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ORIGINAL ARTICLE

Increased replication initiation and conflicts with transcription underlie Cyclin E-induced replication stress

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It has become increasingly clear that oncogenes not only provide aberrant growth signals to cells but also cause DNA damage at replication forks (replication stress), which activate the ataxia telangiectasia mutated (ATM)/p53-dependent tumor barrier. Here we studied underlying mechanisms of oncogene-induced replication stress in cells overexpressing the oncogene Cyclin E. Cyclin E overexpression is associated with increased firing of replication origins, impaired replication fork progression and DNA damage that activates RAD51-mediated recombination. By inhibiting replication initiation factors, we show that Cyclin E-induced replication slowing and DNA damage is a consequence of excessive origin firing. A significant amount of Cyclin E-induced replication slowing is due to interference between replication and transcription, which also underlies the activation of homologous recombination. Our data suggest that Cyclin E-induced replication stress is caused by deregulation of replication initiation and increased interference between replication and transcription, which results in impaired replication fork progression and DNA damage triggering the tumor barrier or cancer-promoting mutations.

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Keywords: Cyclin E; replication fork; origin firing; DNA damage; homologous recombination

INTRODUCTION

Faithful replication of the genome is essential to maintain genomic stability and prevent cancer-promoting mutations. If the progression of replication forks is impaired, this can lead to replication-associated DNA damage, also known as replication stress. It is now recognized that replication stress induced by oncogenes is an important factor that underlies the activation of the ataxia telangiectasia mutated (ATM)- and p53-mediated tumor barriers of senescence and apoptosis.[1–7] There is currently no coherent model to explain how oncogenes induce replication stress and DNA damage. Oncogenes of the growth factor signaling pathways generally activate cell proliferation by deregulating the transition from G1 to S phase of the cell cycle.[8] Deregulated S-phase entry leading to aberrant DNA replication is therefore likely a principal mechanism of oncogene-induced replication stress. It has been reported that overexpression of the oncogenes HPV-16 E6/E7 and Cyclin E leads to S-phase entry in the presence of insufficient nucleotide pools, leading to impaired DNA replication fork progression and DNA damage.[9] However, the underlying mechanism for the nucleotide depletion observed in the presence of oncogenes is not clear, and not all oncogenes that deregulate S-phase entry induce DNA damage.[10] The mechanisms of aberrant DNA replication and oncogene-induced replication stress therefore deserve further scrutiny.

During a normal cell cycle, not only entry into S phase but also the timing of DNA replication initiation during S phase is carefully regulated by cell cycle and checkpoint signaling pathways. Initiation of replication takes place at sites termed replication origins, which are defined by the loading of the pre-replication complex (ORC1-6, Cdc6, Cdt1 and MCM2-7) onto the DNA in a process called replication licensing.[11] Metazoan origins are organized into initiation zones or clusters that are activated at different times during S phase and contain many more licensed origins than are used during each round of replication.[12,13] Origin firing is activated by the Cyclin-dependent kinases Cdk2 and Cdk1, in complex with Cyclin E or Cyclin A, and by Cdc7/Dbf4.[14] These kinases do not activate all licensed origins at least partly due to the action of the ATR and Chk1 checkpoint kinases, which downregulate Cdk activity even during unperturbed S phase.[15,16] Loss of checkpoint regulation of replication initiation causes slow speeds of replication fork progression and accumulation of DNA damage, and mouse models suggest that ATR and Chk1 act as haplo-insufficient tumor suppressors.[17–20] These observations suggest that increased levels of replication initiation during S-phase cause replication stress and genomic instability.

Importantly, experimental overexpression of the oncogenes Cyclin E, c-Myc and h-Ras increases levels of replication initiation.[1,3,7] Although Cyclin E directly activates Cdk2, c-Myc directly interacts with replication origins and c-Myc and h-Ras promote expression of Cyclin E, Cdc6 and other proliferation factors.[1,21] This suggests that high levels of replication initiation may contribute to oncogene-induced replication stress. Several mechanisms by which increased origin density might perturb replication fork progression can be envisaged. The increase in

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active replication forks may deplete necessary replication factors, such as nucleotides. Changes in origin density could also interfere with the spatial coordination of replication initiation and transcription, which may increase collisions between replication and transcription complexes, thereby causing DNA damage and genomic instability.[11,22–25]

As it is downstream of major oncogenic signaling pathways, Cyclin E seems likely to be an important mediator of oncogene effects on replication. Cyclin E is tumor promoting itself if overexpressed in mice and is found overexpressed in a variety of cancers, which correlates with low disease-free survival.[8,21,26] Cyclin E overexpression shortens G1 phase and increases Cdk2 activity.[27–29] Cyclin E overexpression further causes increased origin firing, altered replication fork progression and induces DNA damage, and eventually cellular senescence.[1,2,9] In cells that do not enter senescence, Cyclin E overexpression leads to chromosomal instability.[30,31]

Here we used an established human culture cell model for ectopic overexpression of Cyclin E[1,2] to investigate the importance of increased replication initiation and replication-transcription conflicts for Cyclin E-induced replication stress. We report that downregulating replication initiation in Cyclin E-overexpressing cells rescues perturbed replication fork progression and DNA damage. We further show that a subset of the replication stress induced by Cyclin E can also be reversed by inhibiting transcription, and that Cyclin E induces transcription-associated recombination, suggesting that Cyclin E-induced replication stress results from increased origin firing and increased interference between replication and transcription.

RESULTS

Cyclin E overexpression increases replication initiation and causes replication stress that activates RAD51-mediated recombination

U2OS cells harboring a tetracycline-repressible Cyclin E expression construct[2] were grown in absence of tetracycline to induce Cyclin E overexpression (Figure 1a). Overexpressed Cyclin E often occurs in two lower molecular weight forms around 39 and 44 kDa that have been shown to be functional.[27] The Cyclin E expression levels in the cells used are similar to those identified in cancer cell lines, which can display similar lower molecular weight bands of Cyclin E.[2,32] In agreement with previous reports, 3 days of Cyclin E overexpression reduced the percentage of cells in G1 phase and increased the percentage of cells in S phase (Supplementary Figures S1A and B), and caused accumulation of micronuclei, indicators of genomic instability (Supplementary Figure S1C).[2,27,31] As previous reports had shown that Cyclin E-overexpressing cells cease cycling and enter senescence after 4–6 days,[1] 3 days of Cyclin E induction were chosen for all subsequent experiments. First, we tested the effects of Cyclin E overexpression on replication initiation. Changes in Cdk activity predominantly affect the number of replication clusters and have less effect on the number of active origins within clusters.[33] To test whether this was true for increased Cdk2 activity caused by Cyclin E overexpression, we quantified the numbers of replication foci, which correlates with the number of active replication clusters.[12,34] Cells were pulse-labeled with the thymidine analog iododeoxyuridine (IdU) for 20 min, fixed, and IdU was detected by immunostaining (Figure 1b). We quantified the number of IdU foci in early S-phase cells as identified by the pattern of small, evenly dispersed foci typical for this stage of S phase (Figure 1b). In the presence of Cyclin E, more early S-phase cells displayed high numbers of replication foci (average foci/cell: $-$Cyclin E, 609 ± 22, $n = 77$; $+$Cyclin E, 707 ± 29, $n = 83$; $P = 0.005$) (Figure 1c). We next measured the distance between adjacent replicons within replication clusters using DNA fiber analysis. Cells were sequentially labeled with chlorodeoxyuridine (CldU) and IdU for 20 min each and DNA fibers were spread out. CldU and IdU were detected by immunostaining using specific antibodies (Figure 1d). The fiber-spreading method produces long unbroken tracks of DNA,[12] allowing the measurement of distances between adjacent replication origins. Similar to the reported effects of Cdk activity on replication initiation,[33] Cyclin E overexpression did not measurably change the distances between origins within replication clusters (average kb: $-$Cyclin E, 51.1 ± 3.3, total $n = 108$; $+$Cyclin E, 45.12 ± 3.7, total $n = 105$, $P = 0.19$; Figure 1d). We next measured the lengths of ongoing replication tracks (labeled with CldU followed by IdU) to determine speeds of replication fork progression. Cells overexpressing Cyclin E displayed reduced replication fork speeds (Figure 1e and Supplementary Table S1) in agreement with a previous report.[9] Cells overexpressing Cyclin E displayed a small but significant increase in the ratio of CldU to IdU track length (Figure 1f), suggesting slower fork progression during the second label that probably indicates increased fork stalling.[35]

To analyze the induction of spontaneous DNA damage, cells were immunostained for DNA damage markers. Cyclin E overexpression led to increased amounts of spontaneous DNA damage and double-strand breaks (DSBs) as demonstrated by an increase in both phospho-Serine 139 H2AX (γH2AX) and 53BP1 foci (Figure 1g). DSBs formed at replication forks activate homologous recombination.[36,37] We observed increased spontaneous formation of RAD51 foci in Cyclin E-overexpressing cells, suggesting that homologous recombination is activated to repair damage caused by oncogene-induced replication stress (Figure 1h). RAD51 foci formation was replication-dependent, as it could be prevented by a short 100 min treatment with the replication inhibitor hydroxyurea (Figure 1h). Re-addition of tetracycline to the medium reversed slow replication fork progression and γH2AX formation, showing that both were dependent on the presence of overexpressed Cyclin E (Supplementary Figure S2).

Downregulation of replication initiation rescues replication fork progression in Cyclin E-overexpressing cells

To downregulate replication initiation, cells were treated with the Cdc7/Cdk inhibitor PHA-767491 (PHA, 10 μM)[38] or small interfering RNA (siRNA) against the origin licensing factor Cdc6 before and during DNA-fiber labeling. Both treatments did not affect Cyclin E overexpression itself (Figures 2a and b). PHA-767491 reduced new origin firing as measured by DNA fiber analysis, both by quantification of new origin signals (Figure 2c) and measurement of inter-origin distances (Figure 2d). Cdc6 depletion also strongly increased inter-origin distances, consistent with its role in replication origin licensing (Figure 2d). Treatment with PHA-767491 rescued replication fork speeds in cells overexpressing Cyclin E (Figures 2e and f, Supplementary Table S1). PHA-767491 also slightly increased forks speeds in the absence of Cyclin E; however, this effect was less significant (Figure 2g and Supplementary Table S1). Cdc6 depletion also rescued replication fork speeds in cells overexpressing Cyclin E (Figure 2h), but did not increase replication fork speeds in control cells (Figure 2i, Supplementary Table S1). Similar results were obtained when downregulating replication initiation using the Cdk inhibitor roscovitine in U2OS-Cyclin E cells (Supplementary Figures S3A-C), and PHA-767491 also significantly rescued replication fork speeds in human MRC5 fibroblasts transiently overexpressing Cyclin E (Supplementary Figures S4A-D and Supplementary Table S1). Taken together, these data support the idea that high levels of replication initiation promote Cyclin E-induced replication stress.

Inhibiting DNA resection does not rescue replication fork speeds in presence of Cyclin E

Cdk inhibition can affect many DNA transactions in addition to replication initiation, such as DNA resection. We therefore tested whether rescue of replication fork progression by PHA-767491 or

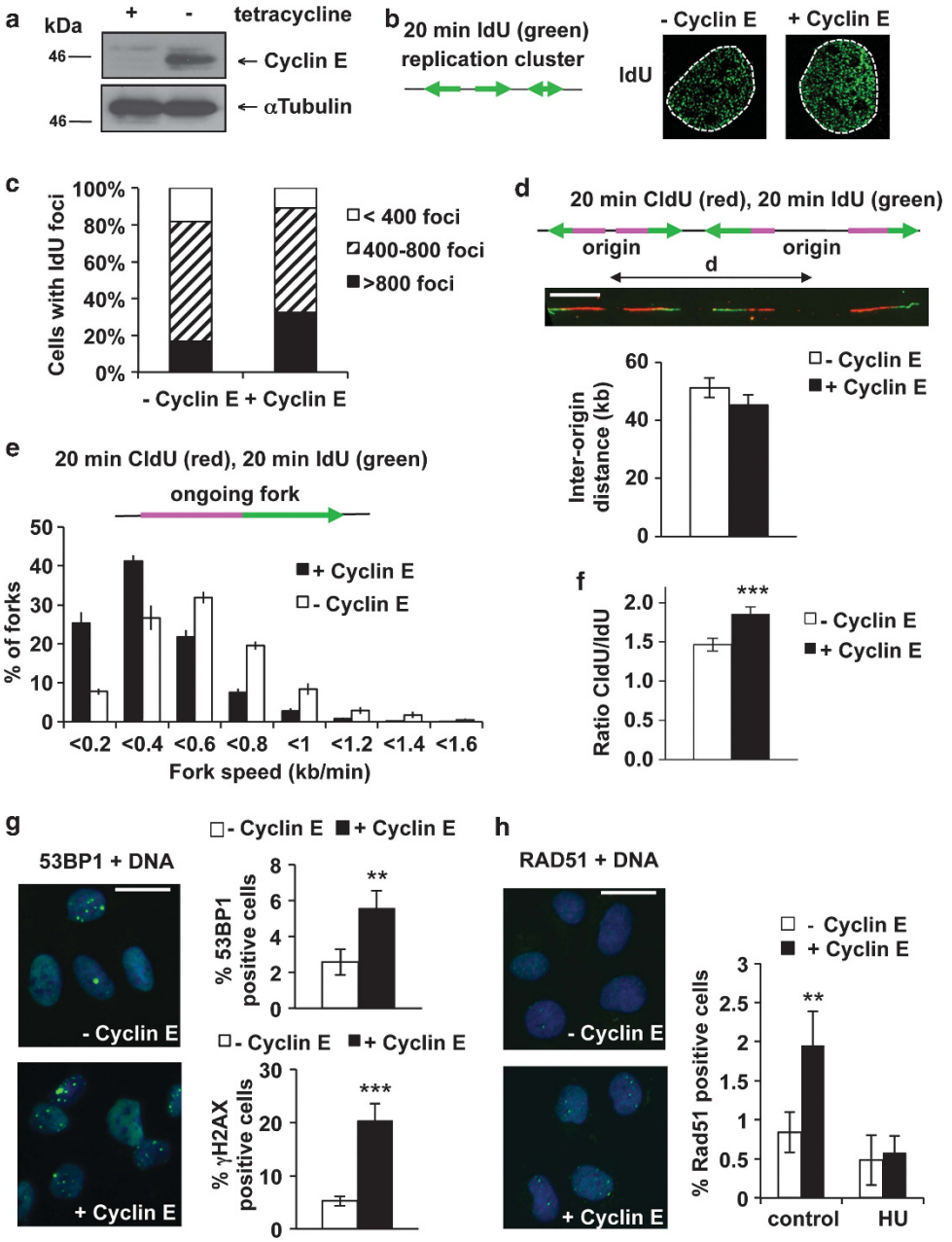

Figure 1. Increased replication initiation and replication stress in cells overexpressing Cyclin E. U2OS–Cyclin E cells were grown for 72 h in presence (−Cyclin E) or absence (+Cyclin E) of tetracycline. (**a**) Protein levels of Cyclin E and α-tubulin (loading control) in whole-cell extracts after removal of tetracycline. (**b**) Cells were pulse-labeled with iododeoxyuridine (IdU) for 20 min and fixed, and active replication clusters were detected by immunostaining for IdU. (**c**) Distributions of replication foci number in early S-phase cells treated as in **b**. (**d**) Cells were sequentially labeled with chlorodeoxyuridine (CldU) and IdU for 20 min each, and DNA fibers were prepared. CldU and IdU were detected using specific antibodies and distances between adjacent origins were measured. Scale bar: 10 μm. (**e**) Cells were treated for DNA fiber analysis as before and lengths of ongoing replication tracks (CldU + IdU) were measured to determine the distribution of replication fork speeds. (**f**) Lengths of CldU and IdU tracks were measured separately, and ratios of first to second label length were calculated. (**g**) Cells were grown on coverslips or in 96-well plates, fixed and immunostained for γH2AX or 53BP1. Representative high-throughput microscopy images of 53BP1 foci (left) and quantification of cells displaying more than 9 53BP1 or 10 γH2AX foci (right). Bar: 20 μm. (**h**) Representative high-throughput microscopy images and quantification of cells displaying more than nine RAD51 foci in the presence or absence of the replication inhibitor hydroxyurea (HU; 2 mM, 100 min). Bar: 20 μm. Means ± s.e.m. (bars) of three to seven independent experiments are shown. Values marked with asterisks are significantly different (Student's *t*-test, $^{**}P<0.01$, $^{***}P<0.001$).

roscovitine was due to inhibition of resection. For this, we used Mirin, an inhibitor of Mre11, a protein that is required for DNA resection.[39] Treatment of U2OS cells with 50 or 100 μM Mirin inhibited camptothecin-induced replication protein a (RPA) phosphorylation, a process that is dependent on resection[40] (Supplementary Figures S5A and B). However, in contrast to PHA-767491 and roscovitine, treatment with 100 μM Mirin did not affect replication initiation and did not rescue replication fork speeds in Cyclin E-overexpressing cells (Supplementary Figures S5C-E and Supplementary Table S1).

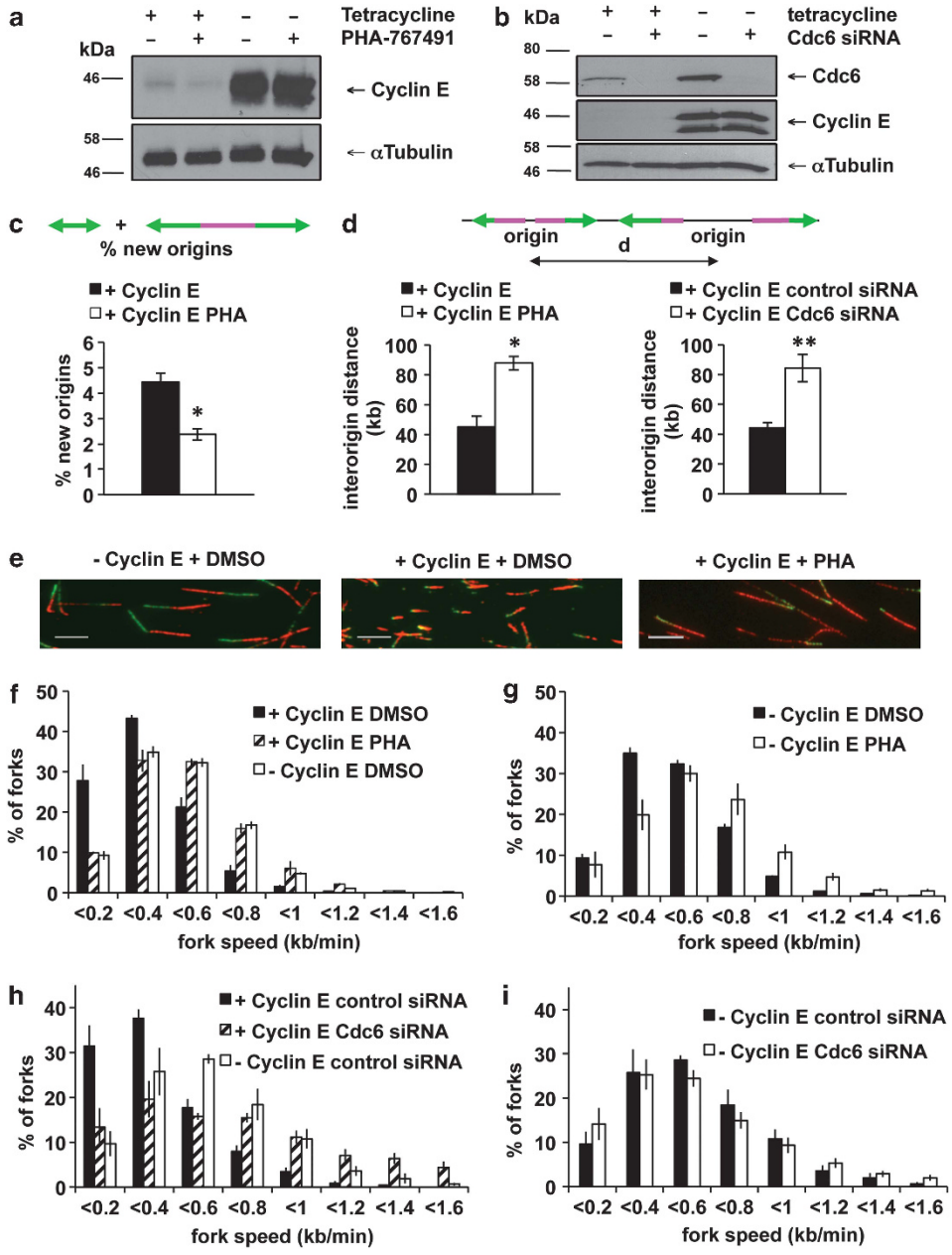

Figure 2. High levels of replication initiation in Cyclin E-overexpressing cells slow down replication fork progression. U2OS–Cyclin E cells were grown for 72 h in the presence (−Cyclin E) or absence (+Cyclin E) of tetracycline, treated with 10 μM PHA-767491 (PHA) for 60 min or transfected with Cdc6 siRNA for 48 h, and labeled with CldU and IdU for 20 min each. (**a**) Protein levels of Cyclin E and α-tubulin (loading control) in whole-cell extracts from cells after treatment with PHA. (**b**) Protein levels of Cdc6, Cyclin E and α-tubulin (loading control) in whole-cell extracts after Cdc6 siRNA transfection. (**c**) New origin firing in cells treated with PHA, shown as percentage of labeled tracks. (**d**) Average inter-origin distances in cells treated with PHA (left) and Cdc6 siRNA (right). (**e**) Representative images of replication tracks from cells treated with dimethyl sulfoxide (DMSO) or PHA as indicated. (**f**) Distribution of replication fork speeds in (+Cyclin E) cells treated with DMSO or PHA. (**g**) Distribution of replication fork speeds in (−Cyclin E) cells treated with DMSO or PHA. (**h**) Distribution of replication fork speeds in (+Cyclin E) cells transfected with control or Cdc6 siRNA. (**i**) Distribution of replication fork speeds in (−Cyclin E) cells transfected with control or Cdc6 siRNA. Means ± s.e.m. (bars) of three to five independent experiments are shown. Values marked with asterisks are significantly different (Student's *t*-test, $^{*}P<0.05$, $^{**}P<0.01$).

Inhibiting transcription rescues replication fork progression in Cyclin E-overexpressing cells

To investigate the possibility that Cyclin E-induced replication fork slowing may depend on conflicts between replication and transcription, we inhibited transcription elongation using the RNA-specific chain terminator 3′-deoxyadenosine (cordycepin).[22] Treatment with cordycepin (50 μM) for 100 min reduced transcription elongation by 50% as measured by incorporation of the RNA-specific labeled nucleoside 5-ethynyluridine (Figure 3a). During the incubation time used in the subsequent experiments, cordycepin treatment did not interfere with Cyclin E overexpression (Figure 3b) and did not impact on replication initiation (Figure 3c). However, cordycepin significantly increased speeds of replication fork progression in cells overexpressing

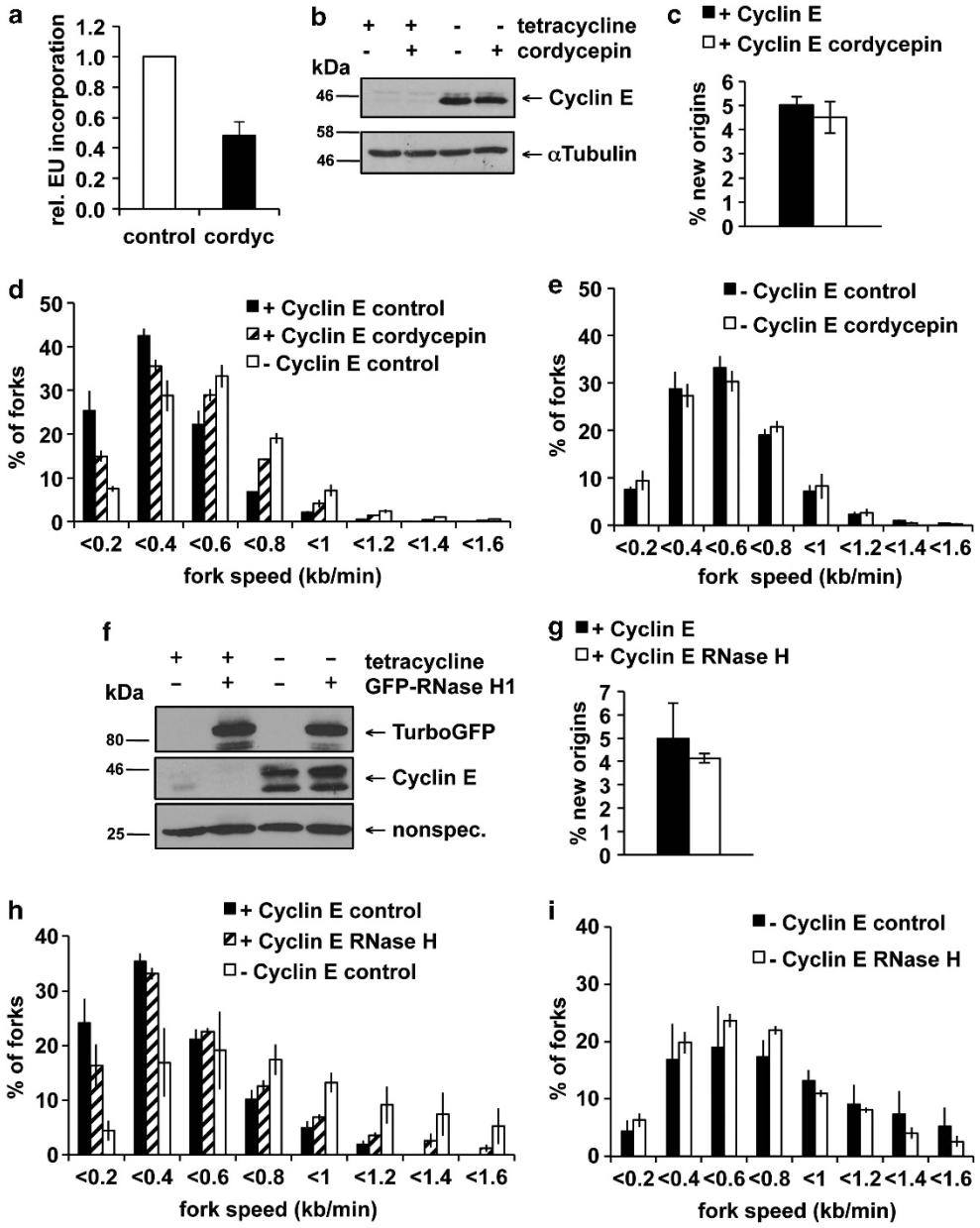

Figure 3. Slow replication fork progression in Cyclin E-overexpressing cells is transcription-dependent. (**a**) U2OS–Cyclin E cells were treated with 50 μM cordycepin (cordy) for 100 min and transcription elongation was measured by incorporation of the RNA-specific labeled nucleoside 5-ethynyluridine (EU). (**b**) U2OS–Cyclin E were grown for 72 h in the presence or absence of tetracycline, and treated with 50 μM cordycepin or H_2O for 60 min before labeling with CldU and IdU for 20 min each. Protein levels of Cyclin E and α-tubulin (loading control) in whole-cell extracts were determined. (**c**) Quantification of new origin firing after treatment with cordycepin, shown as percentage of all labeled tracks. (**d**) Distribution of replication fork speeds in cells treated or not with cordycepin. (**e**) Distribution of replication fork speeds in (− Cyclin E) cells treated or not with cordycepin. Means ± s.e.m. (bars) of three independent experiments are shown. (**f**) U2OS–Cyclin E were grown for 72 h in presence or absence of tetracycline, and transiently transfected with TurboGFP-tagged RNase H1 for 24 h before labeling with CldU and IdU for 20 min each. Protein levels of TurboGFP-RNase H1 and Cyclin E in whole-cell extracts were determined. A nonspecific band from the TurboGFP antibody served as loading control. (**g**) Quantification of new origin firing after transfection with RNase H1, shown as percentage of all labeled tracks. (**h**) Distribution of replication fork speeds after transfection with RNase H1. (**i**) Distribution of replication fork speeds in (− Cyclin E cells) after transfection with RNase H1. Means ± s.e.m. (bars) of three independent experiments are shown.

Cyclin E but not in control cells (Figures 3d and e, and Supplementary Table S1), suggesting that replication fork slowing in the presence of Cyclin E is dependent on ongoing transcription. Inhibiting transcription elongation using cordycepin similarly rescued replication fork speeds in MRC5 fibroblasts transiently overexpressing Cyclin E (Supplementary Figures S4E and F, and Supplementary Table S1). Inhibiting transcription elongation rescued replication fork speeds to a lesser extent than inhibiting replication initiation, suggesting that conflicts between replication and transcription account for a subset of Cyclin E-induced replication stress. During transcription-dependent replication stress, replication forks can be slowed by collision with the transcription machinery itself or by collision with RNA–DNA hybrids (R-loops) left behind by the transcription machinery.[24]

RNase H1 specifically degrades RNA in RNA–DNA hybrids, thus removing R-loops.[22,24] To test the contribution of R-loops to Cyclin E-induced fork slowing, we transiently transfected RNase H1 into Cyclin E-overexpressing and control cells (Figure 3f). RNase H1 transfection had no effect on replication initiation (Figure 3g). With a transfection efficiency of 15–20%, we could observe a small but statistically significant ($P = 0.01$) rescue of replication fork progression by RNase H1 in Cyclin E-overexpressing cells, but not control cells, supporting the idea that the presence of R-loops contributes to Cyclin E-induced replication stress (Figures 3h and i, and Supplementary Table S1).

To test whether origin firing and transcription act in the same pathway of Cyclin E-induced replication stress, we combined siRNA depletion of Cdc6 to downregulate replication initiation with cordycepin treatment to inhibit transcription (Figure 4a). Although each treatment alone increased fork speeds in Cyclin E-overexpressing cells, there was no additional increase in fork speeds if both treatments were combined (Figure 4b and Supplementary Table S1). All three treatments did not significantly increase replication fork speeds in absence of Cyclin E overexpression (Figure 4c and Supplementary Table S1). This is consistent with the hypothesis that in Cyclin E-overexpressing cells, initiation and transcription interfere with replication by acting in the same pathway.

Inhibitors of replication initiation prevent DNA damage in Cyclin E-overexpressing cells

To assess whether high levels of replication initiation were responsible for the induction of DNA damage by Cyclin E, cells were treated with PHA-767491 for the same period as used for fiber analyses (100 min). PHA-767491 reduced the number of Cyclin E-overexpressing cells displaying more than 10 γH2AX foci by more than half (Figures 5a and b). Similar results were obtained using roscovitine (Supplementary Figure S2D). This was not due to changes in the cell cycle profile caused by inhibitor treatment (Figure 5a, right panel) or direct inhibition of H2AX phosphorylation by the inhibitors (Supplementary Figure S6). Furthermore, treatment with PHA-767491 reduced RAD51 foci formation in Cyclin E-overexpressing cells (Figure 5c), indicating that RAD51 has a role in repairing DNA damage arising from slow fork progression caused by increased initiation. Formation of γH2AX in Cyclin E-overexpressing cells was also reduced by depletion of Cdc6 (Figures 5d and e). Because of low cell numbers after siRNA transfection, we used IdU incorporation to verify that Cdc6 depletion did not decrease the percentage of S-phase cells (Figure 5d, bottom panel). This suggests that spontaneous formation of γH2AX and RAD51 foci in Cyclin E-overexpressing cells depends on high levels of replication initiation and large numbers of simultaneously active replication forks.

Inhibitors of transcription prevent DNA damage in Cyclin E-overexpressing cells

It has been reported that collisions between transcription and replication machineries activate homologous recombination.[23] To further confirm the presence of transcription-associated replication stress, we therefore assessed whether conflicts between replication and transcription are the underlying cause for RAD51 foci formation in the presence of Cyclin E. Cells were treated with cordycepin for 100 min to inhibit ongoing transcription and induction of DNA damage markers was analyzed. In contrast to inhibitors of initiation, cordycepin treatment had little effect on the level of γH2AX induced by Cyclin E overexpression, possibly reflecting the partial rescue of replication fork progression in presence of the drug. Interestingly, however, cordycepin treatment completely abolished induction of RAD51 foci by Cyclin E (Figure 6a) and reduced the numbers of

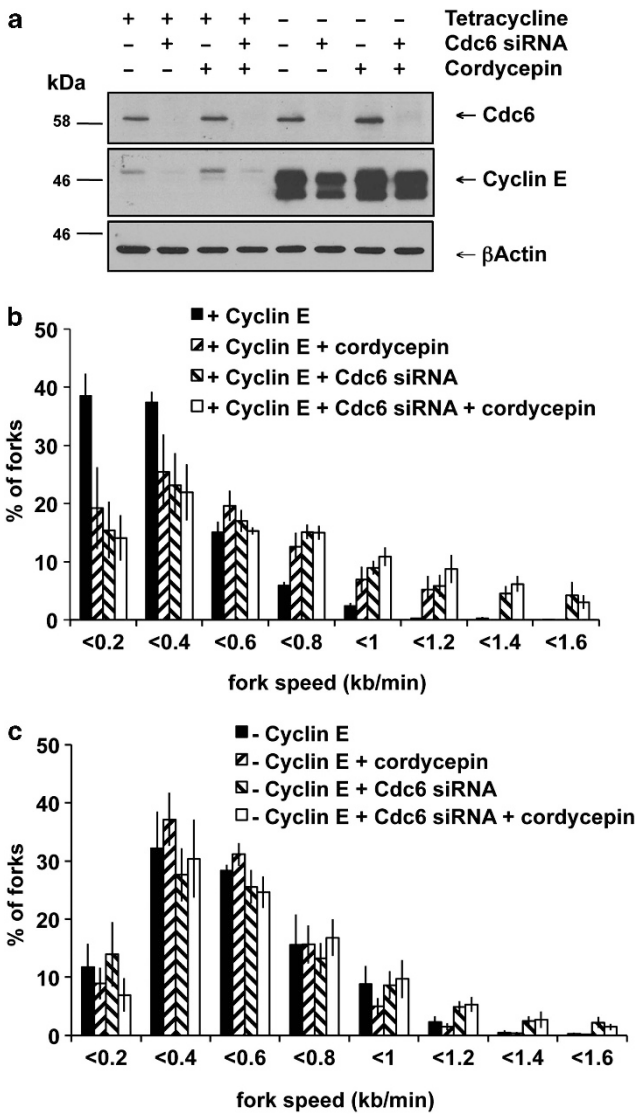

Figure 4. Replication initiation and transcription act in the same pathway to slow replication fork progression. U2OS–Cyclin E grown in absence (+Cyclin E) of tetracycline were transfected with control or Cdc6 siRNA. At 48 h after siRNA transfection, cells were treated with 50 μM cordycepin or H_2O for 60 min before labeling with CldU and IdU for 20 min each. (**a**) Protein levels of Cyclin E, Cdc6 and α-tubulin (loading control) in whole-cell extracts. (**b**) Distribution of replication fork speeds in (+Cyclin E) cells after transfection with control or Cdc6 siRNA and treatment with cordycepin. (**c**) Distribution of replication fork speeds in (−Cyclin E) cells after transfection with control or Cdc6 siRNA and treatment with cordycepin. Means ± s.e.m. (bars) of three independent experiments are shown.

Cyclin E-induced 53BP1 foci (Figure 6b). Cyclin E-dependent induction of RAD51 foci was also reduced or prevented by 100 min treatment with the transcription inhibitors α-amanitin and DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; Figure 6a). This suggests that RAD51 foci in Cyclin E-overexpressing cells depend on ongoing transcription, and that RAD51 has a role in repairing a subset of DNA damage arising from collision of the transcription and replication machineries in cells overexpressing Cyclin E. In addition, there was a slight reduction in RAD51 foci after treatment with α-amanitin, and a reduction in 53BP1 foci after treatment with cordycepin and DRB in control cells. This suggests

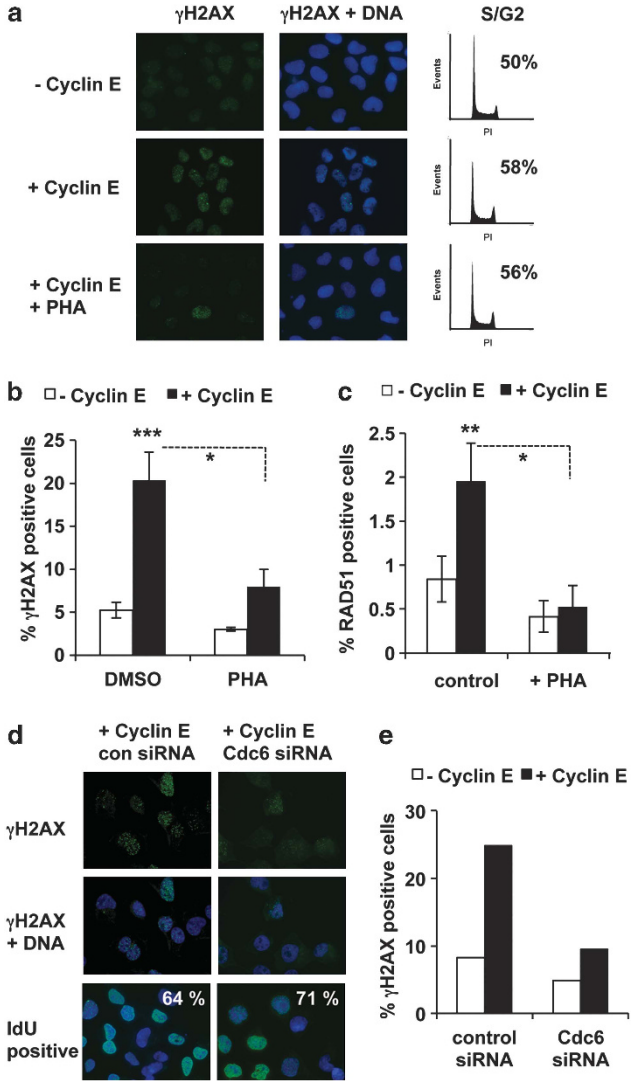

Figure 5. Inhibiting replication initiation prevents DNA damage and RAD51 foci formation in Cyclin E-overexpressing cells. (**a**) U2OS–Cyclin E were grown on coverslips in the presence (−Cyclin E) or absence (+Cyclin E) of tetracycline for 72 h and treated with 10 μM PHA-767491 (PHA) for 100 min. Left: cells were fixed and immunostained for γH2AX (green). DNA was counterstained with DAPI (blue). Right: cells were fixed and stained with propidium iodide for fluorescence-activated cell sorting analysis. (**b**) Percentages of cells displaying more than 10 γH2AX foci. (**c**) Cells were fixed and immunostained for RAD51. Percentages of cells displaying more than nine RAD51 foci were quantified by high-throughput microscopy. Means ± s.e.m. (bars) of at least three independent experiments are shown. Values marked with asterisks are significantly different (Student's *t*-test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$). (**d**) U2OS–Cyclin E were grown on coverslips for in presence or absence of tetracycline and transfected with Cdc6 or control siRNA. Left: 48 h after transfection, cells were fixed and immunostained for γH2AX (green). DNA was counterstained with DAPI (blue). Right: cells were labeled with IdU for 20 min, fixed and immunostained for IdU. Percentages of cells containing replication foci were quantified. (**e**) Quantification of U2OS–Cyclin E cells displaying more than 10 γH2AX foci after treatment as in (**d**). Values from one experiment are shown.

that some spontaneous transcription-associated DNA damage occurs even in the absence of Cyclin E overexpression, but that transcription-dependent DNA damage and especially transcription-associated recombination are greatly increased in presence of overexpressed Cyclin E.

DISCUSSION

In this study, we used an established cell model for Cyclin E overexpression to investigate replication stress induced by this oncogene.[1,2] Our findings support the idea that increased replication initiation caused by Cyclin E overexpression leads to impaired replication fork progression and DNA damage. Our data further suggest that Cyclin E overexpression induces transcription-dependent slowing of replication forks, which is associated with DSB formation and increased transcription-associated recombination.

Our data are in agreement with a previous report that changes in Cdk activity predominantly affect the number of replication clusters and have less effect on the number of active origins within clusters.[33] The activation of replication clusters during S phase normally follows a tightly regulated program,[41] and a situation of increased replication cluster activation can well be described as aberrant replication. Importantly, our findings show that a short inhibition of Cdk activity for less than 2 h can reverse Cyclin E-induced replication stress, supporting the idea that Cyclin E causes replication stress not only by deregulating cell cycle progression but also by disrupting regulation of DNA replication during S phase itself. It has been shown that slow fork progression can cause increased activation of dormant origins,[42] which poses the question as to whether increased origin firing is the cause or consequence of slow fork progression. However, our observations that decreased fork speeds in presence of Mirin (Supplementary Figure S5) and increased fork speeds in presence of cordycepin (Figure 3) are not associated with changes in origin firing support the idea that increased origin firing in Cyclin E-overexpressing cells is the cause and not the consequence of slow replication fork progression. In contrast to the Bester *et al.*[9] study, we did not observe increased dormant origin firing as a result of Cyclin E overexpression. This may possibly be due to our period of oncogene overexpression being much shorter (3 days versus 2–6 weeks), and the change in intra-cluster initiation density resulting from longer-term changes such re-programming of origin selection during mitosis.[43] Another possibility is that Cyclin E overexpression in U2OS cells interferes with origin licensing, thereby reducing the numbers of available dormant origins. Although Cyclin E is required to promote minichromosome maintenance complex loading during exit from quiescence in a Cdk-independent manner,[44] overexpression of Cyclin E has been observed to inhibit minichromosome maintenance complex loading.[29] As activation of dormant origins protects cells from replication stress,[42,45] this might exacerbate the effects of replication fork stalling, incomplete replication, DNA damage and genomic instability (see model in Figure 6c). Incomplete replication causes DNA damage and has been linked to rearrangements at common fragile sites and chromosomal instability, the most common form of genomic instability in sporadic cancers.[45–47]

Higher levels of origin firing mean that more replication forks are active simultaneously, and it appears likely that replication elongation factors such as deoxyribonucelotides (dNTPs) become limiting under these conditions. Indeed, it was recently reported that Cyclin E overexpression decreased dNTP pools, resulting in decreased replication fork progression and replication stress.[9] Our observation that decreasing the number of active replication forks prevents oncogene-induced replication stress is compatible with this, and increased origin firing might be an explanation for the reduced nucleotide levels observed in oncogene-overexpressing cells. However, although sufficient dNTP pools are important to avoid replication stress, their depletion is likely not the only explanation for the DNA damage caused. Indeed, our data suggest that Cyclin E-induced replication stress is also partly due to

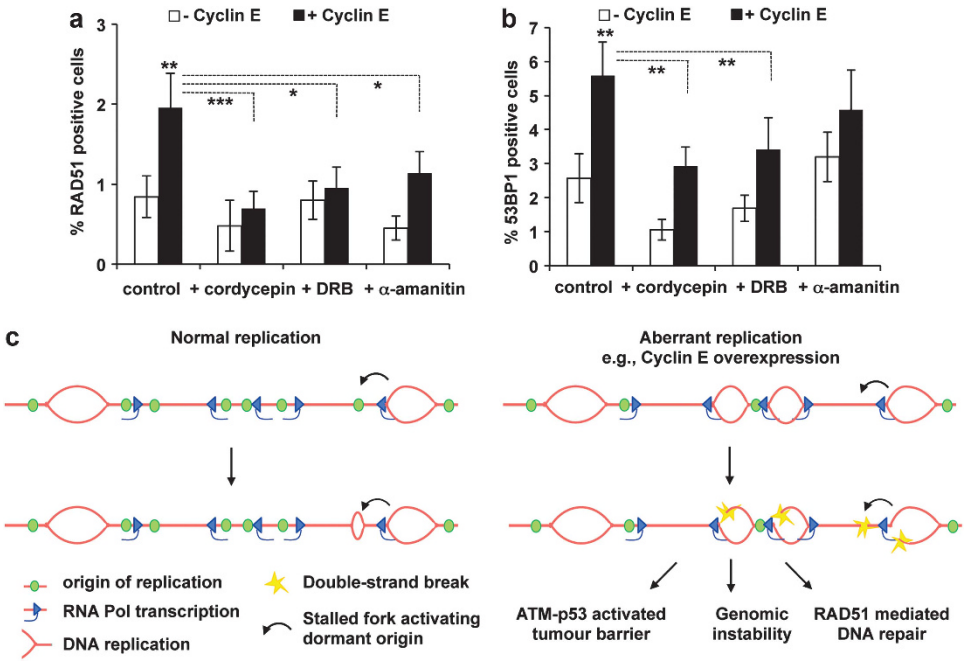

Figure 6. DNA damage and RAD51 foci formation in Cyclin E-overexpressing cells are transcription-dependent. (**a**) U2OS–Cyclin E were grown for 72 h in the presence (−Cyclin E) or absence (+Cyclin E) of tetracycline, treated with 50 μM cordycepin, DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) or 10 μg/ml α-amanitin for 100 min, fixed and immunostained for high-throughput microscopy. Percentages of cells displaying more than nine RAD51 foci. (**b**) Percentages of cells displaying more than nine 53BP1 foci were quantified by high-throughput microscopy. Means ± s.e.m. (bars) of at least three independent experiments are shown. Values marked with asterisks are significantly different (Student's *t*-test, $*P<0.05$, $**P<0.01$, $***P<0.001$). (**c**) Model for Cyclin E-induced replication stress. Cyclin E overexpression increases replication initiation by increasing the number of active replication clusters. More active replication forks deplete deoxyribonucelotide (dNTP) pools and lead to increased conflicts between the transcription and replication machineries. This leads to impaired replication fork progression, DNA damage and increased transcription-associated recombination. Double-strand breaks (DSBs) can form through under-replication of DNA, collisions between replication and transcription, or processing of slow or stalled replication forks. This replication-associated DNA damage activates the ataxia telangiectasia mutated- and p53-dependent tumor barrier, and in absence of ataxia telangiectasia mutated or p53 promotes genomic instability and tumor development.

increased conflicts between transcription and replication. It has been previously reported that collisions between transcription and replication machineries, as well as RNA–DNA hybrids (R-loops) left behind by the transcription machinery, slow replication fork progression and activate homologous recombination.[22–24] Collisions of the replication and transcription machineries have also been implicated in genomic instability at common fragile sites.[25] We speculate that transcription-associated replication stress in Cyclin E-overexpressing cells could be a consequence of increased replication initiation. Changes in origin firing and the regulation of replication clusters could increase conflicts between transcription and replication by interfering with the spatial coordination of replication initiation with gene expression. There is increasing evidence that origin activity correlates with transcription, such that transcribed genes are replicated early in S-phase, active origins are often located near transcription regulatory elements, and that active transcription changes origin activity within the transcribed region.[11] Although the underlying mechanisms for the links between replication and transcription remain to be identified, our data support the idea that correct regulation of origin firing can help prevent transcription-associated replication stress.[48] The DSBs caused by this transcription-dependent replication stress are likely to represent a strong trigger for activation of the DNA damage response. This suggests that preventing transcription-replication interference could affect activation of the tumor barrier. Furthermore, transcription-associated recombination may be an additional mechanism of oncogene-induced genomic instability if the tumor barrier fails.

In conclusion, replication stress in Cyclin E-overexpressing cells likely results from increased replication initiation coupled with conflicts between replication and transcription, which leads to DNA damage and genomic instability through a combination of incomplete DNA replication, DSB formation at stalled replication forks and transcription-associated recombination.

MATERIALS AND METHODS

Cell lines and treatments

Human U2OS osteosarcoma cells were cultured in Dulbecco's modified Eagle's medium and human SV40-transformed MRC5-V1 fibroblasts[49] in minimal essential medium containing 10% fetal calf serum in a humidified CO_2 atmosphere at 37 °C.

U2OS cells containing constructs for tetracycline-repressible overexpression of Cyclin E[28] were grown in the presence of G418 (400 μg/ml), puromycin (1 μg/ml) and tetracycline (2 μg/ml). To remove tetracycline, cells were washed three times in phosphate-buffered saline and transferred into Dulbecco's modified Eagle's medium containing 10% tetracycline-free fetal calf serum (PAA-GE Healthcare, Little Chalfont, UK), G418 and puromycin. Roscovitine (25 μM), cordycepin (50 μM) and camptothecin (1 μM) were from Sigma (Dorset, UK); hydroxyurea (2 mM) was from Fisher Scientific (Loughborough, UK); and PHA-767491 (10 μM) and Mirin (100 μM) were from Tocris Bioscience (Bristol, UK). Cells were irradiated using a Cesium-137 source (2 Gy at 2.3 Gy/min).

DNA fiber experiments

Exponentially growing cells were pulse-labeled with 25 μM CldU and 250 μM IdU for 20 min each. Labeled cells were harvested and DNA fiber spreads prepared as described.[35] Acid-treated fiber spreads were incubated with

rat anti-bromoxyuridine monoclonal antibody (AbD Serotec, Raleigh, NC, USA, 1:1000) and mouse monoclonal anti-bromoxyuridine antibody (Becton Dickinson, Oxford, UK; 1:750) for 1 h at room temperature. Slides were fixed with 4% paraformaldehyde and incubated with anti-rat IgG AlexaFluor 555 and anti-mouse IgG AlexaFluor 488 (Molecular Probes, Paisley, UK; 1:500) for 1.5 h at room temperature. Images were acquired on a Nikon (Kingston upon Thames, UK) E600 microscope using a $\times 60$ (1.3NA) lens, a Hamamatsu (Welwyn Garden City, UK) digital camera and the Volocity package (Perkin Elmer, Cambridge, UK). The lengths of CldU (AF 555, red)- and IdU (AF 488, green)-labeled patches were measured using the ImageJ software (http://rsb.info.nih.gov/ij/) and micrometer values were converted into kilobase using the conversion factor $1\,\mu m = 2.59\,kb$.[12] Termination (red and red–green–red) or origin structures (green and green–red–green) were not measured. Replication structures were quantified using the Cell Counter Plug-in for ImageJ (Kurt De Vos, University of Sheffield, Sheffield, UK).

Immunofluorescence

Cells grown on coverslips were fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 for 5 min before antibody incubations. For high-throughput microscopy, cells were seeded in 96-well plates, fixed in 3.7% formaldehyde + 0.1% Triton X-100 for 20 min and permeabilized with 0.5% Triton X-100 for 10 min. Primary antibodies were mouse monoclonal anti-phospho-Histone H2AX (Ser139; Millipore, Watford, UK; 1:1000), mouse monoclonal anti-bromoxyuridine (detects IdU, 1:50; Becton Dickinson), rabbit anti-RAD51 (1:1000, H-92; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti-53BP1 (1:1000; Bethyl Laboratories, Montgomery, TX, USA). Secondary antibodies were anti-mouse or anti-rabbit IgG AlexaFluor 488 and anti-rabbit IgG AlexaFluor 555 (Molecular Probes). DNA was counterstained with DAPI (4′,6-diamidino-2-phenylindole). For replication foci analysis, cells were labeled with IdU for 20 min, fixed, permeabilized, and DNA was denatured with 2 M HCl for 30 min before immunostaining for IdU. *Z*-stack images (stack size 0.4 μm) were acquired on a Zeiss LSM 510 confocal microscope (Zeiss, Cambridge, UK) using a $\times 100$ (1.4NA) lens. IdU foci numbers of 160 cells were quantified using three-dimensional spots detection by the Imaris software (Bitplane, Zurich, Switzerland). Quantitative high-throughput microscopy images of RAD51 and 53BP1 foci were taken on an IN Cell Analyzer 1000 (GE Healthcare, Little Chalfont, UK) using a $\times 20$ lens, and foci numbers in 500 cells per sample were counted using the IN Cell Investigator software. For γH2AX, 200–2000 cells per sample were analyzed using a Nikon E600 microscope and a $\times 40$ lens.

Transcription assay

The Click-iT RNA assay (Invitrogen, Paisley, UK) was used to incorporate the modified nucleoside 5-ethynyluridine into nascent RNA, followed by fixation and detection using an AlexaFluor azide. Cells were seeded in 96-well plates and treated with 50 μM cordycepin for 100 min. 5-Ethynyluridine (0.2 mM) was added 30 min before fixation in 3.7% formaldehyde + 0.1% Triton X-100. 5-Ethynyluridine incorporation was visualized according to the manufacturer's instructions.

siRNA and DNA transfection

Cdc6 was depleted using siRNA duplex oligonucleotides (Dharmacon, Epsom, UK) against the human Cdc6 target sequence (sense): 5′-AAC UUCCCACCUUAUACCAGA-3′.[50] Allstars Negative Control siRNA (Qiagen, Crawley, UK) was used as non-targeting control. A total of 100 000 cells were grown in 6-well plates for 24 h in the presence or absence of tetracycline, and transfected with 50 nM siRNA using Dharmafect 1 (Dharmacon). Cells were harvested 48 h after transfection. To overexpress human ribonuclease H1, 100 000 U2OS cells grown in 6-well plates for 48 h in presence or absence of tetracycline were transfected with 2.5 μg pCMV6-AC-GFP-RNase H1 (Origene, Rockville, MD, USA) using TransIT-2020 (Geneflow, Lichfield, UK). Antibiotics, except tetracycline, were removed during transfections. For Cyclin E overexpression, 150 000 MRC5 cells grown in 6-well plates for 24 h were transfected with 2.5 μg pcDNA3-Cyclin E[51] using TransIT-2020. Twenty-four hours after transfection, cells were treated and harvested for fiber spreads and western blot analysis.

Western blotting

For protein levels, cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P40, 5% Na-deoxycholate, 0.1% SDS) containing $1\times$ protease inhibitor cocktail (Roche, Burgess Hill, UK). For replication protein a phosphorylation, cells were sonicated in urban transition buffer (50 mM Tris–HCl pH7.5, 150 mM β-mercaptoethanol, 8 M urea). Lysates from $2–5 \times 10^6$ cells per lane were resolved by denaturing polyacrylamide gel electrophoresis and transferred to nitrocellulose. Antibodies were rabbit polyclonal anti-Cyclin E antibody (Santa Cruz Biotechnology; 1:500), rabbit polyclonal anti-phospho-replication protein a (S4/S8) antibody (Bethyl Laboratories; 1:500), rabbit polyclonal anti-TurboGFP antibody (Evrogen, Moscow, Russia; 1:1000), rabbit polyclonal anti-β-actin antibody (Abcam, Cambridge, UK; 1:5000), mouse monoclonal anti-Cdc6 antibody (Santa Cruz Biotechnology; 1:300) and mouse monoclonal anti-α-tubulin antibody (Sigma; 1:500–1:4000). Incubations with primary antibodies were performed at 4 °C over night.

Flow cytometry

U2OS-Cyclin E cells were grown in the presence or absence of tetracycline for 1–5 days. Cells were washed with phosphate-buffered saline and fixed in 70% ethanol at 4 °C. Cells were washed and resuspended in 0.5 ml phosphate-buffered saline containing 10 μg/ml propidium iodide and RNase A for analysis by flow cytometry. Data analysis was performed using the WinMDI software (Joe Trotter, Scripps Research Institute, La Jolla, CA, USA).

Statistical analysis

The means of three to seven independent repeats are shown. Error bars are $1\times$ s.e.m. Statistical significance of differences between means was determined using the Student's *t*-test (one-tailed and paired, or using two-sample with equal variance for unpaired arrays).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)