Chk1 inhibits replication factory activation but allows dormant origin firing in existing factories

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Replication origins are licensed by loading MCM2-7 hexamers before entry into S phase. However, only ~10% of licensed origins are normally used in S phase, with the others remaining dormant. When fork progression is inhibited, dormant origins initiate nearby to ensure that all of the DNA is eventually replicated. In apparent contrast, replicative stress activates ataxia telangiectasia and rad-3–related (ATR) and Chk1 checkpoint kinases that inhibit origin firing. In this study, we show that at low levels of replication stress, ATR/Chk1 predominantly suppresses origin initiation by inhibiting the activation of new replication factories, thereby reducing the number of active factories. At the same time, inhibition of replication fork progression allows dormant origins to initiate within existing replication factories. The inhibition of new factory activation by ATR/Chk1 therefore redirects replication toward active factories where forks are inhibited and away from regions that have yet to start replication. This minimizes the deleterious consequences of fork stalling and prevents similar problems from arising in unreplicated regions of the genome.

Introduction

During S phase of the cell division cycle, the genome must be precisely duplicated, with no regions left unreplicated and no regions replicated more than once. Despite DNA replication being a target of many anti-cancer drugs, it is currently unclear how the program that regulates progression through S phase responds to replicative stresses. Before entering S phase, replication origins are licensed by binding of MCM2-7 hexamers, which provide helicase activity during S phase to unwind DNA ahead of replication forks (Blow and Dutta, 2005; Arias and Walter, 2007). However, many more origins are licensed than are actually used in a normal S phase (Woodward et al., 2006). Only ~10% of licensed origins normally initiate replication in an unperturbed S phase while the rest remain dormant. When replication fork progression is inhibited, dormant MCM2-7 are activated to initiate additional forks to ensure that all of the DNA in the region is eventually replicated (Santocanale et al., 1999; Dijkwel et al., 2002; Anglana et al., 2003; Woodward et al., 2006; Ge et al., 2007; Gilbert, 2007; Ibarra et al., 2008; Doksani et al., 2009; Tuduri et al., 2010).

Existing data are consistent with the idea that activation of dormant origins in response to fork inhibition is a simple consequence of origin activation being a stochastic event (Ge et al., 2007; Blow and Ge, 2009). Dormant origins within an active replicon cluster are usually passively replicated, and thus inactivated, by forks initiated from a neighboring origin. When fork progression is blocked, this inactivation of dormant origins is delayed, thereby increasing the likelihood that a nearby dormant origin initiates. For this process to work effectively, there does not need to be any qualitative difference between the origins that fire and origins that remain dormant in any given cell (Blow and Ge, 2009), and in fact, all available evidence is consistent with the idea that a random subset of origins is selected every S phase.

The inhibition of replication forks also activates DNA damage checkpoint kinases (ataxia telangiectasia mutated [ATM], ataxia telangiectasia and rad-3–related [ATR] kinase, Chk1, and Chk2), which not only stabilize the forks, delay further progression through the cell cycle, and promote lesion repair (Bartek et al., 2004; Branzei and Foiani, 2005; Lambert and Carr, 2005), but also inhibit late origin firing (Santocanale and

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Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and rad-3 related; EdU, ethynyl deoxyuridine; HU, hydroxyurea.

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Diffley, 1998; Shirahige et al., 1998; Dimitrova and Gilbert, 2000; Zachos et al., 2003; Bartek et al., 2004). It is apparently paradoxical that replication stresses can simultaneously activate dormant origins but suppress overall origin initiation.

Adjacent replication origins are clustered and replicated together within replication factories, each of which contains one or more clusters of origins (Jackson and Pombo, 1998; Berezney et al., 2000; Sadoni et al., 2004; Kitamura et al., 2006; Gillespie and Blow, 2010). Different sets of replication factories are activated at different times during S phase, constituting the S phase temporal program of a eukaryotic genome. It has recently been shown in *Xenopus laevis* egg extract that the activation of replication factories can be regulated separately from origin activation within factories by Cdk (Gillespie and Blow, 2010; Thomson et al., 2010). This provides a potential mechanism that may allow dormant origin firing while suppressing overall levels of origin initiation.

In this study, we show that in response to low levels of replication fork inhibition induced by hydroxyurea (HU) or aphidicolin, DNA damage checkpoint kinases (ATR and Chk1) preferentially inhibit the activation of new replication factories while allowing dormant origins to fire within the existing factories experiencing replicative stress. This redirects origin firing away from completely unreplicated regions of the genome and toward active factories, thereby minimizing the deleterious consequences of replication fork stalling.

Results

Replicative stress reduces origin initiation and replication factory number

We first examined how the replication inhibitor HU affected initiation rates in human U2OS tissue culture cells. We measured fork speed 2 h after HU treatment by pulsing cells with BrdU and measuring the lengths of BrdU-labeled tracks by DNA fiber analysis. This showed that HU concentrations from 50 to 500 µM caused a progressive decrease in fork speed down to ∼15% of untreated levels (Fig. 1 a, open squares). In parallel, the overall rate of DNA replication was determined by measuring the incorporation into DNA of the dNTP analogue ethynyl deoxyuridine (EdU). 2 h after HU treatment, there was a reduction in cellular DNA replication rate that was similar to the reduction of fork speed (Fig. 1 a, closed squares). Because the rate of cellular replication is determined by both the fork speed and total number of forks within the cell, we can estimate the relative number of active forks per cell by dividing the cellular replication rate by the fork speed (Fig. 1 b). This showed that 2 h after treatment of cells with 50–500 µM HU, the total number of forks remained fairly constant, rising to a slight peak of 1.7 (relative to untreated cells) at 400 µM. Similar results were obtained when replication rates were measured by BrdU incorporation (Fig. S1, a and b) and when replication forks were inhibited with aphidicolin rather than HU (Fig. 1, c and d).

Forks terminate when they encounter one another, and so lowering fork speed will also reduce the rate at which forks terminate. If the overall origin initiation program during S phase were to remain unaffected by HU or aphidicolin, the slowing of fork speed would lead to an increase in the total number of forks in each cell as the result of a decrease in the termination rate. In other words, 200–400 µM HU, which reduced the fork speed four- to sixfold, should increase the total fork number by four- to sixfold if the rate of origin initiation within the cell was to remain unaffected. Therefore, the observation that the total fork number only increased ∼1.7-fold in the presence of 200–400 µM HU (Fig. 1 b) indicates a strong suppression of overall origin initiation in cells treated with HU. This decrease of total cellular fork number is consistent with previous studies showing that the intra–S checkpoint inhibits initiation at later-firing origins (Santocanale and Diffley, 1998; Shirahige et al., 1998; Dimitrova and Gilbert, 2000; Zachos et al., 2003; Bartek et al., 2004).

Previous studies have shown that HU and aphidicolin also activate additional dormant origins that do not fire efficiently in untreated cells (Ockey and Saffhill, 1976; Taylor, 1977; Anglana et al., 2003; Woodward et al., 2006; Ge et al., 2007; Gilbert, 2007). Typically, an approximate doubling of fork density within active clusters is seen after slowing the fork speed to 25% with 200 µM HU (Ge et al., 2007). Therefore, the inhibition of total cellular initiation rate occurs at the same time as there is an increase in the initiation of dormant origins.

One possible explanation for this behavior is that at low levels of replication inhibition, the activation of new replicon clusters is preferentially inhibited; this would allow dormant origins to initiate within active clusters while also reducing the overall cellular initiation rate (Ge et al., 2007; Gilbert, 2007). The 4–20 replication forks typically active in each replicon cluster colocalize in individual replication factories (Jackson and Pombo, 1998; Berezney et al., 2000; Sadoni et al., 2004; Kitamura et al., 2006; Gillespie and Blow, 2010). To visualize replication factories, we transfected cells with GFP-PCNA and selected for analysis cells expressing only low levels of GFP-PCNA that progressed normally through S phase (unpublished data). Fig. 1 (e and f) shows that 2 h after treating cells with 200 µM HU, the number of replication factories per cell decreased by 30–40%. Similar results were obtained when replication foci were labeled with Cy3-dUTP (Fig. 1 e) or with antibodies against endogenous PCNA (Fig. S1 c). Although there is some variation in total foci number observed by these three different methods, they each gave a similar degree of reduction in factory number after HU treatment. A similar reduction in factory number was also observed when cells were treated with aphidicolin (Fig. S1 c). In addition to U2OS cells, HU reduced factory number in other cell lines including HFF, MRC-5, and IMR90, although the effect was not statistically significant in some transformed cell lines such as Saos2 (Fig. S1 d and not depicted). The weaker suppression of factory number in Saos2 cells might be because of deficient DNA damage checkpoint responses in highly transformed cells. A recent study using high resolution microscopy also reported an increase in factory number after HU treatment of MRC-5 cells (Cseresnyes et al., 2009).

Dormant origin firing occurs within active replication factories

Because 200 µM HU caused a slight increase in total cellular fork number (Fig. 1 b) but a decrease in factory number (Fig. 1 e),

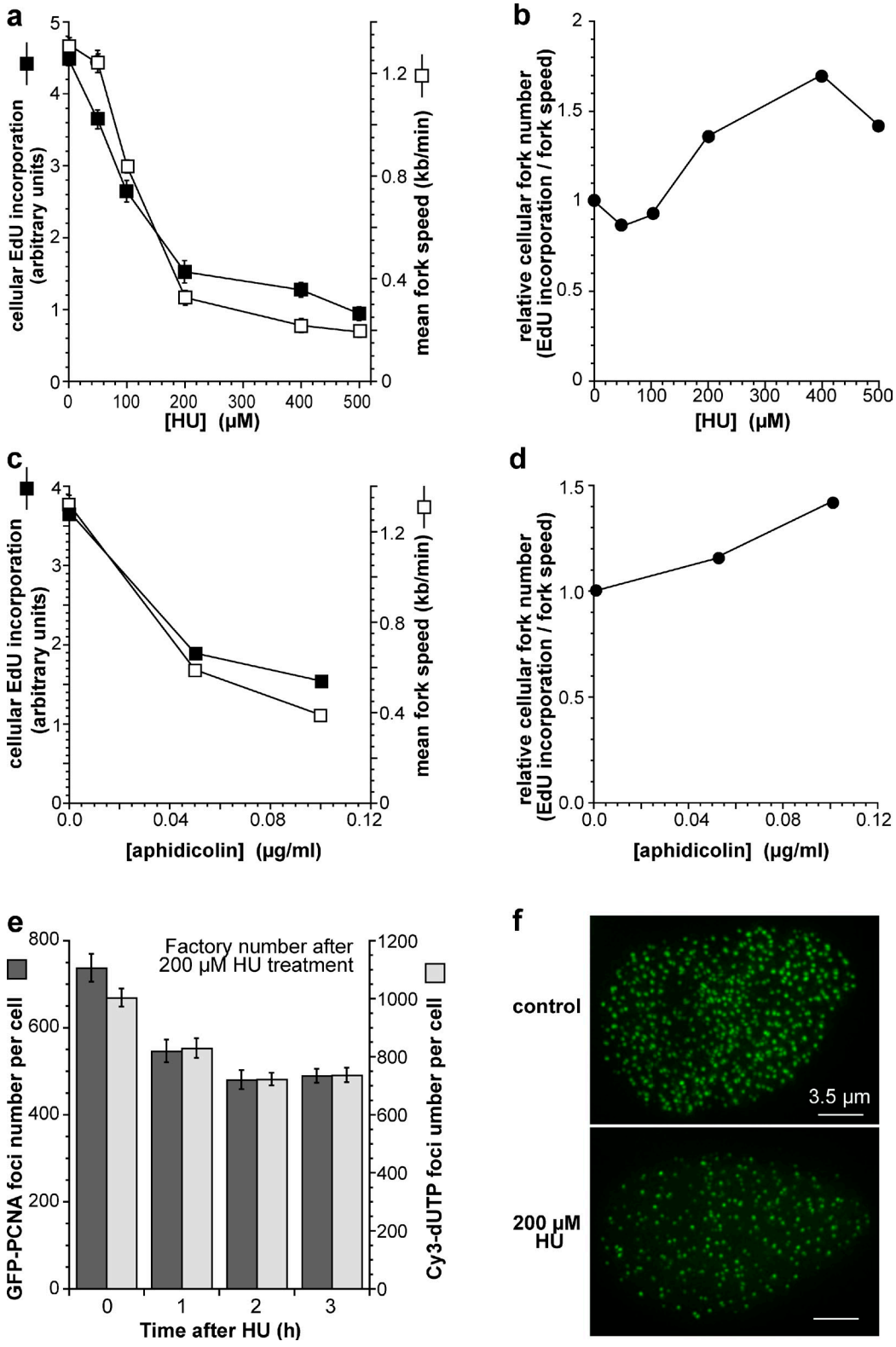

Figure 1. **Overall origin initiation and active replication factories is reduced by HU or aphidicolin.** (a–d) U2OS cells were treated with 0–500 μM HU or 0–0.1 μg/ml aphidicolin for 4 h before pulsing with 10 μM EdU for 30 min. (a and c, closed squares) Cellular EdU incorporation was detected by flow cytometry with mean and SEM calculated from three independent experiments. (a and c, open squares) DNA fiber analysis was performed on parallel samples to determine mean fork speed. The ratio of cellular EdU incorporation to fork speed (b and d) indicates the relative number of forks per cell. (e and f) U2OS cells were synchronized in early S phase by nocodazole shake off followed by incubation with thymidine for 12 h. Cells were released from thymidine for 1 h and treated with HU for 2 h. (e) The number of active replication factories was determined by either transfecting cells with GFP-PCNA 24 h before synchronization or pulsing cells with Cy3-dUTP and fixing after 30 min. Mean cellular foci number and SEM were derived from >50 cells. (f) Representative images of factories labeled with GFP-PCNA.

this suggests that it caused an increase in the number of replication forks in each active factory. To confirm this, we measured the rate of DNA replication within individual replication factories by pulsing cells with Cy3-dUTP after HU treatment (Fig. 2, a and b). The mean Cy3 intensity of foci is plotted in Fig. 2 c (closed squares) and shows that although HU reduced the Cy3-dUTP incorporation into individual replication foci, this reduction was significantly less than the inhibition of fork speed (Fig. 2 c, open squares). The relative number of forks within each factory can be derived by dividing the rate of replication within each factory by the fork speed. This indicates that 200–300 µM HU more than doubled the number of forks within each replication factory (Fig. 2 d). At higher HU concentrations, the number of forks in active factories started to decline. This is consistent with previous DNA fiber experiments showing that 200 µM HU induced dormant origin firing in clusters by about twofold, but at higher HU concentrations, the activation of dormant origins declined (Ge et al., 2007). The decrease in dormant origin initiation at higher concentrations of HU is most likely the result of high levels of the DNA damage checkpoint inhibiting replication initiation. Similar results were obtained when replication foci were pulse labeled with BrdU (Fig. S2, a and b). Treating cells with 0.1 µg/ml aphidicolin (which inhibited fork speed to a similar extent as 200–300 µM HU) also activated more than twice the number of replication forks within each replication factory (Fig. 2, e and f).

As an alternative way of measuring the rate of DNA replication within individual replication factories, we investigated the expulsion of replicated DNA from factories. Cells were transfected with GFP-PCNA to mark active factories (Leonhardt et al., 2000; Sporbert et al., 2002) and pulsed with Cy3-dUTP to label replicon clusters replicated by each factory (Fig. 3 a). 30 min after the Cy3-dUTP pulse, there was significant colocalization of Cy3-dUTP and GFP-PCNA foci. The degree of colocalization between Cy3 and GFP gradually decreased at later times, indicating that DNA replication within the factories was completed (Sporbert et al., 2002; Sadoni et al., 2004). In the presence of 200 µM HU, the rate of separation between Cy3-dUTP and GFP-PCNA foci was 58% of that in controls, whereas the fork speed was ∼25% of controls (Fig. 3, b and c). This is consistent with our previous conclusion that 200 µM HU approximately doubled the number of forks per factory. It should be noted that the length of time taken for separation of the Cy3-dUTP and GFP-PCNA foci is consistent with the idea that each factory replicates the DNA from more than one replicon cluster (Hiratani et al., 2008; Gillespie and Blow, 2010).

To further validate our results, we reduced the availability of dormant origins by titrating in MCM5 siRNA to the maximum level where cells still displayed normal S phase progression, including a normal density of replication forks within origin clusters and normal numbers and intensities of replication foci (Fig. 3 d and not depicted). The percentage of colocalization between PCNA and Cy3-dUTP foci was measured 30 and 120 min after a Cy3-dUTP pulse (Fig. 3 e). At 120 min in the absence of HU, the degree of colocalization was similar between control and MCM5 knockdown cells. However, when cells were treated with 200 µM HU, the MCM5 knockdown cells showed prolonged colocalization between PCNA and Cy-dUTP foci. This is consistent with our previous observation that cells with a partial knockdown of MCM5 initiated significantly fewer dormant origins than control cells after treatment with 200 µM HU (Ge et al., 2007).

DNA damage checkpoints suppress new replication factories

HU and aphidicolin activate DNA damage checkpoint kinases (ATR and Chk1), which have been shown in previous studies to inhibit the initiation of replication origins and inhibit progression through the S phase replication timing program (Santocanale and Diffley, 1998; Shirahige et al., 1998; Dimitrova and Gilbert, 2000; Zachos et al., 2003; Bartek et al., 2004). This behavior would still be consistent with the activation of dormant origins if ATR and Chk1 preferentially inhibited initiation by reducing the number of active replication factories. In agreement with this, the decrease of replication factory number observed in synchronized cells treated with HU or aphidicolin was largely prevented by Chk1 siRNA or caffeine (an ATR/ATM inhibitor; Fig. 4, a and b; and Fig. S1, c and d). In contrast, previous DNA fiber analysis showed that at 200 µM HU, checkpoint kinases reduced origin firing in active clusters by only 11–19% (Ge et al., 2007). We measured the rates of DNA synthesis within factories when the checkpoint had been abolished by Chk1 siRNA or caffeine, both by measuring extrusion of replicated DNA from factories (Fig. 4, c and d) or by measuring Cy3-dUTP foci intensity (Fig. S3). Despite a modest activation of dormant origins when the checkpoint was inhibited, the rates of DNA synthesis within factories actually decreased. This decrease is likely the result of fork destabilization that occurs in the absence of ATR and Chk1 (Dimitrova and Gilbert, 2000; Lopes et al., 2001; Tercero and Diffley, 2001; Zachos et al., 2003). Consistent with this interpretation, replication tracks were observed to shorten when the checkpoint was inhibited (Fig. S3). Collectively, our data support the idea that low levels of checkpoint kinase activity induced by 200 µM HU preferentially reduces the number of replication factories rather than inhibiting the firing of origins within existing factories. This allows the activation of dormant origins as a consequence of stochastic origin firing within existing replication factories where forks are inhibited (Blow and Ge, 2009).

In principle, there are three ways that checkpoints could decrease factory number. The most obvious one is that the checkpoint directly inhibits activation of new factories. Another possibility is that the checkpoint promotes disassembly or abandonment of active factories before they have completed replication. A third possibility is that the checkpoint accelerates the replication of DNA within factories, so that they finish replication faster than normal. This last possibility can be dismissed because HU does not increase the overall rate of replication within factories (Figs. 2 and 3).

Therefore, we devised an assay to determine whether checkpoint kinases reduced factory number by inhibiting new factory activation or by promoting abandonment of partially replicated factories (Fig. 5). GFP-PCNA–expressing cells were synchronized in early S phase and pulsed with Cy3-dUTP to

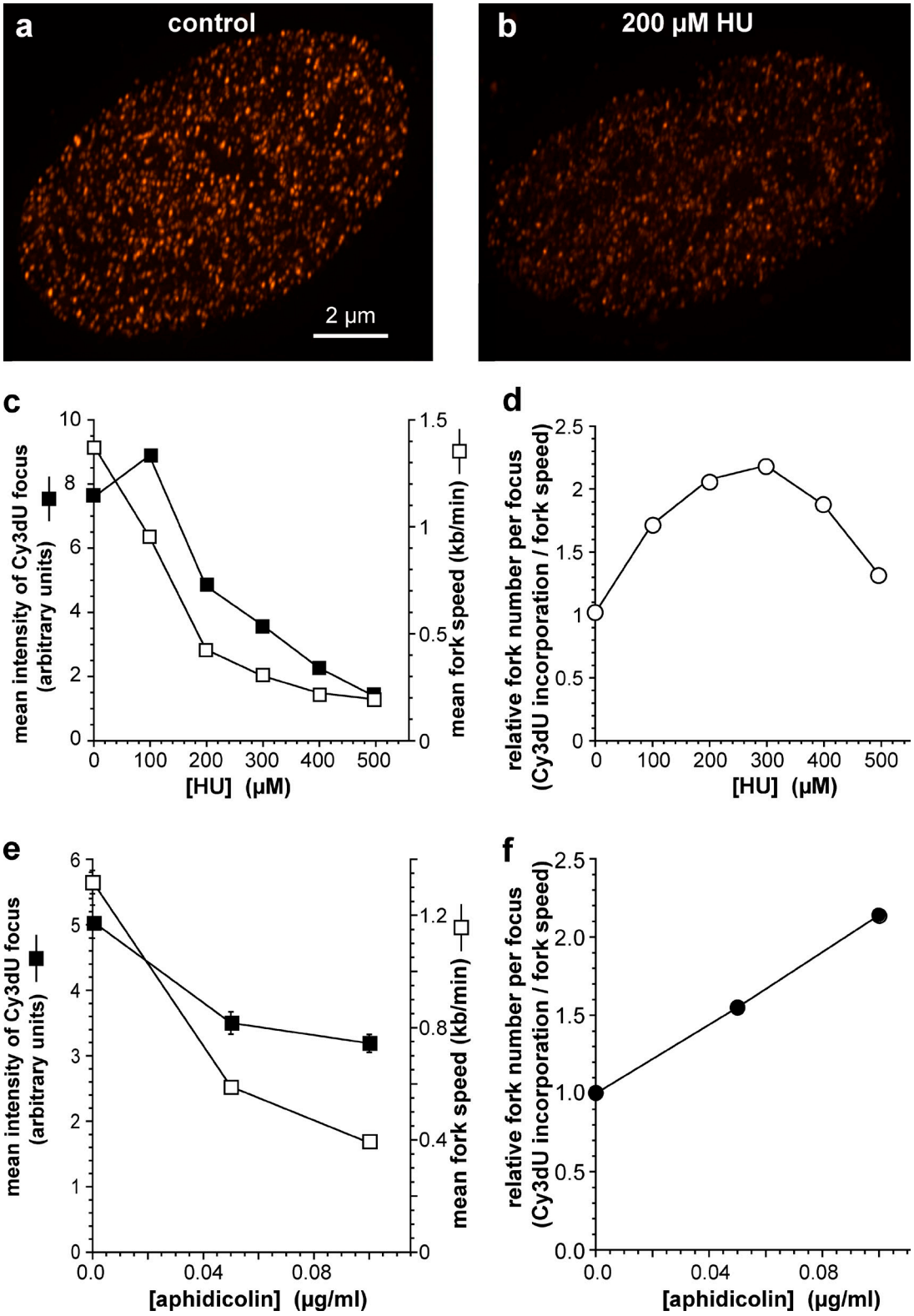

Figure 2. **Dormant origins activated within existing replication factories in response to replicative stress.** U2OS cells were synchronized in early S phase and treated with HU for 2 h as in Fig. 1. Cells were pulsed with Cy3-dUTP for 30 min before fixing. (a and b) Representative images of control (a) and 200 µM HU-treated cells (b). (c–f) The intensity of individual Cy3-dUTP foci was measured and averaged in each cell. (c and e, closed squares) Analysis of 50 cells under each condition gave rise to the overall mean foci intensity with SEM (error bars). (c and e, open squares) The replication fork speed of the samples was measured by DNA fiber analysis. The ratio of mean Cy3-dUTP focus intensity to fork speed (d and f) indicates the relative number of forks within each replication focus.

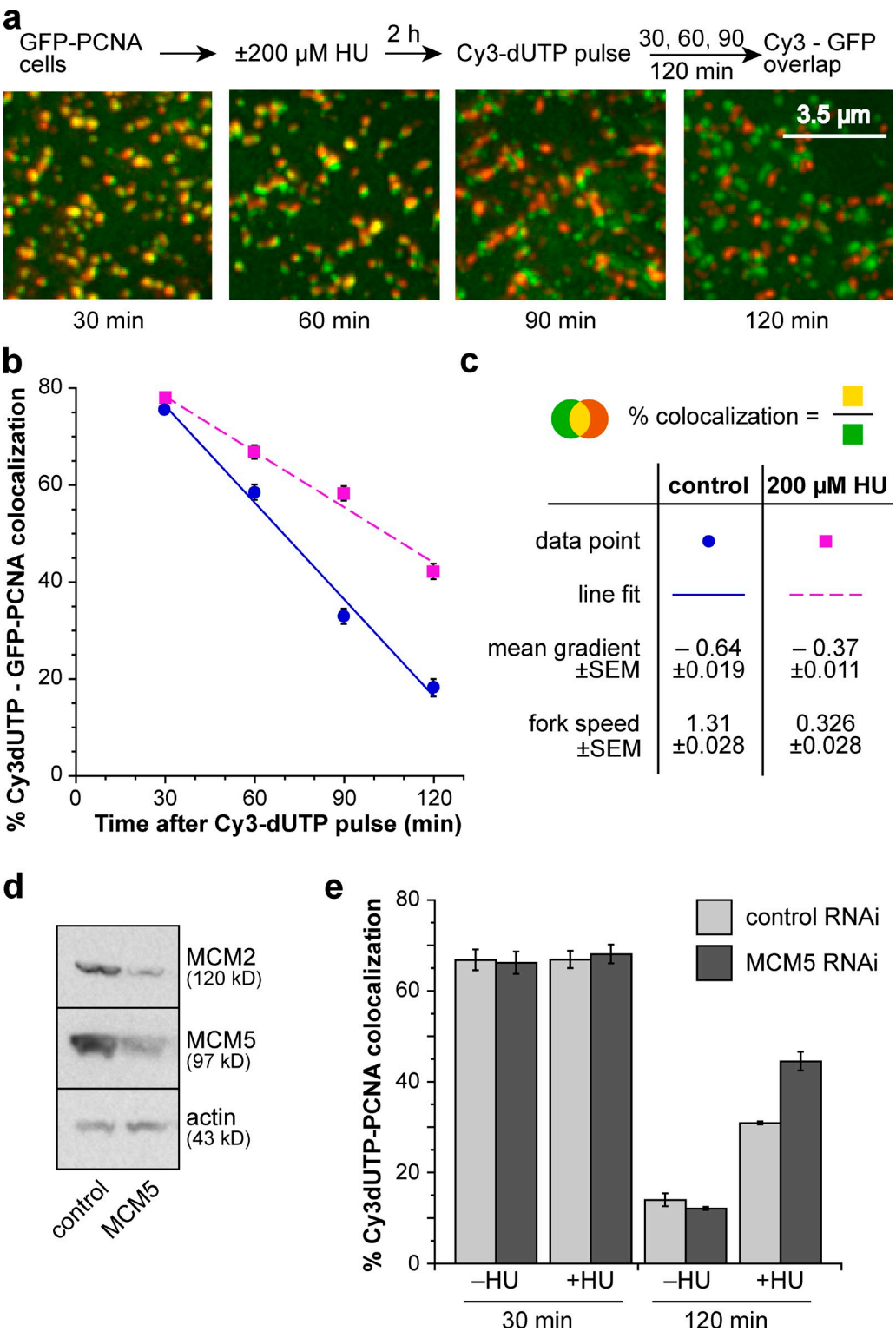

Figure 3. **HU activates dormant origins within active replication factories.** U2OS cells were synchronized in early S phase as described in Fig. 1. (a–c) Cells were transfected with GFP-PCNA 24 h before synchronization in early S phase. After HU treatment, cells were transfected with Cy3-dUTP and fixed for 30, 60, 90, or 120 min to analyze the percentage of colocalization between Cy3-dUTP (red) and GFP-PCNA foci (green). (a) Labeling scheme and representative images are shown. The percentage of colocalization within individual cells was calculated by dividing the colocalized volume of Cy3 and GFP-PCNA foci by the total volume of GFP-PCNA foci. Analysis of >40 cells gave rise to the mean percentage of colocalization at each time point and SEM. (b) Lines were fitted to the data points to calculate the gradient, which indicates the rate of replication within the factories. (c) Composite data from three independent experiments were combined to give a mean gradient and SEM between the three experiments. (d and e) U2OS cells were transfected with MCM5 siRNA 72 h before synchronization in early S phase. (d) Chromatin-bound MCM2 and MCM5 levels were determined by immunoblotting after MCM5 siRNA. After 200 μM HU treatment, cells were transfected with Cy3-dUTP and fixed for 30 or 120 min afterward. (e) The percentage of colocalization between Cy3-dUTP and PCNA foci (as revealed by anti-PCNA immunofluorescence) is shown.

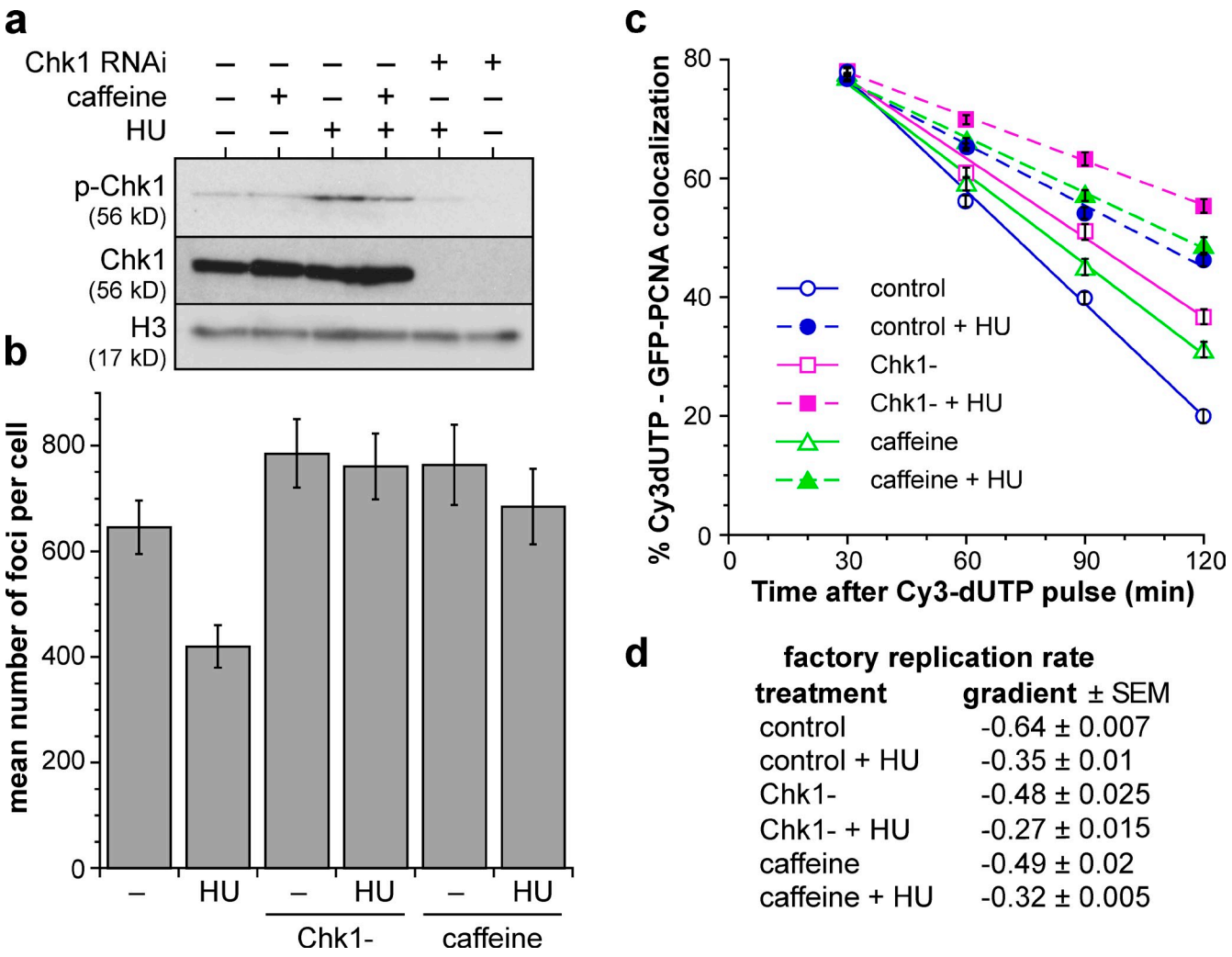

Figure 4. **The effect of checkpoint inhibition on replication factories.** Cells were transfected with GFP-PCNA 24 h before synchronization in early S phase, and the DNA damage checkpoint in these cells was inhibited by either CHK1 siRNA (transfected simultaneously with GFP-PCNA) or 5 mM caffeine. (a) Total cell lysate was immunoblotted for phospho-Chk1, Chk1, and H3. (b) U2OS cells were synchronized in early S phase as described for Fig. 1. After release from thymidine for 1 h, cells were treated with 200 μM HU for 2 h in the presence or absence of caffeine. The number of GFP-PCNA foci in each cell (>50 cells analyzed for each condition) was determined, with error bars representing SEM. (c and d) The rate of DNA replication within factories was measured by the protocol described in Fig. 3, but this time, comparing cells where the checkpoint was inhibited by Chk1 siRNA or caffeine. (c) Data from one set of experiments are shown. Analysis of >50 cells gave rise to the mean percentage of colocalization at each time point and SEM. Lines were fitted to the data points to calculate the gradient, which indicates the rate of replication within the factories. (d) Two independent experiments (n = 2) were performed to give rise to the mean gradient under each condition, and statistical significance is shown as SEM between the two gradients.

label active replication foci. Cells were treated with 200 μM HU for 2 h before analyzing the colocalization between Cy3-dUTP and GFP-PCNA foci. For each cell, we measured the number of foci containing colocalized PCNA and Cy3-dUTP (factories replicating during the Cy3-dUTP pulse that were still active 2 h later), the number of Cy3 foci not associated with PCNA (factories replicating at the time of the Cy3-dUTP pulse but no longer active 2 h later), and the number of PCNA foci not associated with Cy3-dUTP (factories activated only after the Cy3-dUTP pulse). Because the early (type II) S phase replication pattern in U2OS cells lasts >4 h, our experiments should measure replication dynamics within the same early stage of the replication timing program. This was confirmed by the PCNA-only foci still making up an early (type II) replication pattern 2 h after the Cy3-dUTP pulse (Fig. 5 a and not depicted). In untreated cells, around half of the existing factories finished replication in 2 h (therefore becoming Cy3-only foci) with a corresponding increase in the number of newly arisen PCNA-only foci. Treatment with HU reduced the number of Cy3-only foci to ∼60% of control values, which is consistent with the 50–60% reduction in factory replication rates shown in Figs. 3 b and 4 b. Contrary to what would be expected if the checkpoint promoted abandonment of partially replicated factories, treatment with caffeine plus HU further reduced the number of Cy3-only foci to ∼30%. This reflects the even more decreased rate of DNA synthesis within factories in the absence of checkpoint kinases and is likely because of the requirement of checkpoint in stabilizing replication forks (Fig. 4 b and Fig. S3). Two observations strongly suggest that most of the Cy3-only foci had completed replication and were not partially replicated foci whose forks had been disassembled. First, the number of Cy3-only foci in Fig. 5 b matches the rates of factory replication shown in Fig. 4 b,

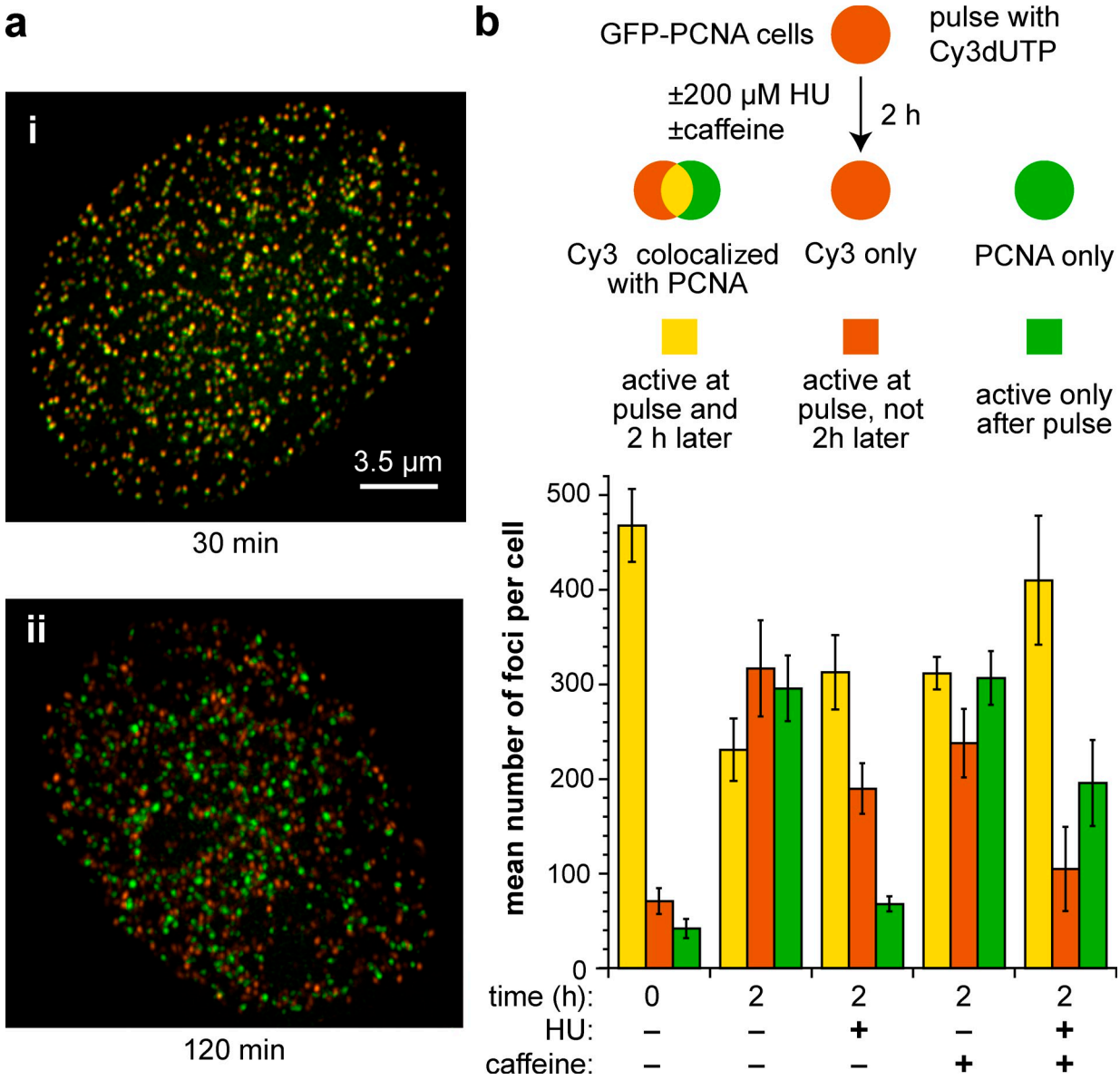

Figure 5. **Checkpoint kinases inhibit new replication factory activation.** Cells were transfected with GFP-PCNA 24 h before synchronization into early S phase as described for Fig. 1. After release from thymidine for 1 h, cells were pulse labeled with Cy3-dUTP followed by treatment with 200 μM HU for 2 h in the presence or absence of caffeine. (a) Representative images of nuclei fixed at 30 (i) or 120 min (ii) after Cy3-dUTP pulse. This shows that cells were in the same early timing stage throughout the experiment. (b) The number of Cy3 foci colocalized with GFP-PCNA foci (>10% volume overlap), Cy3 foci not colocalized with GFP-PCNA foci (<10% volume overlap; Cy3-only foci), and the GFP-PCNA foci not colocalized with cy3 foci (<10% volume overlap; PCNA-only foci) were quantified, and their mean value per cell was represented as yellow, red, and green bars, respectively. Analysis of >50 cells gave rise to mean and SEM (error bars).

as would be expected if the Cy3-only foci had all completed replication. Second, the reduction in Cy3-only foci caused by HU was matched by a corresponding increase in the number of colocalized Cy3 and PCNA foci (Fig. 5 b, yellow bars). These observations suggest that no partially replicated foci had been abandoned. In addition, HU caused a fourfold reduction in the number of PCNA-only foci (newly activated foci; Fig. 5 b, green bars), suggesting that HU inhibited new factory activation. This fourfold inhibition of new factory activation is broadly in agreement with our previous measurements, which showed that although the replication rate in factories was reduced to ∼50%, leading to a doubling of the lifetime of existing factories, the total number of replication factories still dropped by ∼30–40%. When caffeine was added to the cells at the same time as HU, the number of newly activated PCNA-only foci was restored to ∼65% of control levels (Fig. 5 b). Therefore, our data show that checkpoint kinases activated by low levels of replication stress inhibit new factory activation.

Previous work has shown that the regulation of S phase progression can be regulated at three distinct levels: origin activation within individual replication factories, activation of replication factories during individual stage of the replication timing program, and progression from one stage of the replication timing program to another (Krasinska et al., 2008; Gillespie and Blow, 2010;

Thomson et al., 2010). Activation of the intra–S phase checkpoint blocks progression through the replication timing program (Fig. S4; Dimitrova and Gilbert, 2000). However, the checkpoint-dependent inhibition of new replication factory activation shown in Fig. 5 b is not simply a consequence of blocking progression through the timing program because the new factories whose activation is inhibited by the checkpoint are part of the same early type II stage of the replication timing program (Fig. 5 a and not depicted). Therefore, the inhibition of new factory activation is a direct consequence of checkpoint activation.

The inhibition of new replication factories is dependent on ATR-Chk1

HU and aphidicolin, the inhibitors we used in this study, primarily activate ATR and Chk1 checkpoint kinases. Another DNA damage checkpoint pathway involves ATM and Chk2, which are primarily activated by double-strand DNA breaks, e.g., caused by ionizing radiation. Therefore, we examined the replication dynamics after irradiating the cells with γ rays. Fig. 6 (a and b) shows that in contrast to HU and aphidicolin, early S phase cells exposed to ionizing radiation did not reduce the number of replication factories but, instead, reduced the rate of replication within individual factories, possibly the result of an ATM/Chk2-induced lowering of the overall initiation rate.

We next investigated the possible mechanism by which ATR and Chk1 could preferentially inhibit new factory activation in response to replication stress. ATR and Chk1 inhibit the CDC25 phosphatases required for Cdk activation, and recent work using *Xenopus* egg extracts has demonstrated that Cdks play a direct role in promoting the activation of replication factories (Krasinska et al., 2008; Gillespie and Blow, 2010; Thomson et al., 2010). Consistent with this, Fig. 6 c shows that inhibition of Cdk activity by roscovitine reduced the number of active replication factories in U2OS cells. However, inhibition of factory activation in response to HU is unlikely to be mediated by S phase Cdk inhibition in U2OS cells because S phase cells treated with HU or aphidicolin did not significantly reduce total cellular Cdk activity (Fig. 6, d and e). In addition, S phase cells irradiated with UV light did not significantly reduce Cdk activity. These results suggest that ATR/Chk1 reduces factory activation rates by acting on other S phase regulators in addition to Cdks.

Discussion

Despite DNA replication being a major target of chemotherapeutic drugs, remarkably little is known about how the DNA replication program responds to replication inhibition. Our work demonstrates a new way that cells respond to replication stress, which allows cells to activate dormant origins while simultaneously preventing initiation spreading into later-replicating regions of the genome.

To prevent rereplication of DNA, the ability to license new origins of replication by loading MCM2-7 must cease before cells enter S phase. Origin licensing is not activated again until passage into anaphase of the next mitosis (Blow and Dutta, 2005; Arias and Walter, 2007). This means that even if replication forks stall or are abandoned during S phase, cells cannot rectify the problem by licensing new origins but, instead, can only use the origins licensed before entry into S phase. To overcome this limitation, an excess of origins are licensed, from which a small subset is selected for initiation during S phase, with the rest (80–95%) normally remaining dormant (Santocanale et al., 1999; Dijkwel et al., 2002; Anglana et al., 2003; Woodward et al., 2006; Ge et al., 2007; Gilbert, 2007; Ibarra et al., 2008; Doksani et al., 2009; Tuduri et al., 2010). These dormant origins can be activated when replication forks are inhibited, probably by a simple stochastic mechanism, and help cells survive these replicative stresses (Woodward et al., 2006; Ge et al., 2007; Ibarra et al., 2008; Blow and Ge, 2009; Kunnev et al., 2010). Cells lacking sufficient dormant origins are hypersensitive to replicative stress (Woodward et al., 2006; Ge et al., 2007; Kunnev et al., 2010), and mice with a reduced MCM level are cancer prone, probably because of a lack of dormant origins (Pruitt et al., 2007; Shima et al., 2007; Kunnev et al., 2010). However, little is known about how progression through the replication timing program and the use of dormant origins are regulated in response to replicative stresses. One of the major responses to replication fork inhibition is activation of the DNA damage checkpoint kinases ATM, ATR, Chk1, and Chk2 (Bartek et al., 2004; Branzei and Foiani, 2005; Lambert and Carr, 2005). Among other activities, these kinases are known to inhibit initiation of late-firing origins and prevent progression to later stages of S phase (Santocanale and Diffley, 1998; Shirahige et al., 1998; Dimitrova and Gilbert, 2000; Zachos et al., 2003; Bartek et al., 2004). These checkpoint kinases must therefore inhibit late-firing origins while at the same time allowing activation of dormant origins.

We show that ATR and Chk1, when activated in response to fork inhibition, reduce the number of active replication factories. We show that this occurs by a reduction in the rate at which replication factories are activated. A role for Chk1 in negatively regulating factory activation is also supported by the observation that Chk1 inhibition leads to an increase in factory number in the absence of replication inhibition (Maya-Mendoza et al., 2007; this study). We also show that when replication forks are inhibited, most dormant origins are activated in active replication factories. This is consistent with the increased density of replication forks that is seen in replicon clusters by DNA fiber analysis (Ge et al., 2007). Activation of dormant origins within active clusters/factories is likely to occur as a direct consequence of stochastic origin firing (Blow and Ge, 2009). As well as inhibiting factory activation, ATR and Chk1 can also decrease the rate of origin firing in existing factories (Ge et al., 2007), but at low levels of replicative stress, this effect appears to be less strong than the inhibition of factory activation: at 200 μM HU, factory activation rate is reduced by ∼80%, whereas dormant origin activation within active clusters is reduced by <20%. This preferential inhibition of factory activation by ATR/Chk1 allows the firing of dormant origins in active factories in response to low levels of replicative stress. This response is summarized in Fig. 7 a: two factories are shown, the top of which fires before the bottom (Fig. 7 a, left), but in response to replicative stress (Fig. 7 a, right), checkpoints inhibit activation of the second factory while allowing initiation of dormant origins in the active factory.

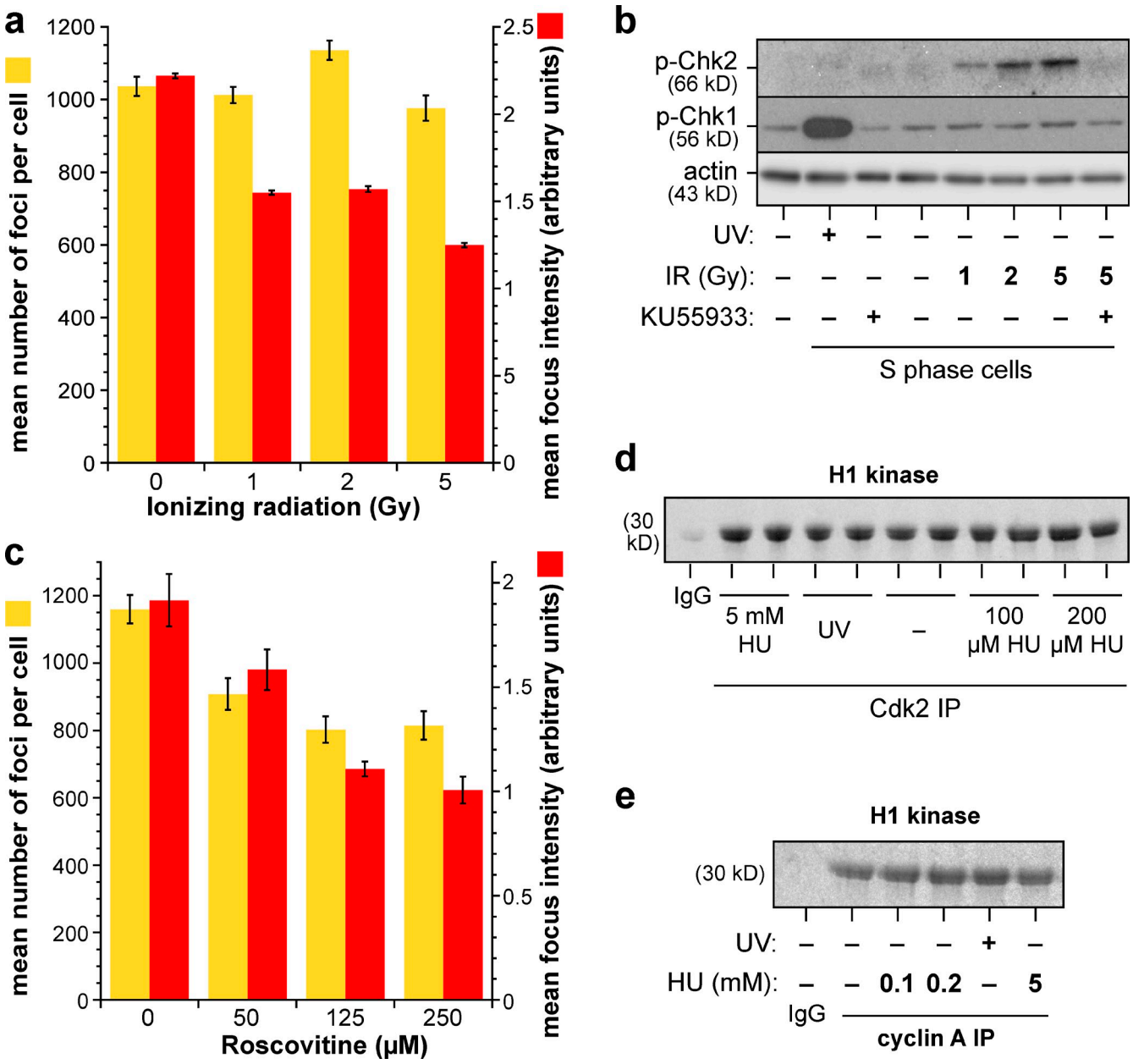

Figure 6. **Effect of different treatments on factory number.** (a) After synchronization in early S phase as described in Fig. 1, U2OS cells were irradiated with 1–5 Gy γ ray. 2 h after irradiation, cells were pulsed with Cy3-dUTP. Both the number and intensity of the Cy3-dUTP foci were measured in each cell. The mean and SEM were derived from >50 cells. (b) 2 h after irradiation with 1–5 Gy γ ray or 20 J/m^2 UV in the presence or absence of 10 μM ATM inhibitor KU55933, total cell lysate was immunoblotted for phospho-CHK1, phospho-CHK2, and actin. (c) After synchronization in early S phase, U2OS cells were treated with 0–250 μM roscovitine for 2 h and pulse labeled with Cy3-dUTP. Both the number and intensity of Cy3-dUTP foci were measured in each cell. The mean and SEM were derived from >50 cells. (d and e) Cells were synchronized in early S phase and treated with HU or 20 J/m^2 UV. 2 h after treatment, cells were lysed for immunoprecipitation with Cdk2 or cyclin A antibody. The associated kinase activity of the immunoprecipitates was assayed on histone H1.

Very little is known about how replication factories are activated and regulated, so we can only speculate about how their activation is inhibited by ATR/Chk1. Recent studies have demonstrated that lowering Cdk activity can preferentially inhibit the activation of new replication factories (Gillespie and Blow, 2010; Thomson et al., 2010). Two possible explanations for this effect have been proposed. One possibility (the "factory substrate" model) is that Cdks may have specialized substrates within a replication factory or focus that must be phosphorylated to activate it; once a factory/focus has been activated, Cdks must then phosphorylate other substrates to promote initiation at individual origins within the active factory. Another possibility (the "founder effect" model) is that it is difficult for Cdks to promote initiation at origins within inactive factories/foci, but when the first origin in a factory has initiated, a change propagates throughout the factory that facilitates initiation at other origins (Gillespie and Blow, 2010; Thomson et al., 2010).

It is known that ATR/Chk1 activity delays progression from G2 into mitosis by promoting inhibitory phosphorylation of Cdk1 (Bartek et al., 2004; Boutros et al., 2007; Timofeev et al., 2010).

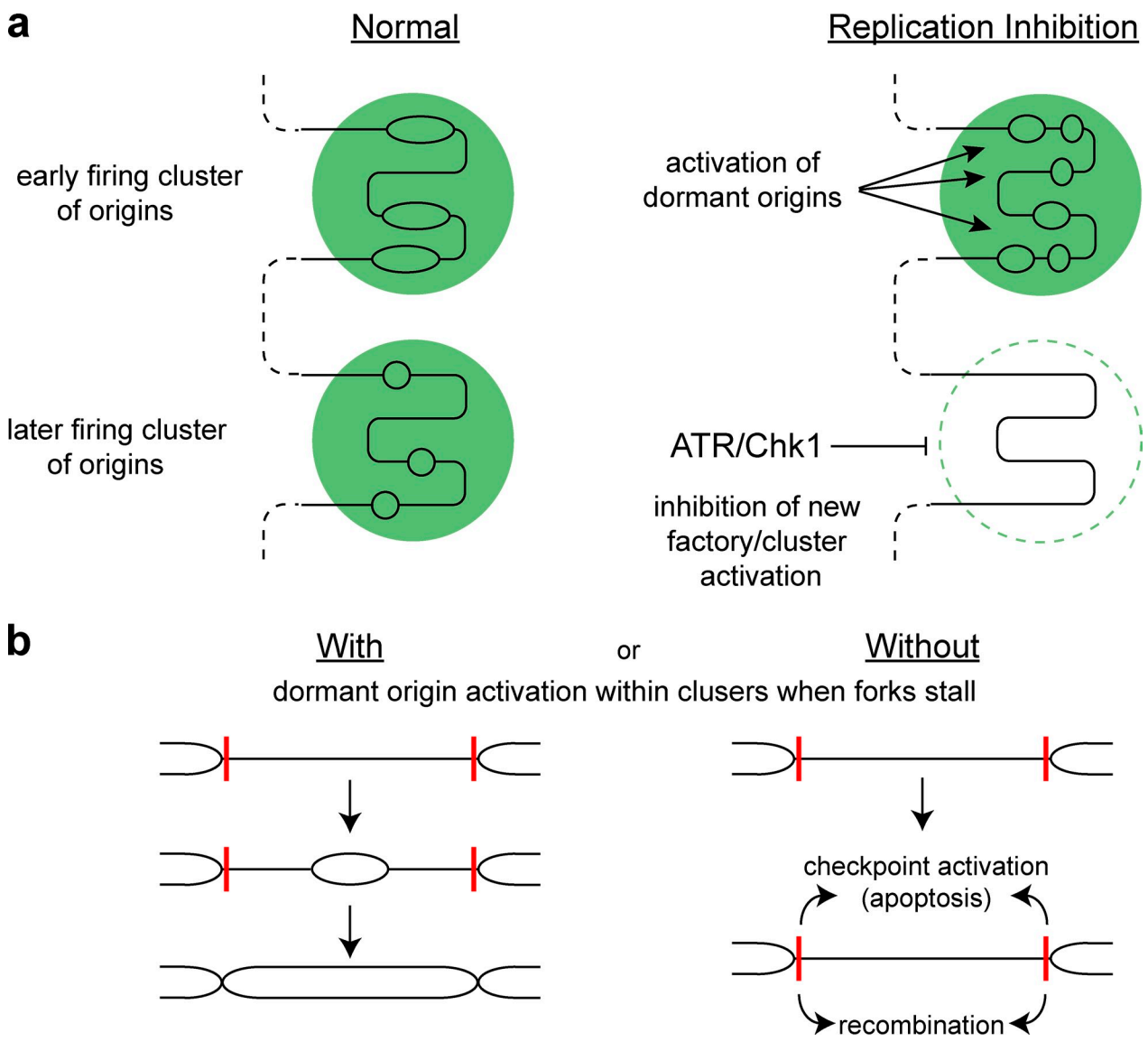

Figure 7. **Model to show how cells respond to low levels of replicative stress.** (a) Two adjacent clusters of origins (factories bounded by green circles) are shown on a single piece of DNA (black lines). Under normal circumstances (left), the upper factory is activated slightly earlier than the factory below, and each initiates three origins. Under low levels of replicative stress (right), replication forks are inhibited in the earlier replicating cluster, which promotes the firing of dormant origins as a direct consequence of stochastic origin firing. Replicative stress activates DNA damage checkpoint kinases, which preferentially inhibit the activation of the unfired later clusters/new factories. (b) A model showing two converging forks on a single piece of DNA (black lines) that have stalled (red bars). If a dormant origin is activated between them, replication can be rapidly rescued (left). If there is no dormant origin firing between the stalled forks (right), the DNA damage response can lead to recombination or induction of apoptosis.

However, we could detect no significant decrease in total levels of Cdk activity when S phase cells were treated with HU or UV. In fact, there is little evidence that in human cells, activation of DNA damage checkpoints causes a lowering of Cdk activity in S phase. Indeed, ionizing radiation has been shown to cause a Chk1-dependent increase in Cdk2 activity (Bourke et al., 2010), and Cdk2 activity has been shown to be required to maintain the G2/M checkpoint in response to DNA damage (Chung and Bunz, 2010). Instead, it is likely that ATR/Chk1 directly inhibits specific Cdk substrates that are required for origin initiation (if the founder effect model is correct) or factory activation (if the factory substrate model is correct). Therefore, artificially lowering Cdk activity mimics one aspect of ATR and Chk1 activation by preferentially inhibiting new factory activation.

The preferential inhibition of new factory activation by ATR/Chk1 causes the redirection of limited replication resources toward active replicon clusters where forks are inhibited and away from regions that have yet to embark on replication. This ensures rapid rescue of stalled forks and minimizes the risk of undergoing inappropriate recombination or apoptosis (Fig. 7 b). This can explain why decreasing the number of dormant origins makes cells hypersensitive to DNA-damaging agents and makes mice cancer prone (Woodward et al., 2006; Ge et al., 2007; Pruitt et al., 2007; Shima et al., 2007; Kunnev et al., 2010). It can also explain why adjacent origins are organized into clusters that initiate simultaneously within factories, as this allows dormant origins to be activated where they are needed and also allows pausing of replication by delaying activation of unreplicated clusters.

These two features work together to ensure the maintenance of genome stability in cells.

Many anticancer drugs target DNA replication, either directly by inhibiting replication fork progression or indirectly by causing damage that has its primary effects during S phase (Blow and Gillespie, 2008). The ability of cells to survive treatment with these drugs depends on the correct regulation of dormant origins (Woodward et al., 2006; Ge et al., 2007). Therefore, this study raises the possibility that if certain cancer cells are incapable of correctly regulating dormant origin and replication factory usage when under replicative stress, they would be more sensitive to chemotherapy drugs.

Materials and methods

Cell lines, synchronization, and siRNA

U2OS, HFF, MRC-5, IMR90, and Saos2 cells were grown in DME containing 10% fetal bovine serum, penicillin, and streptomycin. To synchronize cells in early S phase, cells were treated with 100 ng/ml nocodazole for 10 h before shaking off into 5 mM thymidine for 12 h. Cells were then released from thymidine for 1 h into early S phase and treated with HU, aphidicolin, or 5 mM caffeine for 2 h. MCM5 and CHK1 siRNA were used as described previously (Ge et al., 2007). siRNA oligonucleotides (Applied Biosystems) were made to the following sequences: Mcm5, 5′-GGAGGUAGCUGAUGAGGUGTT-3′; and Chk1, 5′-AAGCAGUCGCAGUGAAGAUUG-3′. Control unrelated oligonucleotide was purchased from Thermo Fisher Scientific. Transfections were performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. For Chk1 knockdown, U2OS cells were transfected with 40 nM siRNA twice within 72 h.

Total cellular rate of DNA synthesis

Asynchronous cells were labeled with 10 µM EdU for 30 min and stained according to the manufacturer's instructions (Click-iT EdU Alexa Fluor 647 flow cytometry; Invitrogen). DNA was stained with 20 µg/ml 7AAD. All samples were analyzed using a flow cytometer (FACSCalibur; BD) and CellQuest software (BD). To quantify EdU incorporation, the geographic mean of incorporated EdU was calculated (because the data are collected on a logarithmic scale), from which the overall rate of DNA synthesis was derived.

Replication foci

To label replication foci, U2OS cells were transfected with 83 µM Cy3-dUTP using FuGENE6 transfection reagent. The cellular incorporation of this amount of Cy3-dUTP into replication foci could last for ∼60 min. Cells were fixed with paraformaldehyde 30 min after transfection and analyzed on the number and intensity of Cy3-dUTP foci. Alternatively, cells were pulsed with 10 µM BrdU for 20 min followed by staining with BrdU antibody. To label replication factories, cells were transfected with GFP-PCNA plasmid using Lipofectamine 2000 (0.7 µg DNA/10^6 cells); only cells expressing low levels of GFP-PCNA were selected for analysis (Leonhardt et al., 2000). Alternatively, endogenous PCNA was labeled with PCNA antibody (PC10) and Alexa Fluor 568–labeled secondary antibodies.

Image acquisition and data analysis

To image replication foci/factories in cells, a wide-field fluorescence microscope (DeltaVision DV3; Applied Precision) was used to collect 0.2-µm serial sections using a 100× NA 1.4 Plan Apochromat objective (Olympus). After deconvolution with SoftWorx (Applied Precision), 3D image stacks were analyzed with Volocity (PerkinElmer) where a threshold-based segmentation was applied to define replication foci. The same degree of threshold was used across different samples to cover 90% of all visually defined foci in each cell. The total voxel intensity of individual focus within a cell was averaged to give rise to cellular focus intensity. More than 120 cells pooled from three sets of experiments were sampled to generate mean focus intensity and SEM under each condition. To calculate cellular foci number, >150 cells pooled from three sets of experiments were used to generate mean foci number and SEM. To analyze the percentage of colocalization between GFP-PCNA and Cy3-dUTP foci, the total colocalized volume was divided by the total volume of GFP-PCNA foci within a cell. More than 50 cells were analyzed under each condition to give rise to the mean percentage of colocalization between the two types of foci and SEM, generating the gradient of separation between Cy3 and GFP-PCNA foci over time. Experiments were repeated multiple times, generating SEM of the gradient.

New factory activation rate

Cells were transfected with GFP-PCNA plasmid, and 12 h after transfection, were synchronized into early S phase using nocodazole and thymidine. Replication foci were then labeled by pulsing the cells with 83 µM Cy3-dUTP. Cells were treated with 200 µM HU in the presence or absence of caffeine for 2 h before fixation and imaging. 0.2-µm serial sections were taken in each cell, and 3D images of the cells were reconstructed using Volocity software as described in the previous paragraph. Using 10% as the cut-off line for colocalization between Cy3 and GFP-PCNA foci, we quantified the number of foci containing Cy3 and GFP-PCNA, Cy3 alone, and GFP-PCNA alone.

Chromatin fractionation and immunoblotting

Immunoblotting was performed as previously described (Ge et al., 2007). To prepare chromatin samples, 10^6 cells washed with PBS were suspended in 2 ml cytoskeleton (CSK) buffer and incubated on ice for 10 min. After centrifugation at 5,000 *g* for 5 min, the pellet was collected, resuspended in CSK buffer, and incubated on ice for 10 min. After a second 5-min spin at 5,000 *g*, the resultant pellet containing chromatin-bound sample was washed with CSK buffer three times and suspended in 100 µl NuPAGE LDS sample buffer (supplemented with 5% 2-mercaptoethanol; Invitrogen). To analyze total cellular proteins, 10^5 cells were washed with PBS and suspended in 100 µl LDS sample buffer. SDS gel electrophoresis and Western blotting were performed according to standard procedures.

H1 kinase assay

For H1 kinase assays, U2OS cells were synchronized into early S phase using nocodazole and thymidine. 2 h after treatment (20 J/m^2 UV; or 0.1, 0.2, or 5 mM HU), 500,000 cells were lysed by lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.5% NP-40) and immunoprecipitated with 2 µg Cdk2 antibody (M2; Santa Cruz Biotechnology, Inc.) or cyclin A antibody (ab39; Abcam). The immunoprecipitates were incubated with 5 µg commercially purified H1 (Millipore) in 25 µl kinase reaction buffer (50 mM Tris HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 5 µM ATP, and 5 µCi γ-[^{33}P]ATP) for 10 min at 30°C. Samples were then analyzed by SDS-PAGE followed by autoradiography.

DNA fiber analysis

DNA fiber spreading was performed as described previously (Ge et al., 2007). Cells were pulsed with 40 µM BrdU for different times depending on the concentration of inhibitor used so that labeled track lengths were more homogenous in size than would occur if the BrdU pulse length was kept constant: 0–100 µM HU, 10 min; 200 µM HU, 20 min; 400–500 µM HU, 30 min; 0.1–0.2 µg/ml aphidicolin, 20 min; and 0.4 µg/ml aphidicolin, 30 min. Cells were then harvested, and DNA fiber spreads were prepared as described previously (Jackson and Pombo, 1998). BrdU-labeled tracks were detected with BrdU anti–sheep antibody (M20105S; 1:1,000; BioDesign, Inc.) using either Cy3– or Alexa Fluor 488–conjugated donkey anti–sheep secondary antibody. Biotin-11–dUTP was detected using a mouse monoclonal antibody (clone BN-34; 1:1,000; Sigma-Aldrich) and an appropriate Cy3-conjugated second antibody. Quality control for spreading DNA was performed using 0.1 µM YOYO (Invitrogen) labeling. Fibers were examined using a 65× microscope (Leica) and 100× confocal microscope (LSM 510; Carl Zeiss, Inc.). The mean and standard deviation of track lengths were determined by measuring the length of labeled tracks that were well separated from other tracks (thereby minimizing the risk that they represented fusions between adjacent replicons).

Online supplemental material

Fig. S1 shows the HU-induced increase in the number of forks per cell when replication rates were measured by BrdU incorporation and the HU-induced reduction in factory number using an anti-PCNA antibody. The changes in factory number are shown by different cell lines treated with HU. Fig. S2 shows the increase in fork number per factory when cells are treated with HU, as measured by BrdU incorporation. Fig. S3 shows focus intensity and fork speed when cells are treated with combinations of HU, Chk1 siRNA, and caffeine. Fig. S4 shows checkpoint-dependent inhibition of progression through the replication timing program in cells treated with 200 µM HU. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201007074/DC1.

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