome duplication (1). We thus hypothesized that a hitherto unidentified pathway might adjust fork speed to the activities of enzymes involved in dNTP synthesis to limit mutagenic nucleotide incorporation or accumulation of unstable replication intermediates that fuel genome instability and cause diseases such as cancer (2, 3).

To test this hypothesis, we combined the iPOND (isolation of protein on nascent DNA) technique with mass spectrometry (MS) to search for replisome components whose gain or loss might illuminate fork speed regulation (Fig. 1, A to C, and fig. S1) (4). In addition to the core replisome subunits, nascent DNA co-purified with TIMELESS, TIPIN, CLASPIN, and AND1 (also known as WDHD1), proteins that are collectively referred to as the replication protection complex (RPC) (5) and whose presence at sites of DNA synthesis was confirmed by immunostaining (fig. S2). Whereas RPC functions have so far been associated predominantly with forks under stress, the yeast RPC orthologs were also implicated in maintaining physiological replisome activity (6). To reconcile these functions, we knocked down RPC components and measured fork speed using the DNA fiber technique (7). With the exception of AND1, depletion of any RPC subunit—CLASPIN, TIMELESS, or TIPIN—led to a robust fork slowdown (fig. S3A). CLASPIN is an established mediator of the S-phase checkpoint; therefore, the reduced fork speed after CLASPIN depletion could be related to a checkpoint defect (8). However, TIMELESS or TIPIN depletion recapitulated the fork slowdown seen after CLASPIN knockdown (Fig. 1D), without detectable impact on checkpoint signaling (fig. S3B) (9). Therefore, we reasoned that depletion of the TIMELESS-TIPIN complex represents a “separation-of-function” condition to study the role of RPC proteins in replisome dynamics. The observed fork slowdown was reproduced by several small interfering RNAs (siRNAs) in a dose-dependent fashion (fig. S4A), and it could be fully rescued by supplementation with siRNA-insensitive TIMELESS (fig. S4B). Fork symmetry was unaffected by TIMELESS or TIPIN depletion (Fig. 1E), indicating that DNA replication continued, albeit with reduced speed. Hence, the mammalian RPC accelerates fork movement in vivo.

To test whether cells exploit the TIMELESS-TIPIN fork accelerator to align replication dynamics with nucleotide synthesis, we set up experimental conditions to mimic a transient metabolic imbalance of ribonucleotide reductase (RNR), whose activity is known to be subject to continuous adjustments during unperturbed cell cycles (10). A short pulse treatment of cells with 50 μM hydroxyurea (HU), an established RNR inhibitor, was sufficient to reduce the pace of DNA replication (Fig. 1, F and G). Although the treatment had little effect on dNTP concentrations (fig. S5A), the slow fork movement was invariably accompanied by a marked dissociation of TIMELESS from the replisome, as observed by iPOND (Fig. 1H) and confirmed by quantitative image–based cytometry (QIBC) (fig. S5B). Dissociation of TIMELESS was already very pronounced upon fork slowing (50 μM HU), and the residual TIMELESS was displaced after fork stalling (1 mM HU), which, unlike fork slowdown, was accompanied by a pronounced dNTP depletion (Fig. 1H and fig. S5A). The latter finding is consistent with a previous report showing that massive fork stalling caused TIMELESS dissociation, albeit with delayed kinetics compared with that for slow forks in our settings (11). This kinetic distinction suggests that replisome activity might be differentially regulated after complete fork stalling (associated with stress signaling) and in response to near-physiological fluctuations of dNTP-generating metabolic pathways, respectively. In support of this reasoning, fork slowdown caused by TIMELESS depletion was not further exacerbated by low HU concentrations (Fig. 1I), indicating that mild perturbation of RNR activity triggers a quantitative biochemical depletion of this fork accelerator from the replisome. Such level of regulation operates without detectable DNA damage signaling measured by H2AX, CHK1, and RPA phosphorylation (Fig. 1F and fig. S5, C and D) or increased fork asymmetry (fig. S6A). Furthermore, treatment of cells with aphidicolin, which blocks DNA polymerases without metabolic interference, did not lead to TIMELESS displacement (fig. S6B), reinforcing the notion that TIMELESS retention at replisomes is coupled specifically to metabolic imbalances.

We first considered limited dNTP supply as the trigger for the TIMELESS dissociation. However, whereas front-loading of cells with dNTP precursors improved DNA synthesis after dNTP depletion and fork stalling induced by high concentrations of HU, it had a negligible effect on slow forks in cells treated with low HU levels (fig. S7A). Together with the minimal fluctuation of dNTP levels in the latter condition (fig. S5A), these data indicated that the dNTP supply per se does not explain the precipitous fork slowdown after mild RNR perturbation. We thus focused on the biochemical mode of action of RNR, which converts ribonucleoside diphosphates (NDPs) to dNTPs through a proton-coupled electron transfer between its R1 and R2 subunits (12). Inhibition of RNR by HU disrupted this process (13), and we reasoned that the resulting redox imbalance could generate a surplus of reactive oxygen species (ROS). Indeed, treatment with 50 μM HU triggered a rapid accumulation of superoxide and hydroxyl radicals to levels comparable to those induced by low concentrations of hydrogen peroxide (H2O2), and this was inhibited by the ROS quencher N-acetyl cysteine (NAC).

RESEARCH

DNA REPLICATION


1 of 6

Downloaded from http://science.sciencemag.org/ on July 18, 2021
Fig. 1. TIMELESS dissociation from chromatin mounts a slow-fork response after mild perturbation of RNR activity. (A) iPOND workflow. (B) Proteins identified on nascent [5-ethyl-2′-deoxyuridine (EdU), green] and mature (thymidine chase, black) chromatin fractions obtained by iPOND and analyzed by MS (n = 3 biological replicates). The data show logarithmized ratios of average protein intensities in the indicated conditions. RPC proteins are highlighted in red. (C) Selected replisome components identified by iPOND and analyzed by Western blotting (WB). (D) (Left) siRNA-mediated depletion of TIMELESS (TIM; “A” and “B” indicate two independent siRNAs) and TIPIN. (Middle) DNA fiber labeling protocol. CldU, 5-chloro-2′-deoxyuridine; IdU, 5-iodo-2′-deoxyuridine. (Right) Replication fork speed in cells treated with the indicated siRNAs (n = 200 fibers for each condition). (E) Fork ratio derived from the data in (D) by dividing the length of DNA tracts labeled by IdU and CldU. (F) QIBC of cells exposed to the indicated doses of HU for 30 min, labeled with EdU and immunostained for γ-H2AX. Nuclear DNA was counterstained by 4′,6-diamidino-2-phenylindole (DAPI) (n = 2500 cells for each condition). The color gradient indicates the mean nuclear γ-H2AX intensity. A.U., arbitrary units. (G) (Left) DNA fiber labeling protocol. (Right) Replication speed in cells treated with the indicated HU concentrations. (H) iPOND-MS analysis of the indicated replisome components after low (50 μM) and high (1000 μM) concentrations of HU compared with mock-treated cells (n = 2 biological replicates). The data show logarithmized ratios of average protein intensities under the indicated conditions. (I) (Left) DNA fiber labeling protocol. (Right) Replication speed of untreated (CldU, red) and HU-treated (IdU, green) forks after indicated knockdowns (n = 200 fibers for each condition).
Fig. 2. TIMELESS and oligomeric PRDX2 form a replisome-based redox sensor. (A) Analysis of intracellular ROS (superoxide plus hydroxyl radicals) by flow cytometry after the indicated treatments, and with or without pretreatment with NAC. Aph, aphidicolin. (B) (Left) DNA fiber labeling protocol. (Right) Replication speed in cells exposed to the indicated treatments with or without NAC ($n = 200$ fibers for each condition). (C) Reciprocal FLAG coimmunoprecipitation (FLAG-IP) followed by Western blotting of extracts from U2OS cells or its derivatives stably expressing FLAG-tagged TIMELESS or PRDX2 as indicated. (D) (Left) Representative images. (Right) Quantification of the sum of PLA focus intensity per cell nucleus obtained with the indicated antibodies. Scale bars, 10 μm. (E) Western blots of purified cellular fractions under reducing (left) and nonreducing (right) conditions (Cyt, cytoplasm; Nuc, nucleus; Chr, chromatin; $n$, number of PRDX2 monomers in a given higher-order assembly). (F) (Top) Western blot of purified cellular fractions under nonreducing conditions from cells exposed to the indicated treatments. (Bottom) Bar graph representing the degree of chromatin-bound higher oligomeric PRDX2 ($n_4$) in the indicated conditions [mean ± SEM (error bars); $n = 4$ technical replicates]. (G) Western blots of coimmunoprecipitated FLAG-TIMELESS under nondenaturing conditions from whole-cell lysates from cells exposed to the indicated treatments.
Fig. 3. Preventing TIMELESS dissociation suppresses the slow fork response and leads to genomic instability. (A) Western blots of iPOND samples prepared as in Fig. 1A from cells treated with siRNAs and HU as indicated. (B) QIBC of TIMELESS chromatin loading in cells treated with siRNAs and HU as indicated. (C) Top) DNA fiber labeling protocol. (Bottom) Replication speed in cells treated as indicated (n = 200 fibers for each condition). (D) Quantification of γ-H2AX in PCNA-positive cells. Data were derived from QIBC analysis of cells treated with siRNAs as indicated; exposed to HU for 30 min; and stained for DAPI, γ-H2AX, and PCNA. (E) (Top) Examples of ultrafine anaphase bridges (UFBs) marked by Bloom’s syndrome helicase (BLM) (red) between anaphase chromosomes counterstained by DAPI (blue). (Bottom) Quantification of UFBs in cells treated with the indicated siRNAs (mean ± range; n = 2 technical replicates). (F) (Left) QIBC of 53BP1 nuclear bodies (NBs) in cells treated with the indicated siRNA and counterstained for cyclin A and DAPI to stratify cell cycle progression (n = 10,000 cells for each condition; colors indicate the number of 53BP1 nuclear bodies per nucleus). (Right) Quantification of 53BP1 nuclear bodies in cells treated as indicated; inset shows examples of G1 cells (cyclin A negative) with 53BP1 nuclear bodies.
H₂O₂ caused an instant fork slowdown and TIMELESS dissociation, similar to the effect of low HU levels; both cases could be rescued by NAC. The DNA fiber data were validated by chromatin immunoprecipitation–quantitative polymerase chain reaction, in which low levels of HU or H₂O₂ displaced TIMELESS from two independent loci. RNR-independent fork slowing by aphidicolin did not elevate ROS levels and could not be rescued by NAC, reinforcing the idea that fork velocity and ROS signaling are coupled. Although prolonged ROS exposure can alter fork progression by oxidative damage (14), this does not seem to explain the instant fork slowdown triggered by natural metabolic fluctuations. A strong support for this assertion is that the slow fork movement induced by mildly elevated levels of ROS remained constant and symmetrical (fig. S6A), indicating direct signaling by ROS rather than indirect oxidative damage of DNA or fork pausing.

Low levels of ROS are beneficial for cell proliferation and survival, in part because of their role as second messenger in various metabolic pathways (15). On the basis of our findings, we hypothesized that ROS may also drive second-messenger signaling at the fork and that a hitherto unknown ROS sensor may be present at active replisomes. To test this hypothesis, we analyzed the TIMELESS interactome, which together with iPOND analysis consistently featured enrichment of peroxiredoxin 2 (PRDX2),...
a member of the evolutionarily conserved peroxiredoxin family that regulates ROS metabolism in diverse physiological settings (fig. S9, A and B) (16, 17). Due to their ability to detect ROS, peroxiredoxins are ideally suited to sense even subtle redox fluctuations at or near replication forks. We validated the MS data by reciprocal coimmunoprecipitation experiments in which TIMELESS interacted with PRDX2 but not with mitochondria-specific PRDX5 (Fig. 2C). As a result of our immunofluorescence studies (fig. S9C) and proximity ligation assay (PLA) (Fig. 2D), we identified a nuclear fraction of PRDX2 that localized to the proximity of proliferating cell nuclear antigen (PCNA), consistent with a role of PRDX2 at active forks.

In line with the cell-based assays, biochemical fractionations resolved on nonreducing gels revealed an exclusive accumulation of oligomeric forms of PRDX2 on chromatin, compared with cytosolic and soluble nuclear fractions (Fig. 2E). The formation of PRDX2 oligomers was thiol-dependent (18) as it was disrupted by mutation of conserved peroxidatic cysteines (fig. S9D). Quantification of these experiments showed that whereas the oligomerized forms of PRDX2 were chromatin-bound under control conditions (i.e., normal forks), low concentrations of HU or H$_2$O$_2$ (i.e., slow forks) triggered disruption of PRDX2 oligomers; their dissociation from chromatin; and accumulation of soluble, faster-migrating forms (Fig. 2F). Both the highly oligomeric as well as the small forms of PRDX2 interacted with TIMELESS (Fig. 2G), indicating that the dissociation of oligomeric PRDX2 from chromatin and the displacement of TIMELESS from replication forks under elevated ROS levels might be mechanistically coupled. The key support for this conclusion was provided by iPOND (Fig. 3A and fig. S10A) and QIBC (Fig. 3B and fig. S10B) studies, which independently confirmed that the HU-induced TIMELESS dissociation from replicating DNA was abolished by PRDX2 depletion. Consistently, the fork slowdown induced by HU or H$_2$O$_2$ was completely suppressed by PRDX2 knocking down (Fig. 3C) or inhibition (fig. S10C). These results confirm that the dNTP pool was not limiting for fork progression under mild RNR fluctuation and are in line with the notion that the fork slowdown reflected dissociation of a replisome accelerator such as TIMELESS. In further support of this conclusion, simultaneous depletion of TIMELESS prevented fork acceleration in PRDX2-depleted cells (Fig. 3C). Mutation of peroxidatic cysteines rendered PRDX2 unable to mediate fork slowdown after low HU levels (fig. S10E), providing an important support to the notion that active PRDX2 oligomers couple cellular redox imbalance to fork speed.

To investigate the effect of mild metabolic stress during dNTP synthesis on genome stability, we treated U2OS cells with low levels of HU. Such treatment alone did not increase replication stress, but the simultaneous depletion of PRDX2 triggered replication-associated DNA damage (Fig. 3D and fig. S11A). Consistently, even in unperturbed cells, where the dNTP metabolic pathways oscillate naturally, depletion of PRDX2 increased the incidence of ultrafine DNA bridges during anaphase (Fig. 3E) and 53BP1 nuclear bodies in G$_1$ cells (Fig. 3F), both established hallmarks of replication-born DNA damage (19, 20). When comparing a panel of normal and cancer-derived cells and cell lines, we noticed that fork velocity tightly correlated with the level of endogenous ROS. Specifically, normal cells showed low levels of ROS and slow forks, whereas cancer cells proliferated with elevated ROS levels and slow forks (fig. 4, A and B). After treatment with low levels of HU, all cell types displayed TIMELESS dissociation from nascent chromatin (fig. S11, B and C) and PRDX2-dependent fork slowdown (Fig. 4C), indicating that the PRDX2-and TIMELESS-executed fork speed adjustment to metabolic perturbations is a fundamental level of replication surveillance. However, we found that PRDX2 depletion specifically sensitized cancer cells to mild replication stress (Fig. 4D), which confirmed that the HU-induced TIMELESS dissociation and fig. S10B) studies, which independently confirmed that the ROS-mediated fork slowdown is key to mitigating replication stress associated with oncogenic transformation.

Collectively, the pathway identified here acts as the first-line surveillance of metabolic fluctuations that, if unaccounted for, could harm replicating genomes (fig. S12A). Under low levels of ROS, oligomeric PRDX2 interacts with TIMELESS at replication forks to mediate fork slowdown (Fig. 4D), which indicates that the ROS-mediated fork slowdown is key to mitigating replication stress associated with oncogenic transformation.

REFERENCES AND NOTES

ACKNOWLEDGMENTS
Research funding was provided by the Novo Nordisk Foundation (grant NNF15CC001189 to J.L. and grant NNF16CC002906 to H.S.). K.S. was supported by the Danish Council for Independent Research (grant 62262). R.G. was supported by a European Molecular Biology Organization long-term postdoctoral fellowship (ALTF271-2014). D.C. was supported by the Halsos Møller Investigator Fellowship from the Novo Nordisk Foundation (NNF14OC0008541) and by the European Union’s Horizon 2020 research and innovation program (grant 648039). We thank J. Bukkeser and G. Karemore from the Protein Imaging Platform for their assistance with microscopy and image analysis, B. Lopez Mendez from the Protein Production and Characterization Platform for help with dNTP quantification, R. Straus for sharing cell strains, and J. Spies and C. Lukas for useful discussions.

SUPPLEMENTARY MATERIALS
www.sciencemag.org/content/358/6364/797/suppl/DC1
Materials and Methods
Figs. S1 to S12
References (23–29)
7 July 2017; accepted 26 September 2017
10.1126/science.aee3172
Redox-sensitive alteration of replisome architecture safeguards genome integrity

Kumar Somyajit, Rajat Gupta, Hana Sediackova, Kai John Neelsen, Fena Ochs, Maj-Britt Rask, Chunaram Choudhary and Jiri Lukas

Science 358 (6364), 797-802.
DOI: 10.1126/science.aao3172

Metabolic regulation of genome stability
Cells respond to metabolic fluctuations by adjusting the speed of DNA replication as a safeguard for genome stability. Somyajit et al. elucidate the cellular mechanisms that align replication fork dynamics with metabolic pathways (see the Perspective by Gómez-González and Aguilera). The elevation of reactive oxygen species (ROS) levels under metabolic stress dissociates a replication accelerator from the replisome and leads to replication slowdown, thus preventing replication stress. Studying this genome surveillance mechanism in cancer cells with elevated ROS levels and increased replication adaptability may provide opportunities to specifically target tumors.

Science, this issue p. 797; see also p. 722