Cdc45 is limiting for replication initiation in humans

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ABSTRACT

Cdc45 is an essential protein that together with Mcm2-7 and GINS forms the eukaryotic replicative helicase CMG. Cdc45 seems to be rate limiting for the initial unwinding or firing of replication origins. In line with this view, Cdc45-overexpressing cells fired at least twice as many origins as control cells. However, these cells displayed an about 2-fold diminished fork elongation rate, a pronounced asymmetry of replication fork extension, and an early S phase arrest. This was accompanied by H2AX-phosphorylation and subsequent apoptosis. Unexpectedly, we did not observe increased ATR/Chk1 signaling but rather a mild ATM/Chk2 response. In addition, we detected accumulation of long stretches of single-stranded DNA, a hallmark of replication catastrophe. We conclude that increased origin firing by upregulated Cdc45 caused exhaustion of the single-strand binding protein RPA, which in consequence diminished the ATR/Chk1 response; the subsequently occurring fork breaks led to an ATM/Chk2 mediated phosphorylation of H2AX and eventually to apoptosis.

Introduction

In higher eukaryotes, initiation of DNA replication is achieved by the ordered assembly of a series of proteins at functionally defined origins of replication. In budding and fission yeasts, the cell division cycle protein 45, Cdc45, increases initial DNA unwinding at replication origins, i.e. origin firing efficiency. In human cells, Cdc45 acts as proliferation-associated antigen. Moreover, recruitment of Cdc45 at origins reflects the temporal replication program, i.e., Cdc45 is loaded at early-firing origins first, whereas it is assembled at late-firing ones late in S phase.

The CDC45 gene product is essential and the protein is part of the replicative helicase, the so-called CMG complex that consists of Cdc45, Mcm2-7, and GINS. The DNA unwinding activity of the undecameric holoenzyme appears to be several hundred-fold stronger than that of the heterohexameric AAA + ATPase Mcm2-7, providing strong evidence that CMG is the functional form of the eukaryotic replicative DNA helicase, whereas Mcm2-7 seems to reflect a dormant form. Activated CMG is required for loading of the single-strand DNA binding protein RPA and DNA polymerase α onto the freshly exposed lagging strand, as well as DNA polymerase ε onto the leading strand, where Dpb2, i.e. the second largest subunit of polymerase ε, forms a complex with the Psf1 subunit of GINS. Then, DNA polymerase ε travels along with the activated CMG helicase on the leading strand.

The number of preformed replication origins per human cell outnumbers the available Cdc45 molecules by far. Therefore, Cdc45 may represent a limiting factor for the establishment and activation of the CMG helicase. In agreement with this view, Cdc45 has been suggested to activate nearby dormant origins of replication after an ongoing replication fork comes to an unscheduled halt. On the other hand, Cdc45 is overexpressed in several tumor cells, where it helps to sustain rapid rounds of cell division. Moreover, Myc-induced loading of Cdc45 into origins puts Cdc45 into a regulatory network of oncogene expression and carcinogenesis. Indeed, there exist several studies that describe overexpressed Cdc45 in human cancer cells. To get a deeper insight into the regulatory functions of Cdc45 for the initiation of DNA replication and for the maintenance of human tumors, we ectopically expressed this putative limiting factor and expected to see a faster S phase. Indeed, we observed increased firing of replication origins, but this caused severe replication stress, an early S phase arrest, replication fork stalling, and eventually cell death by apoptosis.
Results

*Increased levels of Cdc45 induced an early S phase arrest and apoptosis*

Ectopic expression of Cdc45 was achieved in HeLa cells by creating a plasmid vector that encoded N-terminal human Cdc45 fused to a C-terminal enhanced green fluorescent protein (GFP). Cdc45 exclusively localized to the nucleus in essentially all GFP-positive cells inspected (Fig. 1A), whereas transient transfection with a GFP-encoding control plasmid revealed an equal distribution of the control through the nucleus and cytoplasm.

Cdc45-GFP expressing HeLa cells were analyzed by flow cytometry. Since one complete cell division cycle is needed for plasmid accumulation in the nucleus, we analyzed GFP-fluorescence starting at 36 h after transfection. GFP-fluorescent control cells showed a typical cell cycle distribution with only few cells with sub-G1 DNA content (Fig. 1B, upper panel). In the Cdc45-GFP expressing cells, however, we observed an accumulation of cell with a G1 or early S-phase DNA content and a prominent sub-G1 peak indicating DNA fragmentation, a sign of apoptosis that became even more prominent 48 h after transfection (Fig. 1B, lower panel). Flow cytometric detection of cleaved caspase confirmed that apoptosis had been induced by Cdc45 overexpression. Concordant with an early S-phase arrest, cells positive for a cleaved caspase 3-response displayed predominantly a G1 and early S-phase DNA content (Fig. 1C, right panel).

To characterize the events leading to cell death, we inspected DNA replication more directly. Cells were therefore pulse-labeled with the thymidine analog ethynyl deoxyuridine (EdU). Visualization of nascent DNA by fluorescent click conjugation of incorporated EdU revealed that GFP-transfected HeLa cells were normally passaged through S phase (Fig. 2A-C). In contrast, a considerable proportion of cells with S phase DNA content incorporated nucleotides more slowly and apparently had difficulties to progress through S phase (Fig. 2D-F).

**Accumulation of γH2AX after Cdc45 transfection**

To further explore the problems with S phase progression, we probed Cdc45-GFP transfected cells for the presence of the DNA damage marker γH2AX. GFP-Cdc45 transfected cells displayed a prominent, granular nuclear γH2AX signal (Fig. 3A, upper panels), whereas cells transfected with GFP alone (middle) or untransfected controls (lower panels) showed only residual DNA damage. Abundant Cdc45 thus clearly provoked DNA damage. Since background correction of the immunofluorescence images may exaggerate the γH2AX levels in Cdc45-transfected cells a more quantitative treatment by FACS was utilized. This revealed a twofold induction of the average γH2AX levels by transfection with Cdc45-GFP over transfection with GFP (Fig. 3B). γH2AX induction was also confirmed in green monkey CV-1 cells expressing HA-tagged Cdc45 under a tetracyclin-inducible promoter (Supplemental Fig. 1).

In an attempt to timely allocate this type of phosphorylation within the cell cycle, we transfected cells with either GFP or GFP-Cdc45 and analyzed the levels of γH2AX by flow cytometry (Fig. 4). 36 hours after transfection, in most GFP-only transfected cells no damage signal was visible over the entire cell cycle, as the γH2AX signal of GFP-positive cells (green dots) overlaid perfectly with that of mock-transfected cells (Fig. 4A). The Cdc45-GFP expressing cells, however, accumulated γH2AX signals just after entry into

![Figure 1. Ectopic expression of Cdc45 caused apoptosis. (A) Transfection of HeLa cells with a vector containing Cdc45-GFP revealed an exclusive nuclear localization of the protein in transfected cells, whereas transfection with a vector carrying GFP alone led to staining of the entire cell. (B) The fraction of cells with a subgenomic DNA content increased dramatically between 36 and 48 h after transfection with Cdc45-GFP (lower panel), but not with GFP alone (upper panel), indicative for apoptotic DNA condensation and fragmentation. Both attached and detached cells were collected and stained with propidium iodide. The histograms represent the complete cell population from the FSC-SSC dot plot. (C) Accumulation of cleaved caspase 3 in Cdc45-GFP expressing cells 2 d after transfection. The dot plots represent only the GFP-positive (i.e., transfected) subpopulations.](https://example.com/figure1.png)
S-phase (green dots in Fig. 4D, the blue dots represent GFP-negative cells from the same culture). This is consistent with the apparent difficulties of cells with elevated Cdc45 levels to traverse S phase to reach G2. In some contrast to this, addition of the ribonucleotide reductase inhibitor hydroxyurea led to an increase of γH2AX foci over the entire S-phase and a particular accumulation of foci in early/mid-S (Fig. 4B). From these experiments we conclude that Cdc45 exhibits its deleterious effects mainly at the G1-S transition, maybe by affecting firing of replication origins.

**Cdc45 induced disproportionate firing of replication origins**

To determine whether Cdc45-GFP had any influence on the number of fired origins, DNA fiber assays were employed. Asynchronous cells were consecutively pulse-labeled with the thymidine analogs CldU and IdU for various times, mostly 45 min each. After stretching of the fibers and immunological detection of the halogenated analogs, we identified the fraction of forks initiated during the CldU pulse and determined the
distance between neighboring origins (Fig. 5A). There appeared to be an increase in initiation events in Cdc45-GFP transfected cells (Fig. 5). 22,23 In control HeLa cells and in cells overexpressing GFP, we measured comparable inter-origin distances of 115 kb and 129 kb, respectively, which is in accordance with estimates from other groups. 21,24,25 In cells transfected with Cdc45-GFP this distance shrank to 75 kb (Fig. 5), indicating an increased firing of neighboring origins when Cdc45 was increased.

Cdc45-mediated over-firing decreased the elongation rate of replication and induced fork asymmetries

Unexpectedly, the lengths determination of IdU tracks 21 unveiled an about twofold decline of the progression rate of elongating forks in Cdc45-GFP transfected cells (Fig. 5). 22,23 In control HeLa cells and in cells overexpressing GFP, we measured comparable inter-origin distances of 115 kb and 129 kb, respectively, which is in accordance with estimates from other groups. 21,24,25 In cells transfected with Cdc45-GFP this distance shrank to 75 kb (Fig. 5), indicating an increased firing of neighboring origins when Cdc45 was increased.

Signaling in the course of Cdc45-induced S phase arrest

Since S phase replication control and checkpoint response is ascribed to the ATR/Chk1 protein kinase pathway, we firstly inspected this signaling cascade. While 5 h treatment with HU or irradiation with 2 Gy provoked phosphorylation of serine 345 (S345) of Chk1, no such modification was observed after Cdc45-GFP expression (Fig. 7A). We rather observed some increase in Chk1 phosphorylation in all 3 samples 2 d after transfection, which probably reflected some replication stress. Noteworthy, this was not stronger in samples transfected with Cdc45-GFP compared to vector- or GFP-transfected cells (Fig. 7A). Also immunofluorescence microscopy confirmed that Cdc45-GFP-transfected cells had Chk1 levels equal or lower than GFP- or mock-transfected cells (Supplemental Fig. 2A). Surprisingly, treatment of Cdc45-GFP-transfected cells with HU for one hour provided a normal Chk1 response, indicating that Cdc45-GFP does not compromise the response to nucleotide depletion (Supplemental Fig. 2B). There were also no apparent differences of phosphorylation of S428 of the upstream kinase ATR among the 3 different vectors (Fig. 7B). However, we observed a slight but robust ATM response manifested by the phosphorylation of S1981 of ATM and T68 of Chk2 with Cdc45-GFP, but not with the controls (Fig. 7B-D). Considering that only about one third of the cells were transfected to overexpress GFP or Cdc45-GFP, respectively, the
magnitude of response observed in Cdc45-GFP likely represents an underestimate.

**Accumulation of single-stranded DNA in Cdc45-GFP expressing cells**

Considering the phenotype of our Cdc45-overexpression experiments we wondered why the observed difficulties in traversing S-phase, the γH2AX response and subsequent apoptosis apparently went along with ATM/Chk2 signaling but not with the expected ATR/Chk1 cascade. In a recent publication, the Lukas group demonstrated that depletion of the pool of the ssDNA binding protein RPA led to a “replication catastrophe,” which phenotypically was very similar to the effects observed here. Since RPA is an essential component of ATR signaling, a relative exhaustion of this factor by too many ongoing replication forks or by possible sequestration by soluble Cdc45 might have been responsible for the observed effects. We therefore asked whether Cdc45-GFP transfected cells displayed more ssDNA compared to mock or GFP transfected cells. To this end, cells were grown for 30 h in the presence of BrdU. Prior to transfection, BrdU was removed. To detect cells with ongoing DNA synthesis, EdU was added shortly before fixation. Exposure of ssDNA regions was visualized by anti-BrdU immunofluorescence without the canonically used DNA denaturing step. Cells that were transfected with GFP-Cdc45 displayed significantly more BrdU signal (Fig. 8), whereas transfection with GFP alone or mock transfected controls showed only residual amounts of ssDNA. Most important, most GFP-Cdc45 expressing cells with EdU-incorporation exhibited more ssDNA than the corresponding control (Fig. 8A & B). Moreover, there was generally a strong negative correlation between EdU incorporation and ssBrdU exposure: The more cells actively synthesized DNA, the less ssDNA was present (Fig. 8B). The quantitative evaluation of individual mock transfected or GFP-transfected controls revealed a largely undisturbed EdU-incorporation and only sporadic ssDNA exposure. In striking contrast, there was a pronounced ssDNA accumulation in Cdc45-GFP transfected cells (Fig. 8C & D). This was particularly reflected by the average BrdU fluorescence intensity, which was increased more than 2-fold in Cdc45-GFP transfected cells, compared to GFP alone (Fig. 8C), where about 15% of the Cdc45 transfected, but less than 2% of the GFP control cells were ssDNA-positive (Fig. 8).

**Discussion**

Human Cdc45 is a helicase co-factor within the CMG complex, but its comprehensive functions during eukaryotic DNA replication are still not well understood. Upon initiation of replication, Cdc45 becomes loaded into origins, where it helps establishing the Mcm2-7 helicase function. Apparently, the intracellular amount of Cdc45 is carefully controlled, since there are only 30,000 to 40,000 molecules per (growing) cell, which is not enough to start all 250,000 preformed replication origins. On the other hand, many tumor cells display a higher expression of Cdc45, which may contribute a growth advantage due to a higher propensity of origin firing. Therefore, one important issue of this study was to analyze the effects of an increased number of intracellular Cdc45 molecules on cell growth.

Cdc45-GFP-transfected HeLa cells displayed a strict intranuclear localization. After transfection, cells entered the next S phase but had difficulties in reaching the following G2 state. Cells largely remained in S phase for up to 96 h. But already 24 to 36 h after transfection many cells underwent apoptosis, which became visible as a largely broadened sub-G1 peak during FACS analyses, and a dramatically increase of cleaved caspase 3 (Fig. 1). This was preceded by a significant increase in γH2AX formation indicative for double-strand breaks and/or disrupted replication forks. Obviously, the firing of too many origins caused difficulties to initiate DNA synthesis of both strands (Fig. 6). But surprisingly, this did not provoke an ATR/Chk1 response, which is in contrast to replication stalling by e.g. HU-induced nucleotide depletion (Fig. 7A) and ref.35. Commonly, ATR signaling inhibits both the initiation and elongation step of DNA replication, thereby limiting the adverse effects of fork stalling. On the other hand, we did observe a significant increase in ATM/Chk2 signaling as well as accumulation of γH2AX, indicative for double-strand breaks. Thus, firing of too many origins at a time provoked difficulties to complete DNA synthesis of both strands leading to subsequent fork disruption and breakage.

This scenario was directly supported by our observations of an accumulation of uncovered ssDNA in about 15% of all cells transfected with Cdc45-GFP, whereas only 1–2% of the controls displayed this type of DNA. Furthermore, the origin-to-origin distances (Fig. 5A) as well as the average origin-to-origin distances as well as the average origin-to-origin distances (Fig. 5B) were considerably increased in Cdc45-GFP transfected cells (Fig. 7).

Figure 5. Cdc45 overexpression increased origin usage. (A) Representative DNA fiber spreads. Fiber assays were performed after pseudotransfection (mock) or transfection with a GFP- (GFP), or with a Cdc45-GFP-encoding vector (Cdc45), respectively. (B) The distribution of origin-to-origin distances as well as the average origin-to-origin distances (inlet) were evaluated from about 200 representative tracks by using a stretching factor of 2.59 kb/μm.
origin distance shrank from 120 kb to about 75 kb indicating that about twice as many origins have been fired after ectopic expression of Cdc45. Comparably, a threefold increase in the number of replication foci has been observed after microinjection of Cdc45 into Chinese hamster ovary cells. Both results confirm earlier data from Xenopus extracts and fission yeast, where Cdc45 was rate limiting for the initiation of DNA replication. Intriguingly, after Cdc45 transfection the tracks indicating fork elongation were about 50% shorter than those in mock- or GFP-transfected control cells, suggesting that a twofold increase of origin usage resulted in an about twofold diminished elongation rate of individual forks.

This might have been due to an exhaustion of components of the DNA replication machinery or precursors for DNA replication, such as the deoxyribonucleotides (dNTPs). Accordingly, cell treatment with the ribonucleotide reductase inhibitor HU is known to slow polymerase activity and cause γH2X formation but, different to the data reported here, activates the ATR-induced intra-S-phase checkpoint. Moreover, dNTP depletion seems to be unlikely because the

Figure 6. Diminished elongation rates and increased fork stalling in Cdc45-GFP expressing cells. (A) Depiction of a representative track displaying the method. (B) Summary of the measured elongation rates after mock transfection and transfection with a GFP- or a Cdc45-GFP-encoding vector. The elongation rates of mock-, GFP-, and Cdc45-GFP transfected cells were evaluated from about 600 representative tracks for each condition as the lengths of IdU tracks divided by the length of the IdU pulse time. (C) Depiction of representative tracks displaying the method. The shortened track in the Cdc45-GFP sample revealing fork stalling is indicated by an arrow. (D) Mock- and GFP-transfected cells displayed track-length differences of about 1.25 kb/min. After transfection with Cdc45-GFP the mean track-length difference increased to 2.1. Analysis was performed from the same set of images as used in Fig. 5.
ribonucleotide reductase is a self-regulatory enzyme that counteracts this situation.\textsuperscript{43}

If not dNTP depletion, another factor may have become limiting. Because of the shortage of this component, the replication forks halt and this in turn provoked fork regression and fork collapse. Both events, however, should have been accompanied by severe ATR activation, as seen e.g., by DNA polymerase inhibition with aphidicolin (reviewed by ref. \textsuperscript{44}). A notable exception to this scenario is the relative depletion of RPA. RPA is required for stabilizing DNA after strand opening catalyzed by the CMG complex, and at the same time is implicated as a central component of the ATR activation cascade.\textsuperscript{45} When too many origins are opened at a time, RPA may become short to cover all newly formed single-strands. This effect could be augmented if the surplus Cdc45 sequesters RPA independent of DNA (Szambowska et al. manuscript in preparation). Even though we cannot exclude that the excess ssDNA detected by native anti-BrdU staining may be in part covered by RPA, the results are consistent with a RPA exhaustion. Also the observed partial co-localization of ssDNA with sites of EdU incorporation (Fig. 8), which seems paradoxical, makes sense in the light of a RPA limitation. Mainly the freshly displaced lagging strands would suffer from insufficient amounts of RPA\textsuperscript{45}. This in turn diminished both the loading,\textsuperscript{46} and the catalytic rate of the DNA polymerase $\alpha$-primase complex,\textsuperscript{47,48} which would explain the deceleration of lagging strand DNA synthesis and the subsequent accumulation of ssDNA. At the same time, leading strand DNA synthesis should not be directly affected. RPA depletion would also explain the failure of ATR signaling. This, together with an increased amount of Cdc45 would allow the here observed initiation of novel origins elsewhere. In consequence, more and more uncovered single-strands were produced (as observed) that were prone for attacks by nucleases and hydroxyl radicals.

What then is the phenotype of RPA depletion without Cdc45 overexpression? In two studies RPA depleted cells passed rather slowly through S- and accumulated in G2-phase, where they halted and induced an ATM-, but not an ATR-dependent checkpoint response with subsequent apoptosis.\textsuperscript{49,50} In the third study, an apparently stronger RPA depletion provoked a cell cycle arrest in early S-phase. Moreover, these cells accumulated stalled replication forks and double-strand breaks as indicated by $\gamma$H2AX foci, and finally underwent apoptosis.\textsuperscript{51} These features amazingly parallel the phenotypes of Cdc45-GFP expression. Most strikingly, the Lukas group demonstrated recently that exhaustion of the RPA pool by different means led to a ”replication catastrophe” with a very similar phenotype as described here.\textsuperscript{27}

Recently, the Gautier group reported on Myc-induced replication stress in the \textit{Xenopus} oocyte replication system that was attributed to a Myc-facilitated loading of Cdc45 into replication origins.\textsuperscript{18} Addition of purified Myc-protein to \textit{Xenopus} extracts approximately doubled the amount of fired replication origins and caused severe fork asymmetries indicating halted replication forks. These authors also observed $\gamma$H2AX foci and an ATM response indicating stress-induced fork collapses, again confirming our data. Particularly the recent findings put Cdc45 into a regulatory network of oncogene expression and carcinogenesis. Indeed, there exist several studies that describe overexpressed Cdc45 in human cancer cells.\textsuperscript{2,17,19,20} How can this be
Figure 8. Accumulation of ssDNA in Cdc45 overproducing cells. After one cell cycle in medium supplemented with BrdU, cells were transfected with vectors encoding GFP or Cdc45-GFP or empty vector. Newly synthesized DNA was labeled by a 15 min pulse of EdU immediately before fixation. ssDNA was visualized by immunofluorescence against BrdU under non-denaturing conditions (without the HCl step), and newly synthesized DNA was detected by fluorescent click conjugation of incorporated EdU.

(A) Representative image of ssDNA foci in a Cdc45-GFP overexpressing cell. (B) Cdc45-induced increase of ssDNA foci as DNA synthesis decreases. Native BrdU fluorescence intensities of individual nuclei were plotted against the EdU-staining representing the DNA synthesis rate. (C) Average native nuclear BrdU fluorescence was increased in Cdc45-GFP transfected cells. Average fluorescence intensities were calculated relative to mock transfected cells (to allow comparison of multiple independent experiments). GFP-transfected cells displayed a mean fluorescence comparable to the control, whereas staining of the Cdc45-GFP transfected cells increased by a factor of two. (D) About 15 % of the Cdc45-GFP transfected cells showed elevated ssDNA levels, compared to 1-2 % of cells in the respective controls (D). At least 300 cells per treatment were analyzed. Error bars represent standard deviation between at least 3 independent experiments.
accomplished when too much Cdc45 causes cell death? Since tumor cells are usually selected to divide rapidly, it is of advantage to start as many replication origins as possible. This study shows that Cdc45 can become rate limiting for initiation of replication. Therefore, an overexpression of this protein in tumor cells comes as no surprise. This however works only up to a 2- to 3-fold excess, as long as no other factor becomes limiting. Interestingly, an elevated expression of RPA has been widely observed in many malignant tumors (see e.g. 52-54). Therefore, it will be of interest to find out whether Cdc45-overproducing tumors also overproduce RPA. Is it then possible to further overexpress Cdc45 in cancer cells to drive them into apoptosis? Since Cdc45 expression is largely under the control of the E2F promoter, this might be technically difficult. However, the Cdc45 protein contains several PEST and KEN boxes that facilitate cell cycle-specific degradation. Indeed, the half-life of Cdc45 has been determined to be about 10 h, just enough for one passage through S phase. Therefore, inhibiting ubiquitylation and/or degradation of Cdc45 might be a promising strategy for combatting cancer.

Materials and methods

Cloning and vectors

For the preparation of vector pCdc45-eGFP, the complete ORF of human Cdc45 was amplified using primers Cdc45-1F_Xho (5'-GAC CTC GAG ATG TTC GTG TCC GAT TTT CC) and Cdc45R_N1Bam (5'-GAC GGA TCC CGG GAC AGG AGG GAA ATA AG). The PCR product was then cloned into the XhoI and BamHI sites of vector pEGFP-N1 (Life Technologies, Darmstadt, Germany). The vector constitutively expresses Cdc45 with a C-terminal eGFP fluorescent protein tag (Cdc45-GFP) in mammalian cells. For the preparation of vector pcDNA4/TO-Cdc45HA, the complete ORF of human Cdc45 was amplified using primers Cdc45-1F_Xho (5'-GAC CTC GAG ATG TTC GTG TCC GAT TTT CC) and Cdc45R_N1Bam (5'-GAC GGA TCC CGG GAC AGG AGG GAA ATA AG). The PCR product was then cloned into the BamHI and XhoI sites of vector pcDNA4/TO (Life Technologies). The vector expresses Cdc45 with a C-terminal haemagglutinin (HA) tag under the control of a tetracycline inducible vector. All used expression vectors were verified by Sanger sequencing before use.

Cell culture

HeLa (ATCC-CCL-2) were cultivated in Dulbecco’s Modified Eagle’s Medium supplemented with 10 % fetal bovine serum (PAN Biotech GmbH, Aidenbach, Germany) at 37°C, with 5% CO2 and 95% relative humidity. Six-well plates were seeded with 2×10^5 cells per well and incubated overnight. For transient transfection, cells were transfected using FuGene HD according to the instructions of the manufacturer (Roche Applied Science, Mannheim, Germany) and incubated for the times indicated. Where indicated cells were incubated with 10 μM EdU 15 min before harvesting.

Flow cytometry

Cells were trypsinized, washed with phosphate buffered saline (PBS), fixed with 4% formaldehyde for 10 min at room temperature (RT) and then stored at −20°C in 70% ethanol. After washing with PBS, the cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) in PBS for 1 h followed by incubation with the primary antibody in PBS supplemented with 0.1% triton X-100 and 1% BSA (TPB) for 2 h at RT (see Table 1 for a list of antibodies used). The cells were washed 3 times with PBS containing 1% BSA and incubated for 1 h at RT with appropriate secondary antibodies in TPB followed by washes as above. EdU detection was performed with the Click-iT® EdU Alexa Fluor® 647 Flow Cytometry Assay Kit according to the instructions of the manufacturer (Life Technologies, Darmstadt, Germany). DNA was visualized with 4’,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) or Cell Cycle 405 Blue

| Target            | Clone/Name | Species and Type      | Reference          | Application |
|-------------------|------------|-----------------------|--------------------|-------------|
| β-Actin           | AC-15      | Mouse monoclonal      | Sigma Aldrich      | IB          |
| ATM-p51981        | D6H9       | Rabbit monoclonal     | Cell Signaling Technology | IB |
| ATR-p5428         | #2853      | Rabbit monoclonal     | Cell Signaling Technology | IB |
| BrdU              | BU1/75     | Rat monoclonal        | Abd Serotec        | FA          |
| BrdU              | Clone 44   | Mouse monoclonal      | BD Biosciences     | FA          |
| β-Tubulin         | KMX-1      | Mouse monoclonal      | Merck Millipore    | IB          |
| Cdc45             | 3G10       | Rat monoclonal        | (Bauerschmidt et al. 2007) | IB |
| Chk1 p5345        | 133D3      | Rabbit monoclonal     | Cell Signaling Technology | IB |
| Chk2 p568         | C13C1      | Rabbit monoclonal     | Cell Signaling Technology | IB |
| Cleaved Caspase 3 (Asp175) | #9661 | Rabbit monoclonal     | Cell Signaling Technology | FC |
| H2AX p5139        | JBW301     | Mouse monoclonal      | Merck Millipore    | IB          |
| RPA2-p533         | A300-246A  | Rabbit monoclonal     | Bethyl Laboratories | IF          |
| anti-rat-IgG-Alexa Fluor® 555 |             | Goat monoclonal       | Life Technologies  | FA,F        |
| Anti-mouse-IgG-Alexa Fluor® 488 |             | Goat monoclonal       | Life Technologies  | FA,F        |
| Anti-mouse-IgG-DyLight 649 |             | Donkey monoclonal     | Jackson Immuno Research | FC |
| Anti-rabbit-IgG-PerCP |             | Donkey monoclonal     | Jackson Immuno Research | FC |
| HRPC-conjugated anti-rabbit IgG |             | Goat polyclonal       | Jackson ImmunoResearch | IB |
| HRPC-conjugated goat anti-mouse IgG |             | Goat polyclonal       | Jackson ImmunoResearch | IB |
| AP-conjugated goat anti-rat IgG |             | Goat polyclonal       | Jackson ImmunoResearch | IB |

Abbreviations: FA, fiber assay; FC, flow cytometry; HRP, horse radish peroxidase; IB, immunoblot; IF, immunofluorescence.
(Life Technologies) dyes. Flow cytometry was performed with a FACSCanto flow cytometer equipped with violet, blue and red lasers, using the FACSDiva software (BD Biosciences, Heidelberg, Germany) and FlowJo (FlowJo, LLC, Ashland/Oregon, USA).

**Immunofluorescence microscopy**

Cells grown on glass cover slips were washed with PBS, fixed with 4% formaldehyde and stored in PBS. For immunostaining, the cells were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS for 2 min at RT and blocked with 5% (v/v) goat serum (Vector Laboratories, Burlingame, USA). Cells were then mounted in Vectashield containing DAPI (Vector Laboratories, Burlingame, USA). Immunofluorescence studies were performed with an Axio Imager Z1 Apotome and the Axiovision software (Carl Zeiss Jena, Germany).

**Labeling of DNA replication sites and DNA fiber assays**

Twenty-four h after transfection with GFP or Cdc45-GFP encoding vectors, HeLa CCL2 cells were grown in DMEM supplemented with 25 μM iodo-deoxyuridine (IdU, Sigma-Aldrich) for 30 or 45 min. After the first pulse labeling, the medium was removed and fresh DMEM containing 250 μM chloro-deoxyuridine (CldU, Sigma-Aldrich) was added for the second pulse label. Preparation of DNA spreads was done as described with minor modifications. Briefly, after labeling with halogenated nucleotide analogs and resuspension in PBS the cells were washed with ice-cold PBS and diluted to 5×10^5 cells/ml. Approximately 1000 cells (2 μl) were lysed on glass slides by addition of 7 μl lysis buffer (200 mM Tris-HCl pH 7.4, 50 mM EDTA, 0.5% SDS). DNA spreads were generated by slightly tilting the microscope slide and fixed using methanol/acetic acid (3:1) after air drying.

DNA spreads were denatured by incubation with 2.5 M HCl for 75 min at RT. The microscope slides were washed with PBS and blocked with TPB for 1 h. After incubation with a monoclonal rat anti-BrdU antibody (Clone BU1/75, 1:1000) in TBP for 1 h at RT, which binds CldU but not IdU, the slides were washed twice with PBS and once with TBP and subsequently fixed using 4% paraformaldehyde in PBS (10 min, RT), washed again twice with PBS and 3 times with TBP and incubated with goat anti-rat antibody conjugated with Alexa Fluor® 555 (Life Technologies, 1:500 in TBP) for 2 h at RT. DNA spreads were washed again as described above and incubated overnight at 4°C with a monoclonal mouse anti-BrdU antibody (Clone 44, 1:1500) which binds IdU, but not CldU. After washing, an Alexa Fluor® 488 conjugated goat anti-mouse antibody (Life Technologies, 1:500 in TBP) was added for 2 h at RT. The slides were extensively washed again and mounted. Pictures of fluorescent immunolabeled DNA spreads were taken using an Axio Imager.Z1 at 400-fold magnification.

**Detection of ssDNA**

Visualization of ssDNA was performed as described. Genomic DNA of HeLa or U2OS cells (the latter gave lesser backgrounds) was halogenated by growing in DMEM supplemented with 10 μM BrdU for 30 h. BrdU was removed by addition of fresh DMEM before transfection. After 24 h, the replicating cells were pulse-labeled with 10 μM EdU for 15 min, then fixed with methanol for 30 min at −20°C, and shortly rinsed with cold acetone. Inc. BrdU exposed in the single-stranded template strand was detected with a monoclonal rat-anti-BrdU antibody (Clone BU1/75) in TBP for 1 h without a DNA denaturing step, and anti-rat-IgG antibody-Alexa Fluor® 555 as described above. Newly synthesized DNA was stained using the Click-iT® EdU imaging kit with Alexa Fluor® 647 azide (Life Technologies) according to the manufactures instructions. DNA was counterstained with DAPI. Images were taken using the Zeiss Axio Imager.Z1 at 630-fold magnification.

**Data analysis**

DNA fibers were analyzed using ImageJ 1.46r (http://imagej.nih.gov/ij). The pixel to μm ratio was determined and DNA-track lengths were calculated using a DNA stretching factor of 2.59 kbp/μm as described. Nuclear fluorescence intensities for ssDNA were quantified with ImageJ 1.46r using DAPI staining to determine region of interest. At least 3 independent experiments were performed; the given error bars represent standard deviation of the mean values. Significances were determined using 2-tailed Student’s t-test.

**Cell extract preparation and Western analysis**

For Western analysis, cells were lysed directly into Laemmli buffer followed by a brief sonication. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto an Immobilon P PVDF membrane (Merck Millipore, Billerica, USA). Membranes were blocked for at least one hour with 5% skim milk in Tris-buffered saline with Tween 20 (TBST, 10mM Tris–HCL, pH 7.5, 150mM NaCl and 0.5% Tween 20) and washed thrice with TBST, followed by incubation with the indicated primary antibody (Table 1) for at least 2 h. After washing 3 times with TBST, the membranes were incubated with horseradish peroxidase–conjugated secondary antibody (Table 1) for 1 h at RT. Immunoblots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, USA) as described by the manufacturer, using a gel documentation device (G-box, Syngene, Cambridge, UK) or film exposure (Hyperfilm ECL, GE Healthcare, Freiburg, Germany).

**Abbreviations**

AAA+ ATPase associated with various activities
ATM ataxia telangiectasia mutated
ATR ATM and Rad3 related
BrdU Bromodeoxyuridine
Cdc45 cell division cycle protein 45
Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors are grateful to A. Gleiche for technical assistance.

Funding

This study was supported by UFA Grant 3610530016 by the German Federal Office for Radiation Protection as part of the “Kompetenzzentrum für Strahlenforschung.” The Fritz Lipmann Institute (FLI) is a member of the Science Association ‘Gottfried Wilhelm Leibniz’ (WGL) and is financially supported by the Federal Government of Germany and the State of Thuringia.

Author contributions

CK and DK performed all the experiments presented in the manuscript, ACP and KB contributed to the fiber analysis, and AF contributed to immunofluorescence experiments. HP and FG are responsible for this study and contributed to the design and interpretation of the experiments. All authors contributed to the writing of the manuscript.

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