Chk1 Requirement for High Global Rates of Replication Fork Progression during Normal Vertebrate S Phase

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Chk1 protein kinase maintains replication fork stability in metazoan cells in response to DNA damage and DNA replication inhibitors. Here, we have employed DNA fiber labeling to quantify, for the first time, the extent to which Chk1 maintains global replication fork rates during normal vertebrate S phase. We report that replication fork rates in *Chk1*^{−/−} chicken DT40 cells are on average half of those observed with wild-type cells. Similar results were observed if Chk1 was inhibited or depleted in wild-type DT40 cells or HeLa cells by incubation with Chk1 inhibitor or small interfering RNA. In addition, reduced rates of fork extension were observed with permeabilized *Chk1*^{−/−} cells in vitro. The requirement for Chk1 for high fork rates during normal S phase was not to suppress promiscuous homologous recombination at replication forks, because inhibition of Chk1 similarly slowed fork progression in *XRCC3*^{−/−} DT40 cells. Rather, we observed an increased number of replication fibers in *Chk1*^{−/−} cells in which the nascent strand is single-stranded, supporting the idea that slow global fork rates in unperturbed *Chk1*^{−/−} cells are associated with the accumulation of aberrant replication fork structures.

The stability of cellular genomes is maintained in part by cell cycle-specific and DNA structure-specific checkpoints (14, 21). The transducer/effector protein kinases Chk1 and Chk2 have received considerable attention in recent years due to their central role in regulating aspects of cell cycle progression and chromosome metabolism in response to genotoxic stress. In metazoans, deletion or depletion of the Chk1 gene or protein prevents or reduces the delay into mitosis normally imposed in response to DNA damage or if replication is inhibited or blocked (4, 6, 15, 16, 23, 25, 27, 29). Chk1 is also required in metazoans to inhibit origin firing and maintain the stability/viability of stalled replication forks in response to DNA damage or replication inhibitors (5, 26, 27).

In addition to its roles in the response to exogenous cellular stress, Chk1 plays a critical role during normal proliferation because disruption of Chk1 is embryonic lethal in mice and fruit flies and is cell lethal in mouse embryonic stem cells (6, 16, 25). Disruption of Chk1 in chicken DT40 cells is not cell lethal but does confer a pronounced slow-growth phenotype (26). The critical function of Chk1 in unperturbed cells is unclear, but inhibition or depletion of Chk1 has been reported to deregulate origin firing during unperturbed S phase in human cells, leading to elevated levels of single-stranded DNA and DNA breakage (24).

Given that Chk1 is required to maintain replication fork stability in response to DNA replication inhibitors or exogenous DNA damage in metazoans, we considered the possibility that Chk1 may also be required during a normal S phase to facilitate fork progression beyond endogenous lesions or other types of replication fork barrier. Consistent with this idea, the ATR protein kinase that activates Chk1 in response to replication inhibitors or exogenous DNA damage is required for the stability of fragile sites (1). In addition, caffeine, an inhibitor of ATR and ATM, slows replication fork rates in isolated *Xenopus laevis* sperm nuclei (20). However, whether or not Chk1 is required to maintain normal replication fork rates in metazoans has not been examined. Moreover, it is unclear how global any requirement for Chk1 for the progression of replication forks in metazoans might be, given that the number of impediments encountered by replication forks during a normal S phase is unknown.

Here, we have employed a DNA fiber-labeling technique to measure directly the impact of Chk1 on the rate of replication fork progression during normal vertebrate S phase. Strikingly, we report that loss of vertebrate Chk1 reduces global fork rates by half, indicating that Chk1 is a bona fide DNA replication protein that is required for the normal progression of most if not all replication forks.

MATERIALS AND METHODS

Chromatin fiber experiments. For dual labeling of replication tracts, exponential cell cultures of wild-type DT40 cells (clone 18), *Chk1*^{−/−} DT40 cells (26, 27), *Chk2*^{−/−} DT40 cells (27), or HeLa cells were pulse-labeled with 25 μM BrdU for 15 to 20 min followed by 250 μM IdU for 25 to 30 min, as indicated. For single labeling, exponential cell cultures were pulse-labeled with either BrdU or IdU for 45 to 60 min at the concentrations indicated. Where appropriate, cells were

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pretreated with the Chk1 inhibitor UCN-01 (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD) at 300 nM for 1 h prior to labeling and then throughout. To knock down human Chk1, we employed small interfering RNA (siRNA) duplex oligonucleotides (Invitrogen) directed against the Chk1 target sequence (sense) UCGUGAGCGUUUGUUGAAC (30). siRNA duplexes (10 nM) were transfected using siPORT NeoFX reverse transfection reagent (Ambion) according to the manufacturer's protocol. After 48 h, cells were labeled as described above. Labeled cells were harvested and DNA fiber spreads prepared as previously described (11). For immunodetection of BrdU-labeled tracts, acid-treated fiber spreads were incubated with rat anti-BrdU monoclonal antibody (Oxford Biotechnology) at a 1:1,000 dilution for 1 h at room temperature. Slides were then fixed with 4% paraformaldehyde and incubated with AlexaFluor 488-conjugated donkey anti-rat immunoglobulin G (IgG) (Molecular Probes) at 4 µg/ml for 1.5 h at room temperature. To detect both BrdU- and IdU-labeled patches, a sheep polyclonal antibody that recognizes both IdU and BrdU (Biodesign International) was employed at 2 µg/ml overnight at 4°C, followed by a Cy3-conjugated donkey anti-sheep IgG (Jackson Immunoresearch) at 2.5 µg/ml for 1.5 h at room temperature. Fibers were examined using a Zeiss LSM 510 confocal microscope using a 100× (1.4-numerical-aperture) lens, and the lengths of green (AlexaFluor 488)- and/or red (Cy3)-labeled patches were measured using the LSM software. Measurements were recorded from fibers in well-spread (untangled) areas of the slides to prevent the possibility of recording labeled patches from bundles of fibers.

Agarose cell beads and chromosome replication. [³H]thymidine-labeled cells (~3 × 10⁷) were suspended in 3 ml of 0.5% low-melting-point agarose in phosphate-buffered saline (PBS). The cell suspension was overlaid with 2 volumes of mineral oil and mixed vigorously on a vibrating shaker (IKA-VIBRAX-VXR) at full speed before being cooled on ice. Washed cell beads were then incubated in complete medium (37°C) for 2 h to allow cell recovery and resuspended in physiological buffer (100 mM potassium acetate, 30 mM KCl, 10 mM Na₂HPO₄, 1 mM MgCl₂, 1 mM Na₂ATP, 1 mM dithiothreitol, and 200 µM phenylmethylsulfonyl fluoride, pH 7.4) supplemented with 50 mg/ml fatty acid-free bovine serum albumin (Sigma-Aldrich) and 300 µg/ml saponin (Sigma-Aldrich). After permeabilization for 8 min, washed cell beads were resuspended in physiological buffer with bovine serum albumin. For measurement of replication rates, aliquots were mixed with 10× replication mix (10 mM MgCl₂; 20 mM KPO₄ [pH 7.4]; 1 mM each of CTP, GTP, and UTP; 2.5 mM each of dGTP, dCTP, and dATP; 250 µM TTP; 200 mM creatine phosphate; 1 mg/ml creatine kinase) and 30 µCi [α-³²P]TTP (3,000 Ci/mmol; Amersham-Pharmacia). Reactions were incubated at 37°C and stopped at the indicated times by the addition of sodium dodecyl sulfate (SDS). ³H and ³²P counts were quantified via liquid scintillation, and ³²P counts were normalized for cell number by using the ³H counts and converted to pmol TTP incorporated/10⁶ cells. For measuring the percentages of cells in S phase, aliquots were mixed with 10× replication mix as described above but containing 30 µM digoxigenin-11–dUTP (Roche) instead of TTP. Reactions were incubated at 37°C for 30 min, and washed beads were fixed with 4% paraformaldehyde. Digoxigenin-11–dUTP replication foci were immunolabeled with fluorescein isothiocyanate-conjugated antidigoxigenin Fab fragment (1.5 h, 30 µg/ml; Roche), and nuclei were counterstained with DAPI (4′,6′-diamidino-2-phenylindole). S-phase cells were quantified using a Leitz Diaplan microscope.

Immunoblotting. For immunoblotting, harvested cells were lysed in SDS loading buffer and lysates from 3 × 10⁵ to 6 × 10⁵ cells per lane were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Total Chk1 was detected using rabbit polyclonal anti-Chk1 antibody (Santa Cruz Biotechnology) at 4 µg/ml, and Chk1 phosphorylated at Ser³⁴⁵ (phospho-Chk1 Ser³⁴⁵) was detected using rabbit polyclonal anti-phospho-Ser³⁴⁵ antibody at a 1:1,000 dilution (New England Biolabs).

Detection of single-stranded nascent DNA by immunofluorescence. Wild-type (clone 18) and *Chk1⁻/⁻* DT40 cells at 5 × 10⁵ cells/ml were incubated with 25 µM BrdU for 20 min. Cells were then washed three times in cold PBS, and 5 × 10⁵ cells were swollen in 0.075 M KCl for 15 min at 37°C, as previously described (11). Cells were then fixed with methanol-acetic acid (3:1), dropped onto washed microscope slides, and air dried. Slides were acid treated and incubated with sheep anti-BrdU antibody (M20105S; Biodesign) at 4 µg/ml for 1 h and then Cy3-conjugated donkey anti-sheep IgG (Jackson Immunoresearch) at 2.5 µg/ml for 1 h. Nuclei were counterstained with 5 µg/ml Hoechst 33258 (Sigma). For detection of incorporated BrdU in the absence of HCl denaturation, fixed slides were stored at 4°C for several weeks before immunostaining.

For direct labeling of replication tracts with biotin-11–dUTP (Yorkshire Bioscience), cells were washed in cold PBS (3×) and 1 × 10⁶ cells in 10 µl of PBS, transfected with biotin-11–dUTP analogue (1 µl of 50 nM solution) by using FuGene (Roche), and then washed in fresh medium and incubated for 30 min at 37°C. Cells were additionally pulse-labeled with 25 µM BrdU for 20 min either before or after transfection with biotin-11–dUTP. Biotin-11–dUTP was detected using an antibiotin mouse monoclonal antibody (clone BN-34; Sigma) at a 1:1,000 dilution.

RESULTS

Wild-type chicken DT40 cells or DT40 cells lacking the protein kinase Chk1 (26, 27) or Chk2 (27) were pulse-labeled for 20 min with 25 µM BrdU followed by 250 µM IdU for 25 to 30 min, and the length of the labeled DNA replication tracts in DNA fiber spreads was then quantified by indirect immunofluorescence. Dual labeling in this way enables unambiguous identification of replication tracts arising from individual replication forks and establishes their directionality. A visual comparison of DNA fibers from wild-type and *Chk1⁻/⁻* DT40 cells revealed a striking difference in the overall lengths of their replication tracts (Fig. 1A). This difference was also evident when the distribution of fork rates within populations of forks was quantified and plotted, with the entire distribution of fork rates in *Chk1⁻/⁻* cells shifted leftwards, to slower fork rates, during both pulse-labels (Fig. 1B). These data suggest that most if not all replication forks progress in *Chk1⁻/⁻* cells at a slower rate than in wild-type cells. In contrast, we did not observe any replication defect in *Chk2⁻/⁻* DT40 cells, which displayed rates of fork progression similar to that for wild-type DT40 (Fig. 1C).

We noted in the experiments described above that the difference in fork rate distribution between wild-type and *Chk1⁻/⁻* cells was greater during the second pulse-label, employing IdU, than during the first pulse-label, employing BrdU (Fig. 1A and B). Consequently, to examine whether the slow fork rates in *Chk1⁻/⁻* cells were an artifact of employing halogenated nucleosides, we compared the impact of a wide range of BrdU and IdU concentrations on fork rates in experiments in which each of the two halogenated deoxyribonucleosides was employed separately. The extents of fork slowing in *Chk1⁻/⁻* cells were similar in single-label experiments at concentrations of BrdU (Fig. 2A) or IdU (Fig. 2B) ranging from 5 µM to 250 µM, suggesting that the slow fork rate in *Chk1⁻/⁻* cells is unrelated to the use of halogenated nucleosides. Similar experiments revealed that fork rates in wild-type cells were similarly unaffected by concentration of BrdU or IdU and were on average twofold faster than the mean fork rate in *Chk1⁻/⁻* cells at all concentrations examined (Fig. 2C and D). To further rule out that the labeling protocol did not itself induce a requirement for Chk1 activity, we examined the phosphorylation status of Chk1 (13). The level of signal present on anti-phospho-Chk1 Ser³⁴⁵ immunoblots was no greater for cells subjected to dual labeling than the basal level observed for cells that were not subjected to dual labeling, confirming that pulse-labeling does not itself trigger Chk1 activation (16, 18) (Fig. 2E). The basal signal detected in these experiments reflected true phospho-Chk1 Ser³⁴⁵ because it was not observed in cell extract from *Chk1⁻/⁻* cells or *Chk1⁻/⁻* cells harboring nonphosphorylatable Chk1^S345A protein (data not shown). We conclude from these experiments that the slow fork rate observed with *Chk1⁻/⁻* cells is not related to the use of halogenated nucleosides but rather that Chk1 is required for high
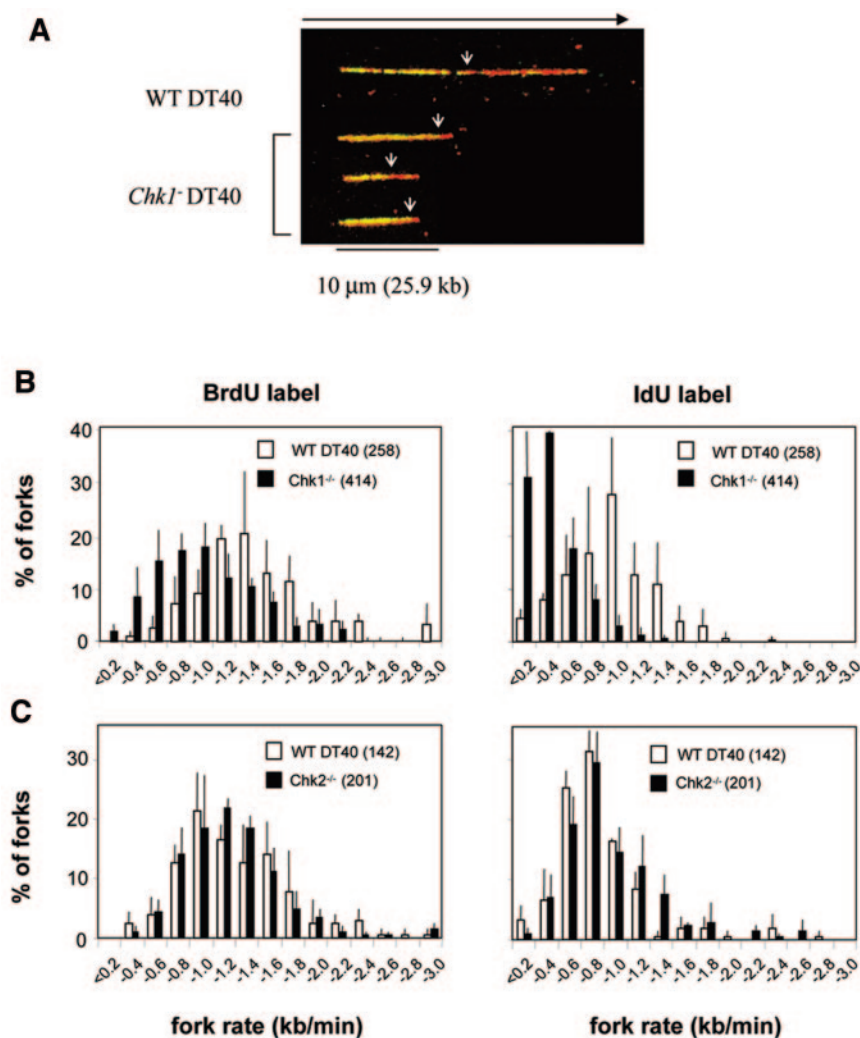
FIG. 1. Replication fibers and fork rates in wild-type (WT) and $Chk1^{-/-}$ chicken DT40 cells. (A) Representative images of replication tracts from wild-type and $Chk1^{-/-}$ DT40 cells pulse-labeled with 25 μM BrdU for 20 min (yellow track) followed by 250 μM IdU for 30 min (red track) and then processed for DNA fiber spreads as described in Materials and Methods. Fork direction is indicated by a black arrow, and the junction between pulse-labels is indicated by a white arrow. (B) Distribution of replication fork rates in wild-type and $Chk1^{-/-}$ DT40 cells pulse-labeled and processed as described above. (C) Distribution of replication fork rates in wild-type and $Chk2^{-/-}$ DT40 cells. For each panel, the distribution of fork rates during the first (BrdU) and second (IdU) pulse-labels is shown. Data bars are the means of three independent experiments, with similar results observed for each, and error bars represent 1 standard deviation. The total number of forks scored for each distribution is indicated in parentheses.

global rates of replication fork progression during unperturbed S phase.

To examine whether the protein kinase activity of Chk1 might be required for its role in maintaining normal fork rates, we examined whether the Chk1 inhibitor UCN-01 (7) also slowed replication fork progression. Indeed, coincubation with UCN-01 during pulse-labeling significantly reduced the rate of replication fork progression in wild-type DT40 cells (Fig. 3A). The impact of UCN-01 was largely Chk1 dependent in these experiments, because the inhibitor had relatively little impact on fork rates in $Chk1^{-/-}$ DT40 cells (data not shown). In addition, the slower fork rates observed with cells lacking Chk1 activity was not due to tonic activation of Chk2, because UCN-01 still decreased fork speeds in $Chk2^{-/-}$ cells (data not shown). Importantly, UCN-01 also reduced replication fork

rates in HeLa cells, suggesting that Chk1 is also required for normal global fork rates in human cells (Fig. 3B). This notion was confirmed by experiments in which Chk1 was depleted in HeLa cells by use of siRNA, in which we again observed reduced global rates of replication fork progression (Fig. 3C).

We next examined whether the requirement for Chk1 for maintaining high fork rates during normal S phase could be recapitulated in vitro. We thus employed a fork extension assay in which cells are encapsulated in agarose microbeads, permeabilized, and then incubated in a physiological buffer containing exogenous deoxynucleoside triphosphates (9, 10, 12). To enable us to normalize the rate of deoxynucleoside triphosphate incorporation for the fraction of cells in S phase, aliquots of encapsulated cells were labeled with digoxigenin-11–dUTP and DAPI in parallel (Fig. 4A). Remarkably, these experi-
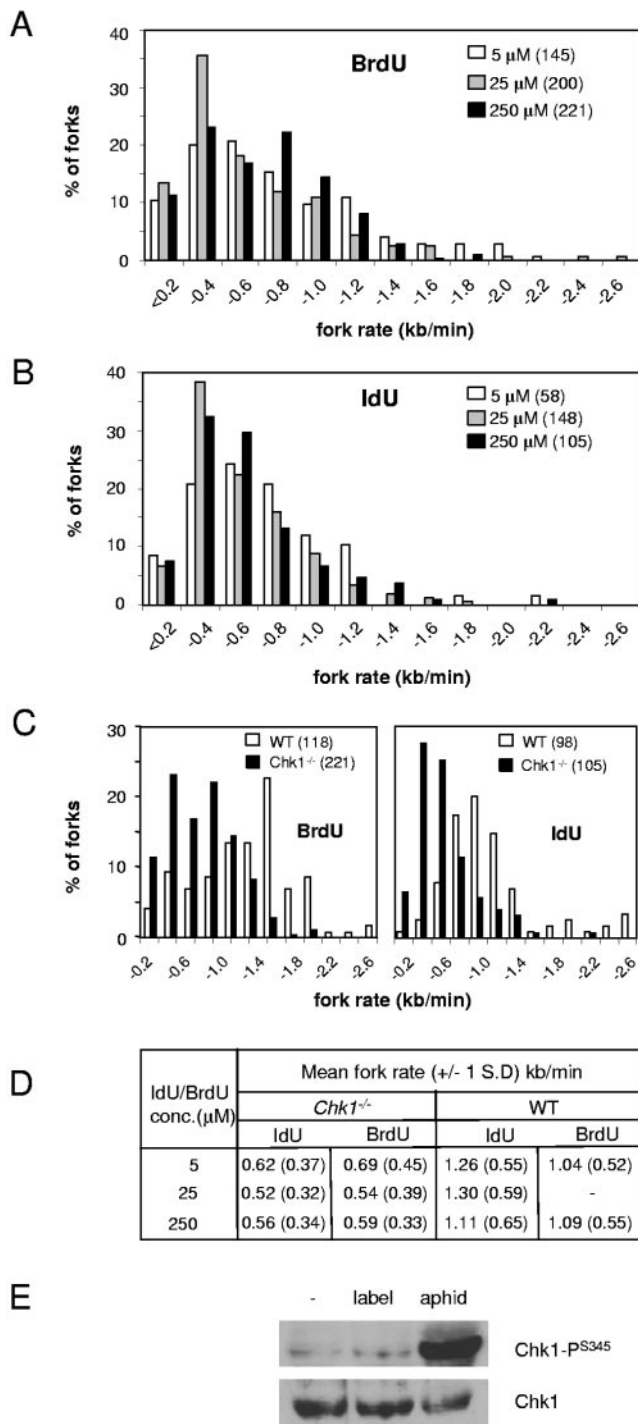
FIG. 2. Impact of BrdU and IdU on replication fork rates and Chk1 Ser[345] phosphorylation in vertebrate cells. (A) Fork rates in $Chk1^{-/-}$ DT40 cells incubated with 5 to 250 μM BrdU for 60 min. The total number of forks scored for each distribution is indicated in parentheses. (B) Fork rates in $Chk1^{-/-}$ DT40 cells incubated with 5 to 250 μM IdU for 60 min. (C) Comparison of fork rate distributions obtained for $Chk1^{-/-}$ cells and wild-type (WT) DT40 cells incubated with 250 μM BrdU or 250 μM IdU for 45 to 60 min. (D) Tabulated average fork rates for $Chk1^{-/-}$ and wild-type DT40 cells incubated with 5 to 250 μM BrdU or IdU. Mean fork rates are calculated from the data shown in panels A and B and are from one experiment for each deoxyribonucleoside. Note that the standard deviation (SD) values reflect the intrinsic variation in progression rates of different rep-

ments revealed that permeabilized $Chk1^{-/-}$ cells exhibit initial fork extension rates that are ~60% of those observed with wild-type cells (Fig. 4B), confirming that the slower rates of fork progression observed with intact cells are also evident in vitro.

We have reported previously that rates of fork progression are actively slowed in the presence of UV or cisplatin damage by homologous recombination (HR), due most likely to the time taken for HR reactions during DNA replication (8). We thus considered the possibility that Chk1 might maintain normal rates of fork progression during unperturbed S phase by inhibiting or preventing unnecessary HR reactions at paused or stalled forks, thereby minimizing delays in fork progression. To address this question, we compared the effect of UCN-01 on fork progression rates in wild-type and $XRCC3^{-/-}$ (HR-defective) DT40 cells. We reasoned that if Chk1 does maintain high fork rates by suppressing HR, then it should be redundant in cells that lack HR. However, UCN-01 triggered fork slowing in both wild-type and $XRCC3^{-/-}$ DT40 cells (Fig. 5), suggesting that the requirement for Chk1 for normal fork rates during unperturbed S phase is not suppression of HR. Once again, we noted in these experiments that the apparent influence of Chk1 on fork rate was greater during the second pulse-label than during the first.

In *Saccharomyces cerevisiae*, Rad53 is required to prevent the formation of replication fork intermediates that contain extensive regions of single-stranded DNA during hydroxyurea treatment (17). Consistent with Chk1 perhaps fulfilling a similar role in vertebrates, Chk1 inhibition has similarly been shown to result in increased levels of single-stranded DNA in human cells (24). However, because cells were labeled with BrdU for 24 h in these experiments, it is not clear whether the single-stranded DNA was located near replication forks. To examine this question directly, we fixed cells immediately after a short pulse-label (20 min) with BrdU and then immunostained with anti-BrdU antibodies in the absence of DNA denaturation. Whereas incorporated BrdU was detected in wild-type DT40 cells only if DNA was first denatured with HCl, BrdU was detected in large numbers of $Chk1^{-/-}$ cells even in the absence of denaturation (Fig. 6A). We noted that storing of fixed slides for several weeks at 4°C was necessary to unveil incorporated BrdU in $Chk1^{-/-}$ cells in the absence of HCl, presumably to enable dissociation of replication protein A from the single-stranded DNA. We also note that, because of the very short pulse-labeling period employed in these experiments, the single-stranded DNA observed here was comprised of nascent strands.

To further colocalize the single-stranded DNA with DNA replication forks, we immunostained chromosome fiber spreads in the absence of HCl denaturation (Fig. 6B). To achieve this, we pulse-labeled replication forks with BrdU for 20 min followed by biotin-11–dUTP for 30 min to tag the forks. These experi-

lication forks. conc., concentration. (E) Levels of total Chk1 and phospho-Chk1 Ser[345] (Chk1-P[S345]) in wild-type DT40 cells mock labeled (−) or dually labeled (label) with BrdU (20 min) followed by IdU (30 min) or incubated with 20 μM aphidicolin (aphid) for 240 min as a positive control for Chk1 phosphorylation.
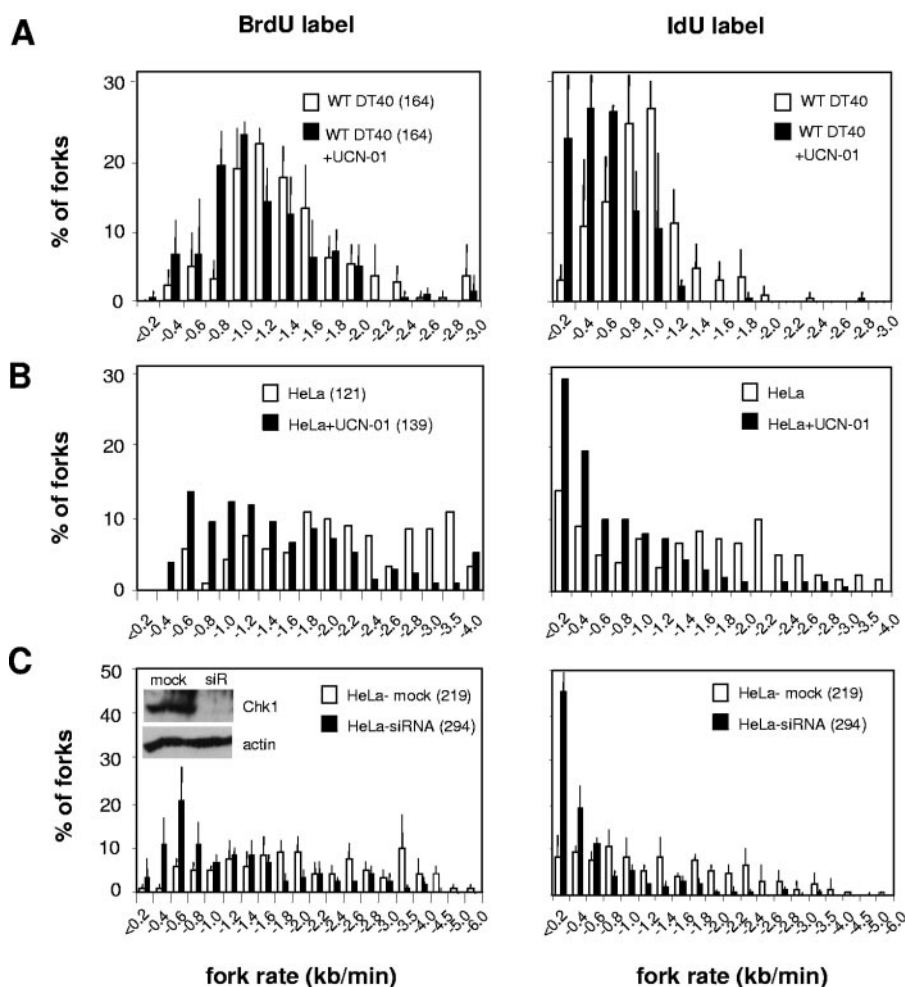
FIG. 3. Impact of the Chk1 inhibitor UCN-01 on replication fork rates in wild-type (WT) DT40 and HeLa cells. (A) Distribution of replication fork rates in wild-type DT40 cells pulse-labeled with 25 μM BrdU for 20 min followed by 250 μM IdU for 30 min in the absence or presence of 300 nM UCN-01. (B) Distribution of replication fork rates in HeLa cells pulse-labeled for 15 to 20 min in BrdU followed by 20 to 25 min in IdU in the absence or presence of 300 nM UCN-01. (C) Distribution of replication fork rates in mock-treated or Chk1 siRNA-treated HeLa cells pulse-labeled as described for panel B. (Inset) Chk1 and actin levels in total cell extract from mock-treated or Chk1 siRNA-treated (siR) HeLa cells. In panels A and C, data are the means of three independent experiments and error bars represent 1 standard deviation. In panel B, data from two independent experiments are combined. For each data set, similar results were observed in each experimental repeat. The total number of forks scored for each cell line is indicated in parentheses.

ments revealed the presence of three- to fivefold more replication tracts containing single-stranded DNA in $Chk1^{-/-}$ cells than in wild-type cells and identified the nascent DNA strands as components of the single-stranded material. Together, these data associate the reduced global fork rates in cells lacking Chk1 protein or activity with the accumulation of abnormal DNA replication structures.

DISCUSSION

Chk1 is required in metazoans in response to DNA damage or replication inhibitors to maintain replication fork stability, suppress unscheduled firing of replication origins, and prevent premature entry into mitosis (4–6, 15, 16, 23, 25–27, 29). However, Chk1 also fulfills a role during the proliferation of unperturbed cells because disruption of *Chk1* is embryonic lethal

in mice and fruit flies and is cell lethal in cultured mouse embryonic stem cells (6, 16, 25). In addition, $Chk1^{-/-}$ chicken DT40 cells exhibit increased doubling times and elevated frequencies of apoptosis (26). One role for Chk1 in unperturbed cells is the suppression of futile or late replication origins (22, 24), and it has been suggested that loss of this function and the consequent increase in DNA replication result in increased chromosome breakage (24).

Here, we demonstrate that Chk1 is also required to maintain high global rates of replication fork progression during normal S phase. It is unlikely that the impact of Chk1 on fork rates is an indirect effect of the elevated level of apoptosis and reduced growth rate reported to occur in these cells (26). This is because we observed fork slowing even during short incubations with Chk1 inhibitor and thus in the absence of any detectable cell death or impact on overall cell cycle distribution. In addi-
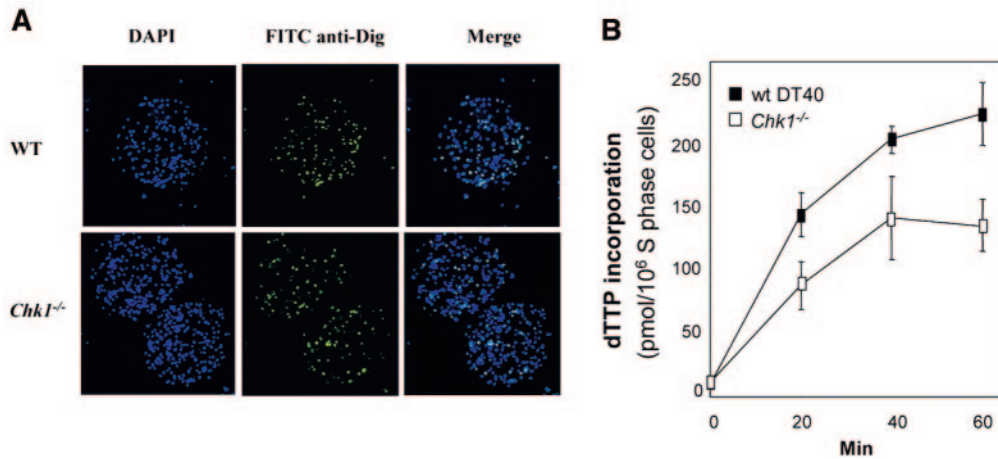
FIG. 4. Replication fork rates in permeabilized wild-type (WT) and $Chk1^{-/-}$ DT40 cells in vitro. (A) Wild-type and $Chk1^{-/-}$ cells were encapsulated in agarose microbeads and permeabilized, and aliquots were then pulse-labeled for 30 min with digoxigenin-11–dUTP. Agarose beads were stained with DAPI to identify nuclei and with fluorescein isothiocyanate (FITC)-tagged antidigoxigenin (anti-Dig) antibody to identify S-phase cells. The fraction of S-phase cells in each population was calculated from multiple microscopic fields and used to normalize replication fork rates, as shown in panel B. The fractions of cells in S phase were 43% ($\pm$3%) and 24% ($\pm$4%) for wild-type and $Chk1^{-/-}$ cells, respectively. Note that a single agarose bead is present in the top panels and two are present in the bottom panels. (B) Replication fork rates were quantified in permeabilized wild-type and $Chk1^{-/-}$ cells encapsulated in agarose microbeads, as described in Materials and Methods, in the presence of [^{32}P]TTP. Results are the means of three independent experiments, with error bars representing 1 standard deviation.

tion, in experiments in which we employed consecutive pulse-labeling with BrdU and IdU to label each replication tract, we consistently observed a greater impact of Chk1 deletion or inhibition during the second pulse-label. This differential im-

pact of Chk1 within individual replication forks is unlikely to be attributable to an indirect effect of Chk1 on cell growth or cell cycle distribution.

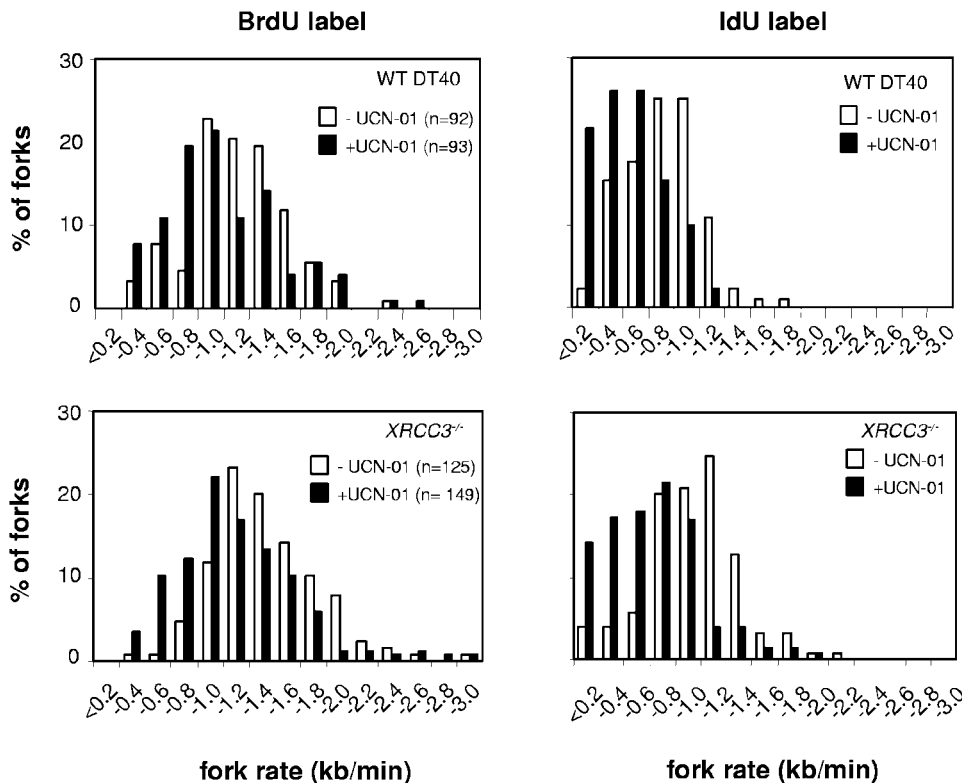A role for Chk1 in maintaining replication fork progression

FIG. 5. Replication fork rates in DT40 cells lacking both Chk1 activity and HR capacity. (A) Wild-type (WT) and $XRCC3^{-/-}$ DT40 cells were pulse-labeled with 25 μM BrdU for 20 min followed by 250 μM IdU for 30 min in the absence or presence of UCN-01 (300 nM) and then processed for DNA fiber spreads. Results are combined from two independent experiments with the same result observed in each. The total numbers of forks scored are indicated in parentheses.
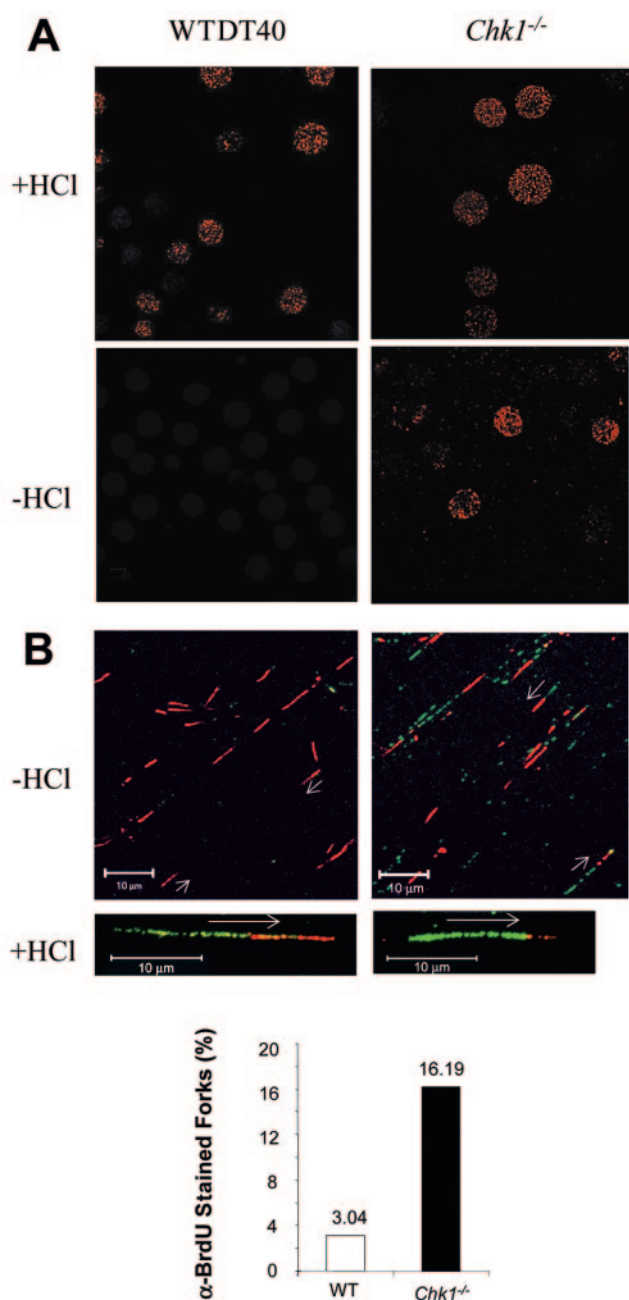
FIG. 6. Accumulation of single-stranded nascent DNA in $Chk1^{-/-}$ DT40 cells. (A) Wild-type (WT) or $Chk1^{-/-}$ DT40 cells were pulse-labeled with BrdU for 20 min, fixed, and then either denatured with HCl and immunostained with anti-BrdU antibodies (+HCl) or stored at 4°C before immunostaining in the absence of HCl denaturation (−HCl). Representative images of multiple cells are presented. Where evident, cells were counterstained with Hoechst to identify nuclei. (B) Wild-type or $Chk1^{-/-}$ DT40 cells were pulse-labeled for 20 min with BrdU (green) followed by transfection for 30 min in the presence of biotin-11–dUTP (red). Samples were then processed for DNA fiber spreads in the absence (top micrographs) or presence (bottom micrographs) of HCl denaturation. The fractions of biotin-labeled forks (n = 164 wild-type forks; n = 142 $Chk1^{-/-}$ forks) that stained with anti-BrdU (α-BrdU) antibody in the absence of HCl denaturation are shown graphically. Data are from a single experiment. White arrows indicate the direction of fork movement. In the case of forks in which only the biotin label is visible, directionality is indicated by tailing of the fluorescent signal, due to exhaustion of the transfected biotin-11–dUTP.

during normal S phase has not been demonstrated previously. However, such a role is consistent with previous reports that ATR, a protein kinase that activates Chk1, is required for the stability of endogenous chromosomal fragile sites in mammalian cells (1) and for normal replication fork rates in isolated *Xenopus* sperm nuclei (20). In addition, termination of Chk1-mediated checkpoints may be facilitated by a feedback mechanism involving Chk1 polyubiquitination and degradation that is triggered once Chk1 is activated by phosphorylation at S345 (28). Since Chk1 polyubiquitination appears to occur even in unperturbed cells, it seems likely that Chk1 is active during normal S phase, a notion supported by our observation of low levels of phospho-Chk1 Ser[345] in unperturbed DT40 cells (Fig. 2E).

Strikingly, the average rate of replication fork progression during a single 60-min pulse-label dropped by half in the absence of Chk1, from ~1.2 kb/min to 0.6 kb/min, suggesting that Chk1 is routinely required by most if not all replication forks during a normal vertebrate S phase. This conclusion is supported by a comparison of the fork rate distributions for wild-type and $Chk1^{-/-}$ cells, in which the majority of the replication forks in Chk1 populations shifted leftwards to slower rates (e.g., see Fig. 2C). Consequently, we conclude that Chk1 is a bona fide DNA replication protein, the activity of which is required by most if not all replication forks.

Despite their slow rate of fork progression, the cell cycle of $Chk1^{-/-}$ cells is not lengthened (26). This suggests that the decrease in fork rate in $Chk1^{-/-}$ cells may be compensated for by an increase in the number of active origins. Increased origin activation has been observed to occur in metazoans in the absence of ATR/ATM activity (22) and in Chk1-depleted or -inhibited cells following chemical perturbation (5, 24, 26). Also, we observed greater numbers of bidirectional, recently initiated replication forks in $Chk1^{-/-}$ cells than in wild-type cells, supporting the presence of increased numbers of active origins (data not shown). We also frequently observed single fibers that contained multiple bidirectional forks in close proximity in $Chk1^{-/-}$ cells. However, despite the increase in number of active origins, we still observed an overall reduction in the level of nucleotides incorporated into permeabilized Chk1 cells in vitro. This would not be expected if the reduced fork rate in $Chk1^{-/-}$ cells were compensated for by increased fork numbers. Perhaps the compensatory activation of secondary origins in living cells is most pronounced towards the end of S phase, once the time allotted to complete replication using the primary origins has expired, whereas our in vitro experiments employed asynchronous populations of permeabilized cells distributed throughout S phase.

What is the role of Chk1 at replication forks during a normal S phase? One possibility is that Chk1 promotes the activity of one or more components of the replication machinery, such that the replisome translocates more slowly in $Chk1^{-/-}$ cells. However, if this were true, then loss of Chk1 should have affected fork rates to a similar extent during the two pulse-labels of our dual-labeling protocol, whereas in fact the apparent impact of Chk1 was greatest during the second pulse-label. A more likely explanation for our data is that Chk1 is required to maintain the stability of most if not all replication forks during normal S phase. Such a role would be analogous to its role following cellular exposure to genotoxins or replication

inhibitors and could explain why the apparent impact of Chk1 was greatest during the second pulse-label. Because we scored only those forks that incorporate both labels during the dual-labeling protocol, forks that stalled irreversibly or for prolonged periods during the first pulse-label and which were thus not active during the second pulse-label were not scored. In contrast, all forks that stalled during the second label, no matter how prolonged or severe the stalling event, were scored because these forks were already dually labeled. Consequently, a role for Chk1 in maintaining global fork stability would have a greater apparent impact on rates during the second pulse-label than during the first, which is what we observed.

It is currently unclear why the stability of replication forks might be threatened so frequently during normal S phase. However, chromosomes are known to contain fragile sites and replication slow zones that require checkpoint proteins for their stability (1, 2). In addition, other physiological sources of replication blockage during a normal S phase are endogenous lesions and regions of the genome containing repetitive sequences, extensive secondary structure, or nucleoprotein complexes. The mechanism by which Chk1 might stabilize replication forks is also unclear. In budding yeast, checkpoint proteins are required to maintain the presence of DNA polymerase α and ϵ at the replisome in response to hydroxyurea (3, 19), though whether they fulfill a similar role in unperturbed cells is not known. In budding yeast, the intra-S-phase checkpoint is required during hydroxyurea treatment to prevent the formation of replication fork intermediates containing extensive regions of single-stranded DNA (17). Recently, single-stranded DNA was also observed to accumulate in human cells incubated with Chk1 inhibitor (24). Our finding that Chk1 is required to suppress the occurrence of single-stranded nascent DNA extends this observation and provides a possible explanation for the slow global rates of replication fork progression in cells lacking Chk1 during normal vertebrate S phase.

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