Kinase-independent function of checkpoint kinase 1 (Chk1) in the replication of damaged DNA

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The checkpoint kinases Chk1 and ATR are broadly known for their role in the response to the accumulation of damaged DNA. Because Chk1 activation requires its phosphorylation by ATR, it is expected that ATR or Chk1 down-regulation should cause similar alterations in the signals triggered by DNA lesions. Intriguingly, we found that Chk1, but not ATR, promotes the progression of replication forks after UV irradiation. Strikingly, this role of Chk1 is independent of its kinase-domain and of its partnership with Claspin. Instead, we demonstrate that the ability of Chk1 to promote replication fork progression on damaged DNA templates relies on its recently identified proliferating cell nuclear antigen-interacting motif, which is required for its release from chromatin after DNA damage. Also supporting the importance of Chk1 release, a histone H2B-Chk1 chimera, which is permanently immobilized in chromatin, is unable to promote the replication of damaged DNA. Moreover, inefficient chromatin dissociation of Chk1 impairs the efficient recruitment of the specialized DNA polymerase η (pol η) to replication-associated foci after UV. Given the critical role of pol η during translesion DNA synthesis (TLS), these findings unveil an unforeseen facet of the regulation by Chk1 of DNA replication. This kinase-independent role of Chk1 is exclusively associated to the maintenance of active replication forks after UV irradiation in a manner in which Chk1 release prompts TLS to avoid replication stalling.

pol eta foci | pol iota foci | DNA fibers | Chk1 inhibitors

The checkpoint kinases ATR and Chk1 are central factors in the DNA damage response (1). During the S phase checkpoint, ATR is activated at single-stranded DNA (ssDNA) and this event, in turn, activates the effector kinase Chk1. Although ATR remains associated with the DNA, activated Chk1 rapidly spreads throughout the whole nucleus. Within the nucleoplasm, Chk1 delays the progression through S phase via phosphorylation of key target genes (2, 3).

Several lines of evidence suggest that the activities of ATR and Chk1 are also required for proper unperturbed S phase progression. In fact, ATR or Chk1 loss leads to embryonic lethality (4–7), and Chk1 heterozygosity is associated with multiple defects, including a miscoordinated cell cycle and increased apoptosis (8).

A contribution of Chk1 to replication fork stability during unperturbed DNA replication was identified and characterized in detail (9–11). Chk1 activity promotes the maintenance of global replication rates by regulating origin firing. In line with these observations, the monoallelic expression of the mutant Chk1 S317A, which is not phosphorylated by ATR, impairs fork elongation (12). Together, these results reveal an unambiguous role of the Chk1 kinase during unperturbed DNA replication.

Intriguingly, recent reports described a kinase-independent effect of Chk1 on DNA replication-associated events. Scorah and colleagues discovered a proliferating cell nuclear antigen (PCNA) binding motif of Chk1 (Chk1_TRFF motif) required for the efficient dissociation of Chk1 from chromatin after UV irradiation (13). Yang et al. also reported a kinase-independent function of Chk1 in the control of UV-induced PCNA ubiquitination (14). This modification of PCNA is known for its contribution to the activation of the DNA replication auxiliary process translesion DNA synthesis (TLS), which triggers the utilization of specialized polymerases (pols) to overcome fork stalling by using DNA lesions as replication templates (15).

Herein, we compared the effect of Chk1 and ATR knockdown on replication fork progression after UV irradiation in U2OS cells. We observed a specific requirement of Chk1, but not ATR, in the maintenance of replication fork progression and for the recruitment of the specialized polymerases η and pol ι into subnuclear foci. Interestingly, neither the kinase domain of Chk1 nor Claspin, a Chk1 regulating factor, were required for the progression of DNA forks and for pol η focal organization. In contrast, we found that the Chk1_TRFF motif is critical for this contribution of Chk1. Similar results were obtained when using a H2B-Chk1 chimera (16) that abrogates Chk1 release from the chromatin fraction. Taken together, our data reveal a kinase-independent contribution of Chk1 to the replication of damaged DNA that strengthens the link between Chk1 and the coordination of TLS.

Results

Chk1 Is Required for Replication Fork Progression After UV Irradiation. Given the multiple roles for Chk1 and ATR in the S-phase checkpoint, we wondered whether Chk1 and ATR modulate the early DNA replication response to UV irradiation. We knocked Chk1 and ATR with specific siRNAs (Fig. 1*A*) and used a DNA fiber spreading technique, a method for labeling tracts of new DNA synthesis in vivo (17). Two consecutive incorporations of different halogenated nucleotides, CldU and IdU, labeled two subsequent periods of DNA synthesis. The DNA molecules that incorporated these analogs can be visualized by fluorescence microscopy (Fig. 1*B*). A shortening of the second track indicates a delay or blockage in the progression of DNA replication after UV irradiation.

Average CldU/IdU ratios of ≈1 were obtained in sham-irradiated conditions independently of the siRNA used (Fig. 1 *C* and *D*). This ratio is expected because the quality of DNA templates is identical for both labeling periods. However, and in agreement with the previous reports (10–12), the rate of incorporation of the thymidine analogs on undamaged DNA of Chk1 and ATR depleted samples was slower than the rate obtained for siLuc transfected samples (Fig. S1 *A* and *B*).

As previously reported, UV irradiation shifted the ratios to greater numbers in all samples. Also, a spread of ratios became evident, which is in line with the increased heterogeneity in the DNA templates caused by UV induced damage (18). Under these conditions, Chk1 knockdown impaired the progression of DNA replication, while the effect of ATR depletion was much less pronounced (Fig. 1*C*). To facilitate a direct comparison the results were plotted as cumulative percentages of forks at each ratio (Fig. 1*D*).

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The authors declare no conflict of interest.

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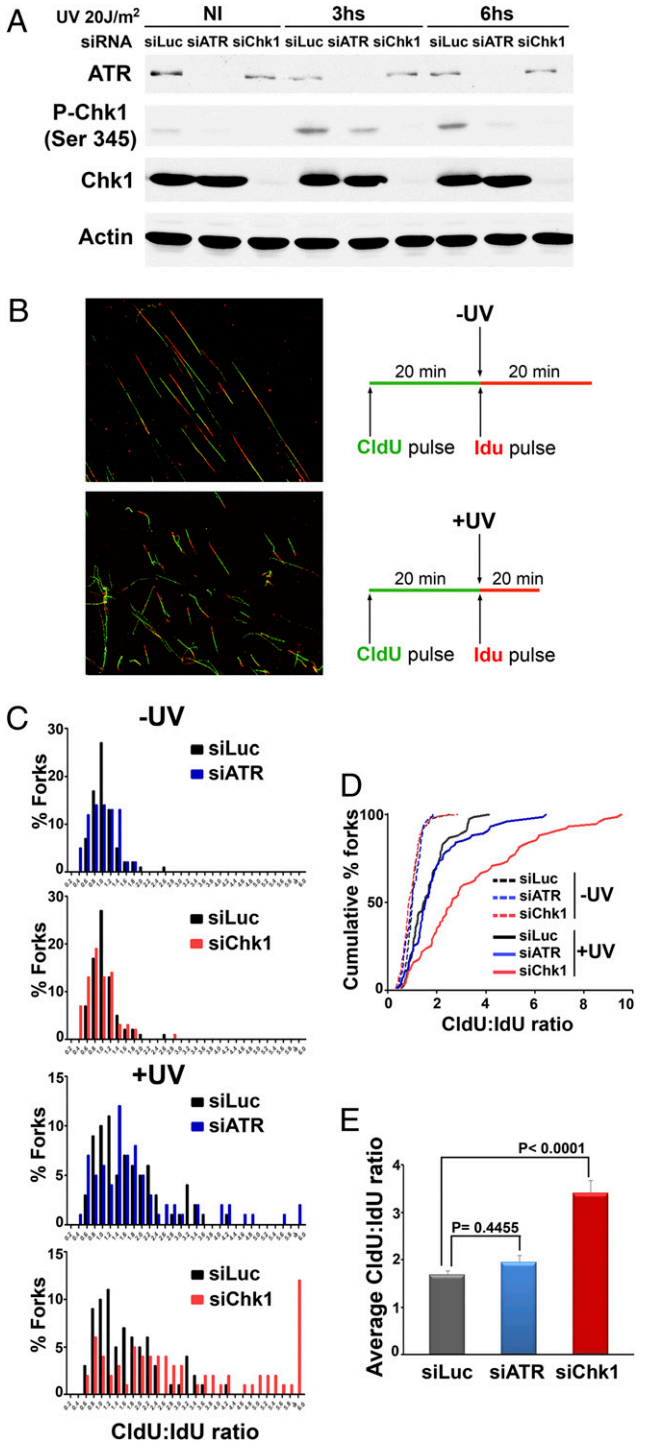

Fig. 1. Chk1 is required to promote the progression of replication forks on damaged DNA. (*A*) U2OS cells were transfected with the indicated siRNA and UV irradiation (20 J/m^2). Samples were used for Western blot analysis by using specific antibodies. (*B Left*) Schematic of the DNA fiber labeling experiment. Unperturbed DNA replication results in ratios of CldU: IdU ≈ 1. Replication stalling during the second labeling period results in higher ratios. Representative images of DNA fibers are shown at *Right*. CldU, chlorodeoxyuridine; IdU, iododeoxyuridine. (*C*) U2OS cells transfected with the indicated siRNA were UV-irradiated (20 J/m^2) when indicated and subjected to the DNA fiber labeling described in *B*. Comparative distribution of ratios obtained in unirradiated controls (-UV) and UV irradiated samples (UV). Ratios higher than 6 were pooled together in the last category. (*D*) The data in *C* were plotted as a cumulative percentage of forks at each ratio. (*E*) Average ratios of data described in *C*.

The comparison of average ratios shows that only the knockdown of Chk1 led to significant differences in replication fork progression (Fig. 1*E*). We conclude that Chk1, but not ATR, knockdown impairs fork progression at early times after UV irradiation.

Chk1 Knockdown Impairs the Recruitment of Specialized Pols to Damaged DNA Sites. Translesion DNA synthesis is central to overcome the stalling of replication forks (15). The recruitment of specialized pols to replication factories can be monitored by analyzing their subnuclear organization by using high-resolution microscopy. This parameter is used as an indicator of TLS activation, given that the percentage of cells with detectable pol η nuclear foci steeply increases after UV irradiation (19–22). To address the impact of ATR and Chk1 knockdown on pol η recruitment, we scored the nuclear pattern of pol η in two arbitrary categories: negative (none to 15 foci per cell) and positive (>15 foci per cell) (Fig. 2). We observed that Chk1, but not ATR, significantly reduced the percentage of cells with pol η foci. Strengthening this result, siChk1 also impaired both PCNA and pol η interaction in chromatin inmunoprecipitation assays and the extent of recruitment of pol η to PCNA foci (Fig. S2). Chk1 knockdown also impaired the organization of the specialized pol ι (Fig. S3). Together, these results show that Chk1 is required at early times after UV to promote recruitment of TLS pols.

Kinase Activity of Chk1 Is Not Required for the Maintenance of Replication Fork Progression After UV Irradiation. Because of the contrasting results obtained after Chk1 and ATR knockdown, we wondered whether the effect of Chk1 on DNA replication after UV could be independent of its kinase activity. We treated cells with Chk1 inhibitors: UCN-01 (23) and Gö6976 (24). As expected, because the kinase activity of Chk1 is involved in DNA replication elongation on undamaged DNA, UCN-01 and Gö6976 treatments reduced the overall length of DNA fibers in sham-irradiated samples (Fig. S1*C*). However, this inhibition of Chk1 kinase activity had no significant shortening effect after UV irradiation (Fig. 3 *A* and *B*). This result indicates that the kinase activity of Chk1 is not required for the replication of damaged DNA. To further test this hypothesis, we used a previously characterized kinase-dead (KD) mutant of Chk1 (25) in the context of residual endogenous Chk1. To this end, a silent mutation within the region homologous to the siChk1 sequence was generated, making this Chk1 mutant [Chk1_KD$_{r(resistant)}$] refractory to the Chk1 siRNA (Fig. 4*A*). As a control, we generated a wild-type construct, also refractory to Chk1 siRNA (Chk1_wt$_r$). Strikingly, Chk1_KD$_r$ was as efficient as Chk1_wt$_r$ to rescue the replication efficiency of UV-damaged DNA when endogenous Chk1 was depleted (Fig. 4 *B* and *C*). Supporting

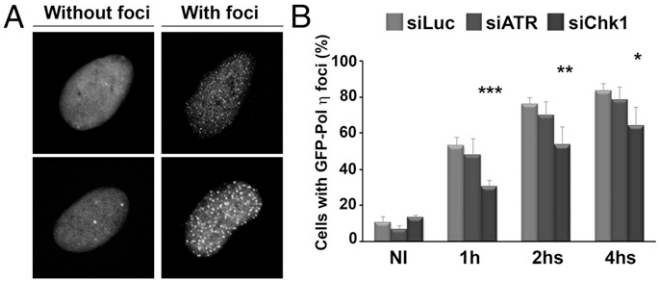

Fig. 2. Chk1 but not ATR is required for efficient focal organization of pol η after UV irradiation. U2OS cells transfected with GFP-pol η and the indicated siRNA were fixed at different time points after UV irradiation (30 J/m^2). (*A*) Representative images of cells with and without focal organization of pol η. (*B*) The subnuclear distribution of pol η was determined in three independent experiments. Two hundred cells per sample were counted. Representative images are shown [significance of the differences between siLuc and each condition ***$P < 0.001$; **$P < 0.01$, *$P < 0.05$, no asterisk, not significant (NS), $P > 0.05$].

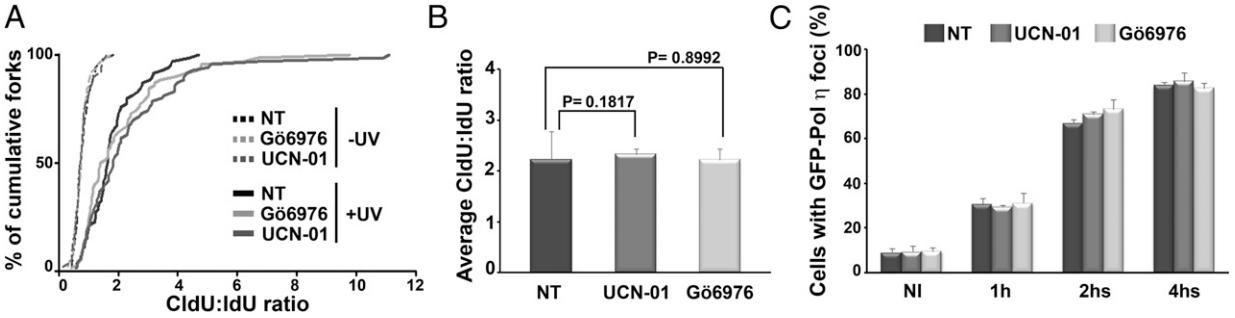

Fig. 3. Chk1 kinase activity is not required for fork progression and pol η focal organization after UV irradiation. (*A*) U2OS cells were treated with Gö6976 and UCN-01 from 90 min before irradiation until the end of the experiment. Cells were subjected to DNA fiber labeling and results expressed as cumulative percentage of forks at each ratio. (*B*) Average ratios for data in *A*. (*C*) U2OS cells treated as in *A* were UV-irradiated (30 J/m^2) and used to determine the percentage of cells with pol η foci. Differences between control and each treatment were not significant ($P > 0.05$).

this finding, we also observed that UCN-01 and Gö6976 had no effect on pol η recruitment (Fig. 3*C*). Furthermore, Chk1_wt$_r$ and Chk1_KD$_r$ equally complemented the defect in pol η foci formation observed when endogenous Chk1 is depleted (Fig. 4*D*). Thus, although Chk1 kinase activity is important for replication of undamaged DNA (Fig. S1*B* and ref. 9–12), this activity is not further required for the specific replication across damaged DNA templates after UV irradiation.

Effect of Chk1 in Replication Fork Progression and Specialized Pols Recruitment After UV Irradiation Is Independent of Claspin. Claspin is a mediator of the checkpoint response in S phase that promotes Chk1 stabilization and activation (26–28). Interestingly, Claspin stabilizes replication forks independently of Chk1 phosphorylation (29), and Claspin and Chk1 were shown to positively regulate PCNA ubiquitination (14). Given this previous evidence, we thought that the kinase-independent role of Chk1 in DNA replication after UV irradiation might depend on Claspin. We used previously described siRNA for Claspin (14). In agreement with previous reports (29, 30), Claspin down-regulation reduced the rate of fork elongation during unperturbed DNA replication (Fig. S1*D*). Also, as previously reported, the knockdown of Claspin reduced Chk1 phosphorylation and partially affected Chk1 levels (Fig. 5*A*). However, Claspin knockdown had no further specific effect on the progression of replication forks after UV damage (Fig. 5 *B* and *C*). Moreover, the recruitment of pol η to damaged sites was not altered by Claspin knockdown (Fig. 5*D*), even after two rounds of siRNA transfection (Fig. S4). Together, these data show that the kinase-independent role of Chk1 on the replication of damaged DNA and the recruitment of pol η does not depend on Claspin.

Timely Release of Chk1 from Chromatin Is Critical to Promote Replication Fork Progression After UV Irradiation. Because the Chk1 role on the replication of damaged DNA is independent of its kinase activity and of its partnership with Claspin, we thought that other Chk1 domains might be involved. A recent report identified a PCNA interacting domain (PIP box) in the carboxyl-terminal region of Chk1 (encompassing residues 374–381, known as Chk1 TRFF motif) (13). We generated a mutation in this domain of Chk1 and made that construct refractory to the siRNA for Chk1 (Chk1_TRAA$_r$; Fig. 6*A*). As controls, we used Chk1_wt$_r$ and Chk1_KD$_r$. In striking contrast to Chk1_wt$_r$ and Chk1_KD$_r$, Chk1_TRAA$_r$ was unable to rescue the defect in fork progression after UV irradiation when endogenous Chk1 was depleted (Fig. 6 *B* and *C*). Also, only Chk1_TRAA$_r$ failed to rescue the kinetics of pol η recruitment after UV irradiation (Fig. 6*D*). Moreover, Chk1_TRAA$_r$ also impaired both PCNA and pol η interaction in chromatin immunoprecipitation assays and the extent of recruitment of pol η to PCNA foci (Fig. S5). Together, these data demonstrate that the Chk1 role on the progression of replication forks and the recruitment of specialized pols requires its TRFF motif.

Because the Chk1 TRFF motif promotes Chk1 release from chromatin after UV irradiation (Fig. S6*A* and ref. 13), we tested whether abrogating Chk1 release after UV has an impact on the replication of damaged DNA. We used a previously characterized Flag-tagged H2B-Chk1 chimera that is unable to dissociate from chromatin (Fig. S6*B* and ref. 16). We mutagenized H2B-Chk1 and its Flag-tagged control, Chk1wt, to be refractory to the siRNA for Chk1 (Fig. 7*A*; FlagH2B-Chk1$_r$ and FlagChk1_wt$_r$). Strikingly, as observed for Chk1_TRAA$_r$, H2B-Chk1$_r$ was unable to rescue the defect in fork progression after UV irradiation when endogenous Chk1 was depleted (Fig. 7 *B* and *C*). Also, H2B-Chk1$_r$ failed to rescue the kinetics of pol η recruitment after UV irradiation (Fig. 7*D*). Together, our findings indicate that alterations in Chk1 levels or in its timely release from replication forks after UV irradiation impair replication forks progression on damaged DNA.

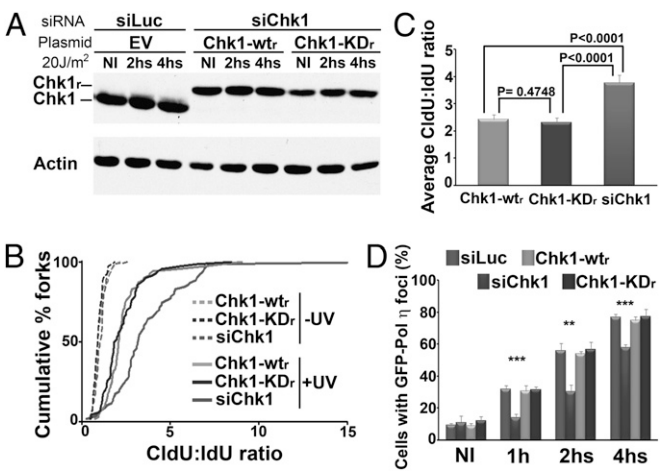

Fig. 4. Chk1_wt$_r$ and Chk1_KD$_r$ efficiently complement Chk1 down-regulation in replication assays and focal organization of pol η. (*A*) U2OS cells transfected with siRNA specific for Chk1 and empty vector (EV) or Chk1_wt$_r$ and Chk1_KD$_r$ were irradiated (20 J/m^2) and used for Western blot by using the indicated specific antibodies. (*B*) U2OS cells transfected with siRNA specific for Chk1 and the indicated Chk1 mutants were subjected to the DNA fiber labeling. Cumulative percentage of forks at each ratio is shown. (*C*) Average ratio of the data described in *B*. (*D*) U2OS cells transfected with GFP-pol η, the Chk1 siRNA, and the indicated Chk1 mutants were used to determine the percentage of cells with pol η foci. The significance of the differences between siLuc and each condition is shown (***$P < 0.001$; **$P < 0.01$, no asterisk = NS, $P > 0.05$).

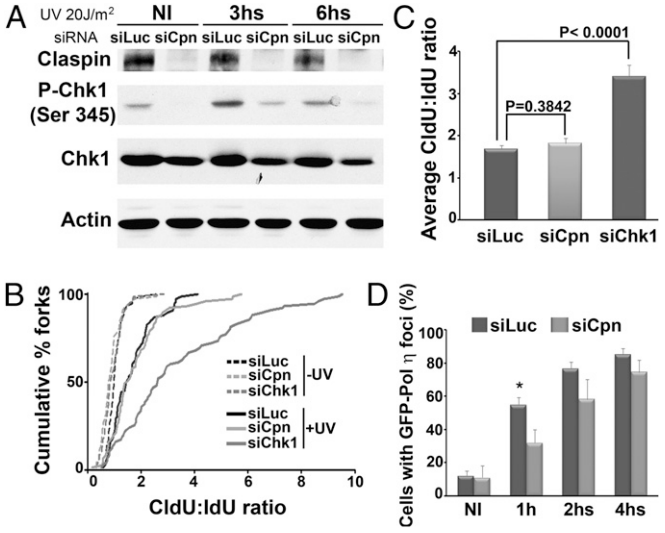

Fig. 5. Claspin is not required for fork progression and pol η focal organization after UV irradiation. (*A*) U2OS cells transfected with the indicated siRNAs were UV-irradiated (20 J/m^2) and used for Western blot analysis by using the indicated antibodies. (*B*) U2OS cells transfected as above were subjected to the DNA fiber labeling assay described in Fig. 1*B*. Cumulative percentage of forks at each ratio is shown. (*C*) Average ratios for the data described in *B*. (*D*) U2OS cells transfected with GFP-pol η, and the indicated siRNA were used to determine the subnuclear distribution of pol η. The significance of the differences between the siLuc and siCpn is shown (*$P < 0.05$).

Discussion

Contribution of Chk1 to the Replication of Damaged DNA. During unperturbed replication, the kinase activity of Chk1 promotes replication fork progression by modulating CDK-dependent origin firing (9–11). In addition, in this manuscript, we show that the replication across DNA lesions caused by UV light requires additional features of Chk1 that depend on its ability to be released from chromatin after UV irradiation.

We provide robust evidence ruling out a role of the kinase domain of Chk1 in fork progression across damage DNA. First, although unperturbed replication elongation is affected by Chk1 inhibitors, UCN-01, and Gö6976, there is no further effect of these inhibitors on the replication of damaged DNA after UV irradiation. Second, the data obtained with the different Chk1 mutants used in this study reveal that the kinase domain of Chk1 does not modulate any specific aspect of the replication across damaged DNA templates. In agreement, the Kannouche laboratory also found no effect of UCN-01 treatment on another replication parameter, fork density, when comparing unirradiated and UV treated conditions (31). Thus, although the kinase activity of Chk1 certainly contributes to the replication of the undamaged DNA, it is irrelevant for the replication across DNA lesions.

In strike opposition to the Chk1_KD$_r$ mutant, the Chk1_TRAA$_r$ and H2B-Chk1$_r$ mutants are incapable of rescuing depletion of endogenous Chk1 after DNA damage (Figs. 6 *B* and *C* and 7 *B* and *C*). This result is particularly interesting because these mutations do not alter the Chk1 kinase domain. In fact, the Chk1 TRAA mutation seems to increase Chk1 kinase activity (13). A number of findings should be taken into account when attempting to decipher the mechanism by which Chk1 aids the replication of damaged DNA templates. First, the retention of Chk1 in chromatin-associated PCNA complexes is not disrupted by the mutation of Chk1 PIP box (13), presumably because of indirect interactions between Chk1 and PCNA (e.g., through Claspin; refs. 14 and 32). Second, both the Chk1 TRAA mutation and the H2B-Chk1 fusion impair Chk1 release to the nucleoplasm after UV damage (13), suggesting that these Chk1 mutants might localize in the vicinity or at replication forks for longer time-frames than Chk1 WT. Third, both the Chk1 TRAA mutation and the H2B-Chk1 fusion fail to restore efficient pol η foci formation (this work), this observation being potentially relevant at the times used during the DNA fiber spreading experiments. These results suggest that the dynamics of Chk1 release and pol η recruitment must be coordinated to influence the ability of the cell to overcome the stalling of replication forks.

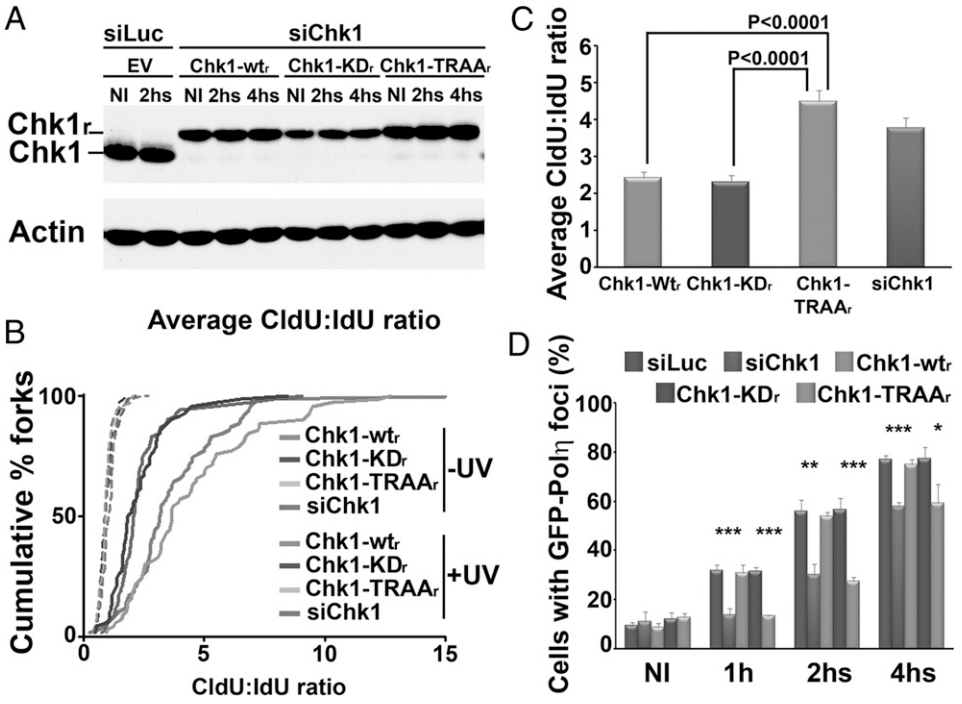

Fig. 6. A PIP domain in Chk1 is required for fork progression and pol η focal organization after UV irradiation. (*A*) U2OS cells transfected with siRNA specific for Chk1 or EV, Chk1_wt$_r$, Chk1_KD$_r$, and Chk1_TRAA$_r$ were irradiated and used for Western blot analysis by using the indicated specific antibodies. (*B*) U2OS cells transfected with siRNA specific for Chk1 and the indicated Chk1 mutants were subjected to the DNA fiber labeling assay described in Fig. 1*B*. Cumulative percentage of forks at each ratio is shown. (*C*) Average ratios for the data described in *B*. Other significance values are reported in Table S1. (*D*) U2OS cells transfected with GFP-pol η, Chk1 siRNA, and the indicated Chk1 mutants were used to determine the percentage of cells with pol η foci. The significance of the differences between siLuc and each condition is shown (***$P <$ 0.001; **$P < 0.01$; *$P < 0.05$, no asterisk = NS, $P > 0.05$).

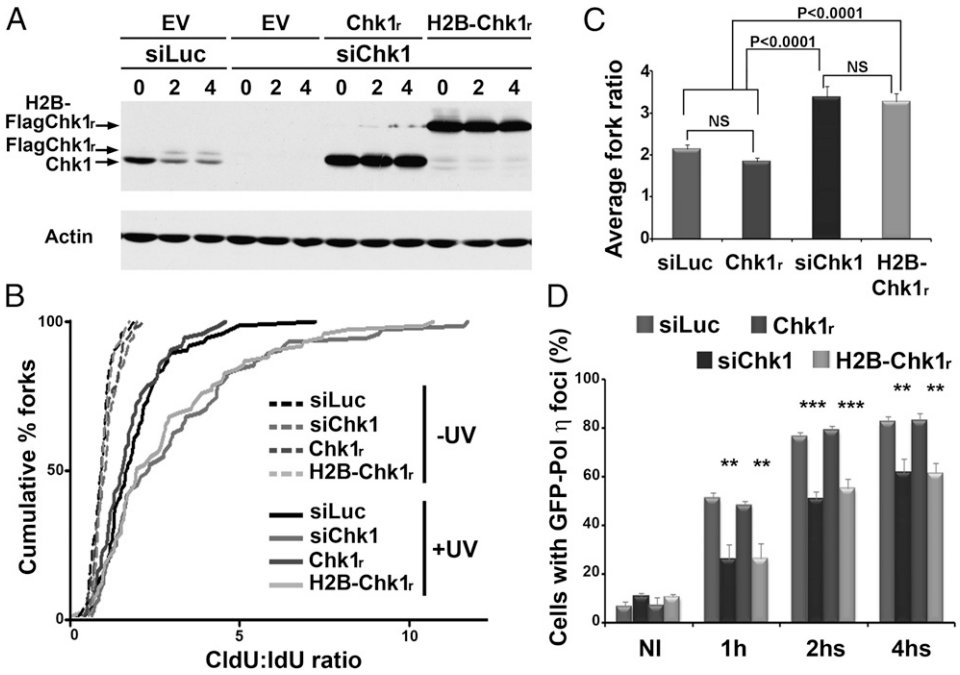

Fig. 7. Chromatin retention of Chk1 impairs fork progression and pol η focal organization after UV irradiation. (*A*) U2OS cells transfected with siRNA specific for Chk1 and EV, FlagChk1_wt$_r$, or FlagH2B-Chk1$_r$ were irradiated, and samples were used for Western blot analysis by using the indicated specific antibodies. (*B*) U2OS cells transfected with siRNA specific for Chk1 and the indicated Chk1 mutants were subjected to the DNA fiber labeling assay described in Fig. 1*B*. Cumulative percentage of forks at each ratio is shown. (*C*) Average ratios for the data described in *B*. NS, not significant, $P > 0.05$. (*D*) U2OS cells transfected with GFP-pol η, Chk1 siRNA, and the indicated Chk1 mutants were used to determine the subnuclear distribution of pol η. The significance of the differences between siLuc and each condition is shown (***$P < 0.001$; **$P < 0.01$; no asterisk = NS, $P > 0.05$).

Coordination Between Checkpoint and TLS Activation. The coordination of the checkpoint and TLS pathways was initially explored in the context of defective TLS. Pol η deficiency results in sustained Chk1 phosphorylation (33) and the accumulation of other checkpoint markers such as ssDNA and H2AX phosphorylation (31). Importantly as well, Chk1 is required for the survival of UV-irradiated cells deficient in pol η expression (31).

Although the above-mentioned data demonstrate that the checkpoint is up-regulated when TLS is defective, the contribution of checkpoint signals to the efficient activation of TLS is much less clear. This interplay between TLS and checkpoint regulating signals is particularly interesting because the activation of both the checkpoint and TLS pathways are spatially associated with DNA replication forks. While we should mention that we did not observed colocalization of Chk1 and pol η both before and after UV irradiation (Fig. S7), the defect in pol η focal organization observed when the Chk1 TRAA mutant is expressed suggests that the release of Chk1 from chromatin might be necessary for the correct kinetics of recruitment of pol η (that, in turn, might impact the progression of DNA replication after UV irradiation). This scenario resembles the case of another PIP box bearing protein, p21, which is degraded after UV, presumably to promote the loading of pol η (34).

A potential link between Chk1 and TLS was proposed when Yang et al. discovered a kinase-independent effect of Chk1 on the ubiquitination of PCNA (14), a modification of PCNA known to promote TLS (15). Interestingly, not only Chk1 but also Claspin potentiates PCNA ubiquitination after UV irradiation (100 J/m^2) (14). Under our experimental settings, although Chk1 affects polη/PCNA interaction and polη focal organization, neither Chk1 nor Claspin affect PCNA ubiquitination (Fig. S8). Although the effect of checkpoint proteins on different TLS markers might not totally overlap in all scenarios and UV doses used, it is clear that kinase-independent functions of Chk1 potentiate the up-regulation of TLS signals. Interestingly as well, Lehmann and colleagues identified an ATR phosphorylation site in pol η that is required for cell survival and modestly contributes to DNA synthesis behind DNA replication forks (21). Thus, both ATR and Chk1 regulate different TLS-related events. Also, a link between PCNA ubiquitination and checkpoint proteins was documented when using other DNA damaging agents. Vaziri and colleagues reported the requirement of RPA, ATR, and Chk1 for PCNA ubiquitination after treatment of cells with the bulky adduct-forming genotoxin benzo(*a*)pyrene dihydrodiol epoxide (20). More intriguingly, not only are ATR and Chk1 required for efficient activation of TLS-associated events, but also the TLS pol η promote efficient Chk1 activation. In fact, different motifs of pol η are required for the efficient phosphorylation of Chk1 at ser 317, a site required for Chk1 activation (21). Taken together, multiple levels of cross-regulation interconnect checkpoint and TLS pathways. In particular, the discoveries presented herein unveil an unexpected level of complexity that involves a nonkinase motif of Chk1, which contributes to the coordination of fork progression and TLS.

Materials and Methods

Cell Culture, Transfection, and UV Irradiation. U2OS cells (ATCC) were grown in DMEM (Invitrogen) with 10% (vol/vol) FCS. Transfections were performed by using Lipofectamine 2000 (Invitrogen) and Jet Prime (VWR). UV irradiation was performed 48 h after transfection. Chk1 inhibitors used were UCN-01 (150 nM; Sigma) and Gö6976 (1 μM; Calbiochem). siRNA duplexes (Thermo-Fisher Scientific) were as follows: siChk1 GAAGCAGUCGCAGUGAAGA, siATR CCUCCGUGAUGUUGCUUGA, siCpn GGAAAGAAAGGCAGCCAGA, and siLuc CGUACGCGGAAUACUUCGA.

The Chk1_wt and Chk1_KD (pcDNA3-myc-Chk1 and pcDNA3-myc-Chk1 D130A, respectively) were gifts from H. Piwnica-Worms (Washington University School of Medicine, St Louis, MO) (25). The FlagH2B-Chk1 and control FlagChk1 were kindly provided by S. Jackson (University of Cambridge, UK) (16). Site-directed mutagenesis used in this study is detailed in *SI Materials and Methods*. GFP-Pol η and GFP-Pol i were gifts from A. Lehmann (University of Sussex, Brighton, UK) and Roger Woodgate (NICDH, NIH, Rockville, MD) respectively

(19). UVC irradiation was delivered with a CL-1000 UV cross-linker equipped with 254-nm tubes (UVP).

Preparation and Immunolabeling of DNA Fibers. DNA fibers were analyzed by using a protocol adapted from De Haro et al. (35). Briefly, exponentially growing cells were pulse-labeled with CldU (20 μM) for 20 min. Cells were washed twice and irradiated with 20 J/m^2 UVC, and IdU (200 μM) was added for additional 20 min. Cells were trypsinized and lysed with 6 μL of 0.5% SDS, 200 mM Tris·HCl (pH 7.4), and 50 mM EDTA buffer onto clean glass slides, that were tilted, allowing DNA to unwind. Samples were fixed in 3:1 Methanol/Acetic acid and denatured with HCl (2.5 M) for 1 h, blocked in PBS 5% (wt/vol) BSA for 15 min, and incubated with the mouse anti-BrdU (Becton Dickinson) to detect IdU, donkey anti-mouse Cy3-conjugated secondary antibody (Jackson Immuno Research), rat anti-BrdU (Accurate Chemicals) to detect CldU, and donkey anti-rat Alexa Fluor 488 secondary antibody (Invitrogen). Slides were mounted with Mowiol 488 (Calbiochem), and DNA fibers were visualized by using a Zeiss Axioplan confocal microscope. Images were analyzed by using Zeiss LSM Image Browser software. Each dataset is derived from measurement of at least 75 fibers.

Immunostaining and Microscopy. Samples were analyzed as described (22). For image documentation, cells were fixed in 2% (wt/vol) paraformaldehyde/sucrose for 20 min followed by 15-min incubation with 0.1% Triton X-100 in PBS. GFP-Pol η was detected by GFP autofluorescense, and DAPI was used to visualize nuclei. Images were obtained with a Zeiss Axioplan confocal microscope. For quantifying the percentage of cells with GFP-Pol η, cells were incubated in ice-cold methanol for 20 min followed by a 30-s pulse of ice-cold acetone. This method allows detection of only well-assembled GFP-Pol η foci.

Protein Analysis. Samples were lysed in Laemmli buffer, and Western blots were performed by using the following antibodies: anti-Chk1 (Santa Cruz Biotechnology; G-4), anti-ATR (Santa Cruz Biotechnology, *N*-19)/ anti-Claspin (Bethyl Laboratories), anti-phospho-(S345)-Chk1 (Cell Signaling); and anti-actin (Sigma-Aldrich). Incubation with secondary antibodies (Sigma) and ECL detection (Amersham GE Healthcare) were performed according the manufacturers' instructions.

Statistical Analysis. Frequency distribution of DNA fiber ratios was analyzed with GraphPad Prism 5 software. Mann–Whitney test was used for statistical analysis. Satistical analysis of Pol η and pol ι foci formation was performed in GraphPad inStat software by using the Student's *t* test. Other calculations and graphics were performed by using Microsoft Excel 2007.

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Supporting Information

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SI Materials and Methods

Generation of Chk1$_r$ Plasmids. Resistance of Chk1 plasmids to the siRNA Chk1 used in this study was achieved by using the following primers: Forward 5′ GTGAATAGAGTAACTGAAGAAGCAGTTGCTGTCAAGATTGTAGATATGAAGCGTG 3′ and Reverse 5′ CACGCTTCATATCTACAATCTTGACAGCAACTGCTTCTTCAGTTACTCTATTCAC 3′. This mutagenesis resulted in silent alterations of three codons. To generate the Chk1_TRAA$_r$, the siChk1 refractory Chk1_wt$_r$ was used as a template. Phenylalanine 380 and 381 were mutated to alanine with forward primer 5′ GGTTGGTCAAAAGAATGACACGAGCCGCTACCAAATTGGATGCAGAC 3′ and reverse primer 5′ GTCTGCATCCAATTTGGTAGCGGCTCGTGTCATTCTTTTGACCAACC 3′.

Chromatin Immunoprecipitation of PCNA. Chromatin immunoprecipitations were performed as described (2). U2OS cells were plated in 60-cm culture dishes, transfected as previously indicated, and irradiated at 40 J/m2. Cells were rinsed twice with PBS and then extracted with 5 mL of CSK buffer (250 mM sucrose, 25 mM KCl, 10 mM Hepes at pH 8.0, 1 mM EGTA, and 1 mM $MgCl_2$) for 12 min. The CSK-extracted cells were fixed with 1% formaldehyde in PBS (4.5 mL) for 10 min. Then, 0.5 mL of 1 M glycine was added for 5 min to quench the cross-linking reaction. The cross-linked nuclei were rinsed with PBS and then lysed in 500 μL of IP lysis buffer (10 mM Tris·HCl at pH 7.5, 25 mM FNa, 20 mM NaCl, 1% Nonidet P-40, 1% Na-Deoxicholate, and 0.1% SDS) freshly supplemented with protease and phosphatase inhibitors. Lysates were scraped from the plates and transferred into 1.5-mL Microfuge tubes. Samples were sonicated and clarified by centrifugation at 10,000 × g for 10 min. PCNA was immunoprecipitated overnight at 4 °C with 5 μL of monoclonal PCNA antibody (PC-10 AC; Santa Cruz Biotechnology). Samples were washed, boiled to revert the cross-link, and resolved in SDS/PAGE for Western blot analysis.

Isolation of the Chromatin Bound Fraction. A protocol published by Petermann et al. was used to isolate the chromatin bound fraction (3). Briefly, U2OS cells were irradiated with UVC (40 J/m^2). After 30 min, the cytoplasmic protein fraction was removed by incubation in hypotonic buffer (10 mM Hepes at pH 7, 50 mM NaCl, 0.3 M sucrose, 0.5% Triton X-100, and protease inhibitor mixture) for 5 min on ice. Nuclear soluble fraction was removed by incubation with nuclear buffer (10 mM Hepes at pH 7, 200 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitor mixture) for 10 min on ice. The remaining insoluble fraction was resuspended in sample loading buffer and was used for Western Blot with mouse monoclonal anti-Chk1 (G-4; Santa Cruz Biotechnology), rabbit polyclonal anti-Rad18 (A301-340 A; Bethyl Laboratories), and goat polyclonal anti-H2B (C-19; Santa Cruz Biotechnology). Using this protocol we found that Grb2 is totally concentrated in the S1 fraction and that H2B is exclusively retained in the chromatin bound fraction. Endogenous Chk1 was released as described (4, 5).

Chk1 Immunodetection. Coverslips were blocked overnight in PBS/2% donkey serum (Sigma) and incubated 1 h with primary antibody anti-Chk1 DCS-310 (Abcam). Secondary anti-mouse Cy3-conjugated antibody was from Jackson ImmunoResearch.

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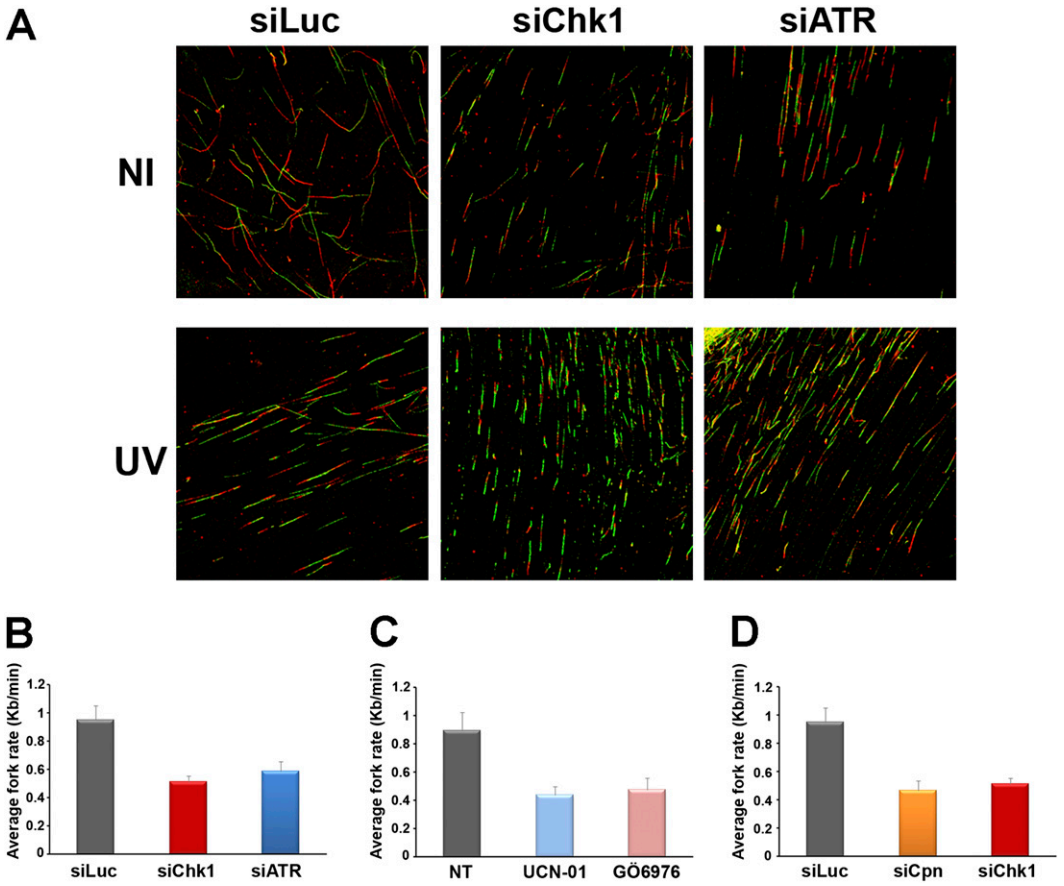

Fig. S1. Chk1 but not ATR is required for the progression of replication forks when the DNA is damaged. (*A*) U2OS cells were labeled with CldU and IdU by following the protocol described in Fig. 1*B*. Representative fields of labeled DNA fibers from U2OS cells transfected with control siRNA, siATR, and siChk1 are shown. (*B*) ATR and Chk1 knockdown impair fork progression rate during unperturbed replication.U2OS cells were transfected with the indicated siRNA and subjected to the DNA fiber labeling protocol described in Fig. 1. Average fork rates of unirradiated samples of the experiments described in Fig. 1*C* were calculated as reported (1). (*C*) Chk1 kinase activity is required for fork progression during unperturbed replication. U2OS cells were treated with the indicated Chk1 inhibitors and subjected to DNA fiber labeling. Average fork rates were calculated as in *A*. (*D*) Claspin knockdown impairs fork progression rate during unperturbed replication U2OS cells were transfected with the indicated siRNA and subjected to the DNA fiber labeling. Average fork rates were calculated as in *A*.

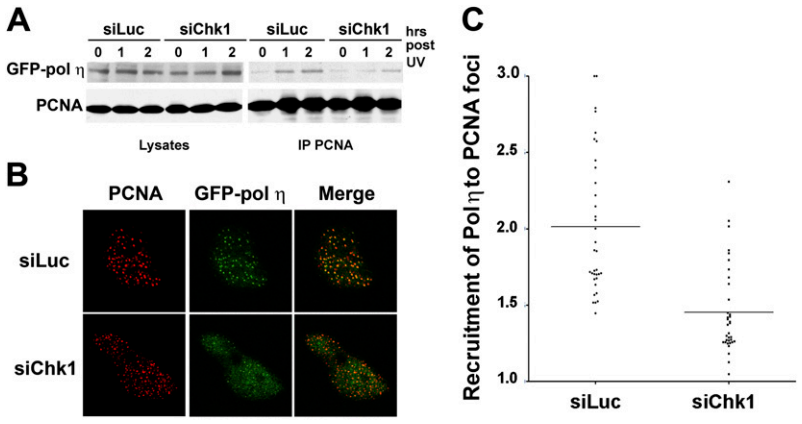

Fig. S2. Chk1 impairs pol η/PCNA interaction and the degree of pol η localization to PCNA foci after UV. (*A*) U2OS cells were transfected with the indicated siRNA and GFP-pol η. After UV irradiation (40 J/m^2), samples were subjected to chromatin inmunoprecipitation by using a monoclonal PCNA antibody as described in *SI Materials and Methods*. (*B*) U2OS cells were transfected with the indicated siRNA and GFP-pol η as in *A* and subjected to UV irradiation (30 J/m^2). Two hours later, cells were fixed with ice cold methanol for 20 min and acetone for 30 s and subjected to inmunostainning with anti-PCNA antibody. Thirty-five confocal images of cells with focal PCNA distribution were documented for each sample. Representative images are shown. (*C*) Images were analyzed by using the Matlab software. Two independent experiments were analyzed. The extent of pol η recruitment to PCNA foci was expressed as the increase in average pol η intensity at PCNA foci with respect to the remaining average pol η nuclear signal.

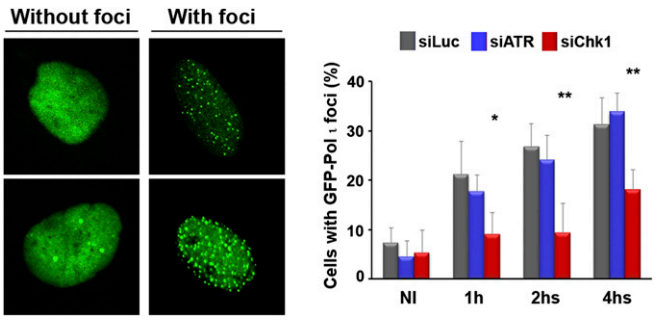

Fig. S3. Chk1 is required for efficient focal organization of pol ι after UV irradiation. (*A*) U2OS cells were transfected with GFP-pol ι and the indicated siRNA for ATR and Chk1. Cells were fixed at different time points after UV irradiation (30 J/m^2) and the subnuclear distribution of pol ι was determined in three independent experiments. Representative images are shown. The significance of the differences between siLuc and each condition is shown (**$P < 0.01$, *$P < 0.05$).

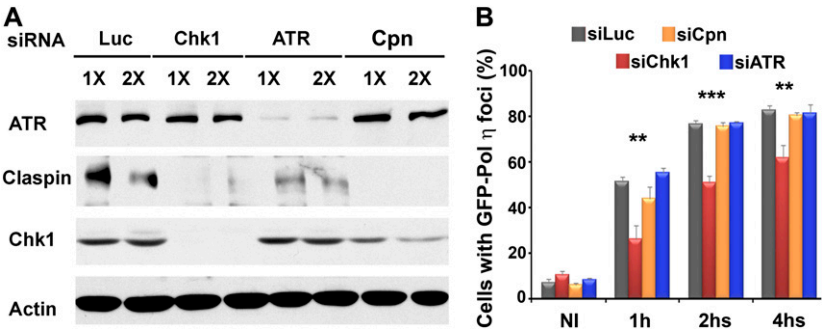

Fig. S4. Chk1 but not ATR or Claspin is required for efficient focal organization of pol η after UV irradiation. (*A*) U2OS cells were transfected with the siRNAs for Chk1, ATR, or Claspin. Twenty-four hours later, samples were transfected with GFP-pol η and the indicated siRNA when indicated (2×). Western blots were performed to document the extent of protein knockdown. (*B*) Samples were transfected twice with the indicated siRNA as reported in *A*. Twenty-four hours after the second transfection, cells were fixed at different time points after UV irradiation (30 J/m^2) and the subnuclear distribution of pol η was determined. The significance of the differences between siLuc and each condition is shown (**$P < 0.01$; ***$P < 0.001$).

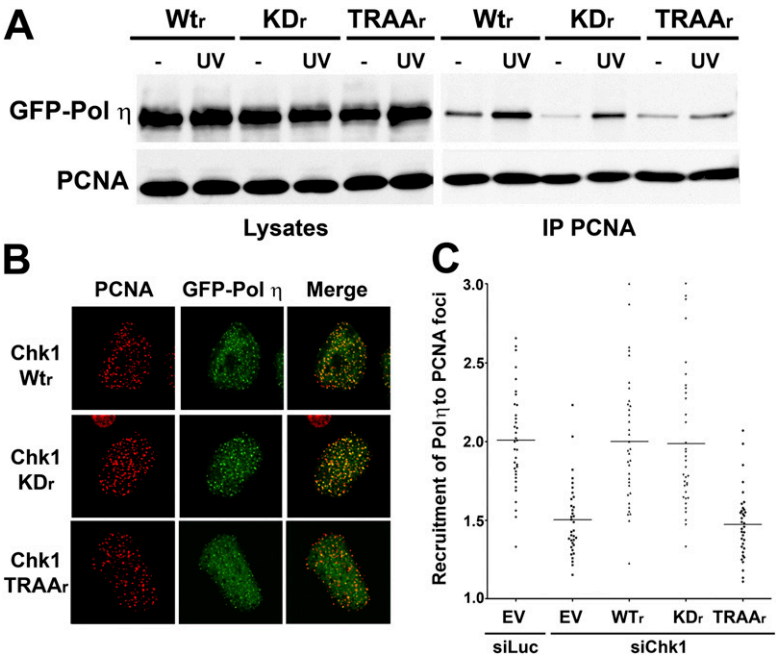

Fig. S5. Chk1_TRAA$_r$ fails to restore polη/PCNA interaction and efficient pol η localization to PCNA foci after UV irradiation. (*A*) U2OS cells were transfected with the indicated siRNA, Chk1 plasmids, and GFP-pol η. After UV irradiation (40 J/m^2), samples were subjected to chromatin inmunoprecipitation as in Fig. S3*A*. (*B*) U2OS cells were transfected with the indicated siRNA, Chk1 plasmids, and GFP-pol η. Cells were fixed, stained with PCNA antibody, and subjected to confocal documentation as described in Fig. S3*B*. (*C*) Images were analyzed by using the Matlab software as described in Fig. S3*C*. Thirty-five nuclei per sample were documented in each experiment. Two independent experiments were analyzed.

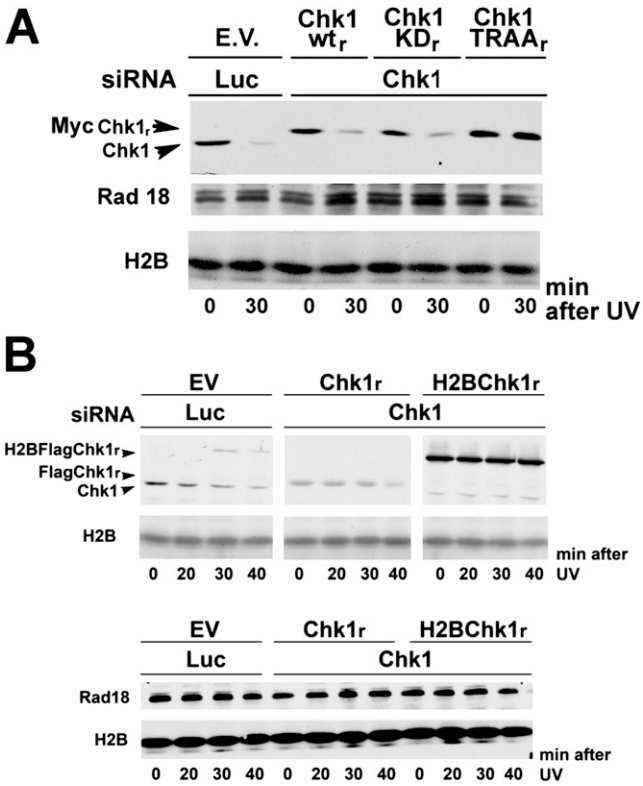

Fig. S6. Chk1-TRAA$_r$ and H2B-Chk$_r$1 are retained in the chromatin fraction after UV irradiation. (*A* and *B*) U2OS cells were transfected with the indicated siRNA and Chk1 plasmids. Samples were UV-irradiated at the indicated times before lysis. Samples were subjected to a three-step extraction procedure as described in *SI Materials and Methods*. The levels of Chk1, Rad 18, and H2B in the chromatin bound fraction were analyzed by Western blot. In *B*, different exposure times were selected for the Chk1 blot to avoid saturation of the CHk1 bands.

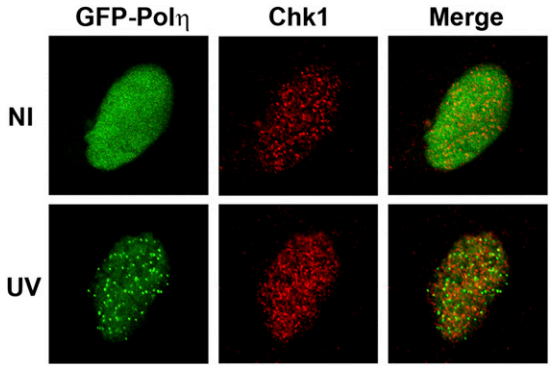

Fig. S7. Chk1 and pol η do not colocalize. U2OS cells were transfected with GFP-pol η. Forty-eight hours later, samples were UV-irradiated (UV) (30 J/m^2) or not irradiated (NI). Cells were fixed at different time points after UV irradiation, and the subnuclear distribution of pol η and Chk1 was determined by confocal microscopy. A representative image showing Chk1 and pol η localization is shown.

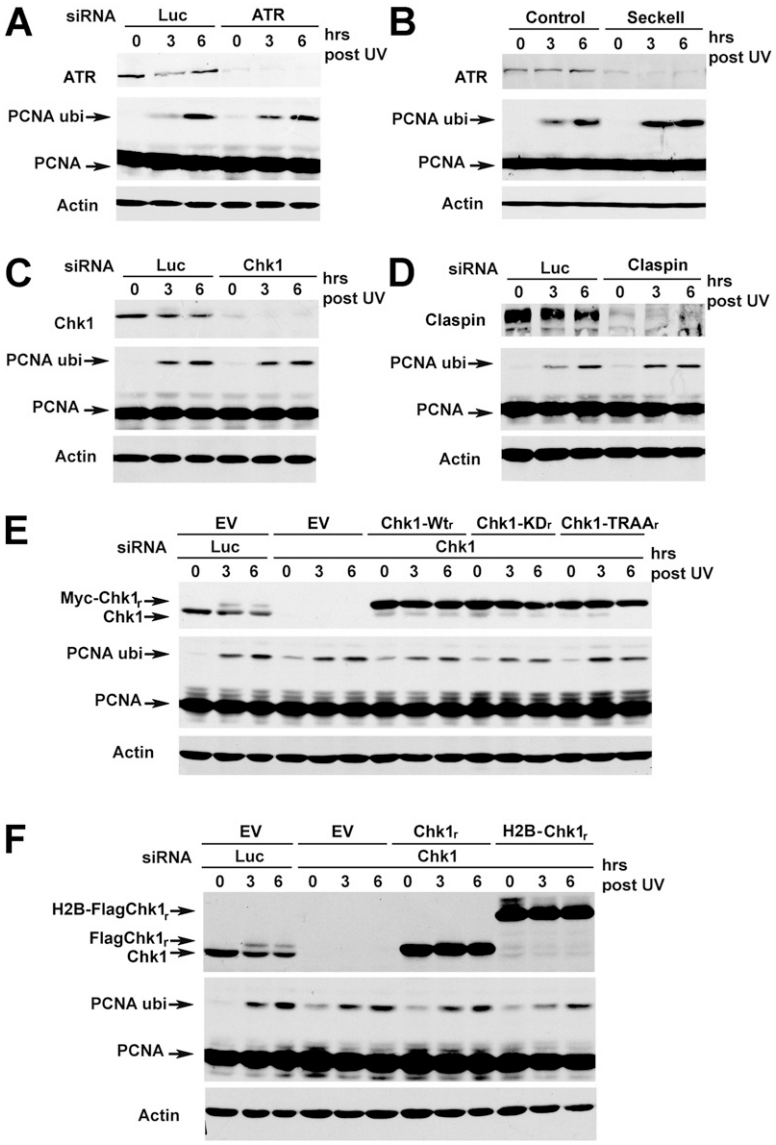

Fig. S8. The levels of PCNA ubiquitination induced by UV irradiation (40 J/m^2) in U2OS cells is not modulated by ATR, Chk1, Claspin, or any mutant used in this study. (*A*, *C*, and *D*) U2OS cells were transfected with the indicated siRNA for ATR, Claspin, and Chk1. Cells were irradiated, lysed at the indicated times, and subjected to Western blot analysis to determine the extent of PCNA ubiquitination. (*B*) Seckell and control cells were UV-irradiated and lysed at the indicated times and subjected to Western blot analysis to determine the extent of PCNA ubiquitination. (*E* and *F*) U2OS cells were transfected with the siRNA for Chk1 and the indicated Chk1 plasmids. Cells were irradiated, lysed at the indicated times, and subjected to Western blot analysis to determine the extent of PCNA ubiquitination.

Table S1. Significance of the differences between all samples used in Fig. 6*C*

Comparison	Significance
Chk1_wt$_r$ vs. siChk1	$P < 0.0001$
Chk1_wt$_r$ vs. Chk1_TRAA$_r$	$P < 0.0001$
Chk1_KD$_r$ vs. siChk1	$P < 0.0001$
Chk1_KD$_r$ vs. Chk1_TRAA$_r$	$P < 0.0001$
Chk1_wt$_r$ vs. Chk1_KD$_r$	$P = 0.4748$
Chk1_TRAA$_r$ vs. siChk1	$P = 0.1196$

Mann–Whitney test was applied to obtain *P* values.