Signaling from Mus81-Eme2-Dependent DNA Damage Elicited by Chk1 Deficiency Modulates Replication Fork Speed and Origin Usage

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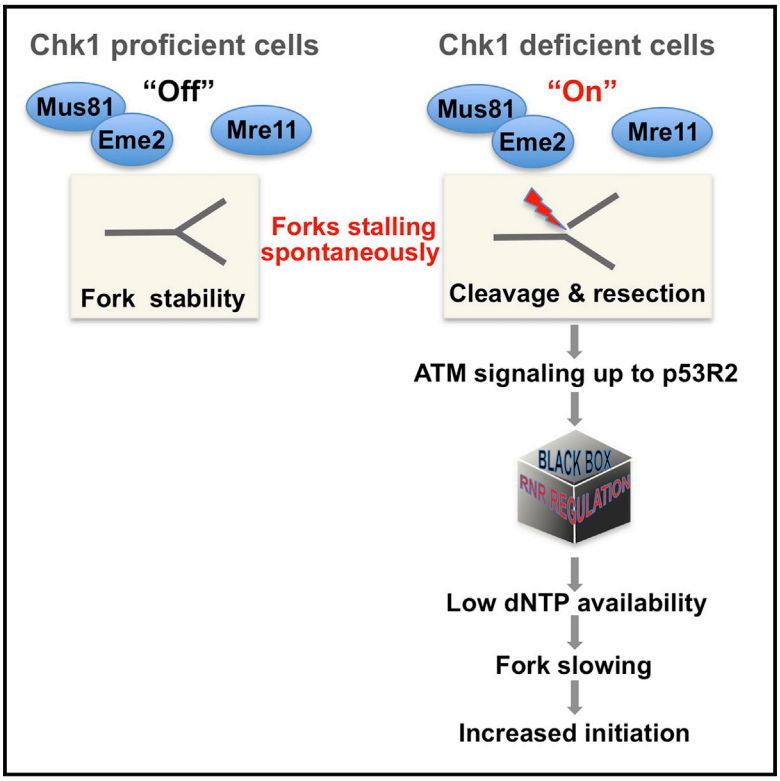
Graphical Abstract



Highlights

- Nuclease-dependent DNA damage events are induced in Chk1-deficient cells
- Activation of the ATM pathway limits DNA precursors available for replication
- DNA precursor starvation elicits replication fork slowing in these cells
- Origin firing is fine-tuned by fork speed independently of Chk1 status

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In Brief

Técher et al. show that modulation of DNA replication dynamics in Chk1-deficient mammalian cells is the consequence of DNA damage arising through unscheduled Mus81-Eme2 and Mre11 activation. Signaling of this damage by the ATM pathway impedes fork progression through dNTP shortage.

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Signaling from Mus81-Eme2-Dependent DNA Damage Elicited by Chk1 Deficiency Modulates Replication Fork Speed and Origin Usage

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SUMMARY

Mammalian cells deficient in ATR or Chk1 display moderate replication fork slowing and increased initiation density, but the underlying mechanisms have remained unclear. We show that exogenous deoxyribonucleosides suppress both replication phenotypes in Chk1-deficient, but not ATR-deficient, cells. Thus, in the absence of exogenous stress, depletion of either protein impacts the replication dynamics through different mechanisms. In addition, Chk1 deficiency, but not ATR deficiency, triggers nuclease-dependent DNA damage. Avoiding damage formation through invalidation of Mus81-Eme2 and Mre11, or preventing damage signaling by turning off the ATM pathway, suppresses the replication phenotypes of Chk1-deficient cells. Damage and resulting DDR activation are therefore the cause, not the consequence, of replication dynamics modulation in these cells. Together, we identify moderate reduction of precursors available for replication as an additional outcome of DDR activation. We propose that resulting fork slowing, and subsequent firing of backup origins, helps replication to proceed along damaged templates.

INTRODUCTION

The DNA damage response (DDR) is a signal transduction pathway that coordinates DNA replication, DNA repair, and cell-cycle progression and eventually triggers cell death and/or senescence (reviewed in Ciccia and Elledge, 2010). Central to this network are two kinases and their major downstream targets, ATM-Chk2 and ATR-Chk1. While the ATM-Chk2 branch of the DDR essentially senses double-strand breaks, the ATR-Chk1 branch detects alterations of replication fork progression (reviewed in Zeman and Cimprich, 2014).

Not surprisingly, deficiency in some components of the DDR leads to accumulation of DNA damage (reviewed in Aguilera and Gómez-González, 2008). In addition, in vertebrates, depletion or inactivation of various proteins involved in genome maintenance, such as ATR (Eykelenboom et al., 2013; Koundrioukoff et al., 2013; Marheineke and Hyrien, 2004; Shechter et al., 2004), Chk1 (Katsuno et al., 2009; Maya-Mendoza et al., 2007; Petermann et al., 2010; Seiler et al., 2007), Claspin (Petermann et al., 2008; Scorah and McGowan, 2009), Wee1 (Beck et al., 2012; Domínguez-Kelly et al., 2011), FHIT (Saldivar et al., 2012), BLM (Chabosseau et al., 2011), NEK8 (Choi et al., 2013), BRCA2 or Rad51 (Wilhelm et al., 2014), or RBBP6 ubiquitin ligase (Miotto et al., 2014), elicits a 20%–40% decrease in replication fork speed and, when determined, an increase in the density of initiation events. Such alteration of the replication dynamics is commonly referred as to ''replication stress'' and is assumed to trigger DNA damage.

Fork movement heavily relies on deoxyribonucleoside triphosphate (dNTPs) availability. In S phase, when the deoxynucleoside triphosphohydrolase activity of SAMHD1 is low (discussed in Stillman, 2013), the amount of precursors mainly relies on the level of ribonucleotide-reductase (RNR) activity and on the so-called ''salvage pathways.'' In mammals, RNR contains two copies of a large catalytic subunit (R1) and two copies of a small regulatory subunit (either R2 or p53R2). While the R2-R1 complex fuels the nuclear replication machinery, the main role of the p53R2-R1 complex is to provide precursors for mtDNA replication and DNA repair in quiescent cells, in which R2 is not expressed (reviewed in Mathews, 2015). Whether fork slowing observed in some DDR-deficient mammalian cells results from modulation of RNR activity is presently unknown. By contrast, slowing of fork movement upon alteration of the salvage network has been documented. Notably, reduced levels of cytidine-deaminase or thymidine kinase 1 have been respectively observed in BLM- or FHIT-deficient cells. Accordingly, addition of exogenous DNA precursors restores fork velocity in these

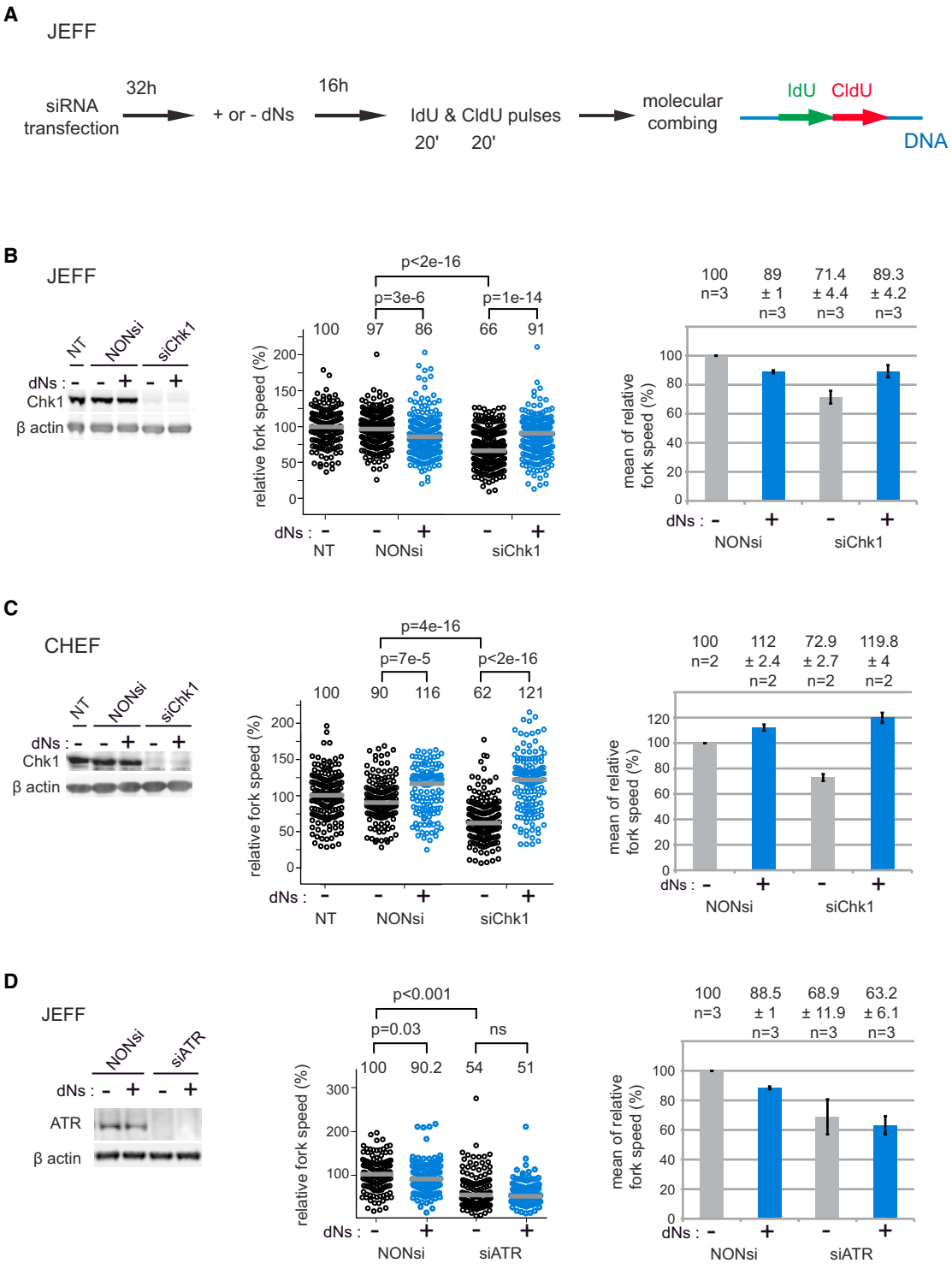


Figure 1. Exogenous dNs Rescue Fork Slowing in Chk1-Depleted Cells

(A) Scheme of the protocol. Cells transfected with small interfering RNA (siRNAs) were complemented or not with dNs. Ongoing forks were pulse-labeled with two thymidine analogs (IdU then CldU). After combing, DNA molecules were counterstained in blue. IdU and CldU were revealed in green and red, respectively (see Supplemental Experimental Procedures).

(B) Impact of dNs addition on Chk1-dependent modulation of fork movement in JEFF cells. Left: western blot analysis of Chk1 depletion. β actin is shown as a loading control. Supplementation or not with dNs is indicated (+ or –). NT, non-transfected; NONsi, transfection with a control siRNA; siChk1, transfection with a set of Chk1 siRNAs. Middle: dot plots of relative fork speed. Addition of dNs and transfection conditions are indicated. Horizontal gray lines represent the median

(legend continued on next page)

genetic backgrounds (Chabosseau et al., 2011; Saldivar et al., 2012).

Here, we studied the mechanism by which Chk1 affects the replication dynamics in the absence of exogenous replication stress. We found that depletion of Chk1 triggers Mus81-Eme2 and Mre11-dependent DNA lesions. Strikingly, fork speed is restored by co-depletion of Chk1 together with the nucleases responsible for such damage, or with intermediates in ATM signaling. In addition, our results showed that supplying DNA precursors rescues fork speed in cells depleted of Chk1 and revealed that the increased density of initiation events observed in these cells strictly inversely correlates with fork speed, independently of Chk1 status. Altogether our data show that modulation of DNA replication dynamics in Chk1-depleted cells is a response to DNA damage arising in this genetic context. Hence we identify a new mechanism of cross-talk between the DNA damage response and replication dynamics.

RESULTS

Precursor Addition Rescues Fork Speed in Chk1- but Not in ATR-Deficient Cells

We used molecular combing to determine fork speed in cells affected either in ATR or Chk1 function (Figure 1A). JEFF (human B-lymphocytes immortalized with Epstein Barr virus) and CHEF (normal Chinese hamster embryonic fibroblasts) cells were depleted of Chk1 by RNA silencing or treated with the Chk1 inhibitor UCN-01 (Syljuåsen et al., 2005). Median fork speeds have been previously shown to be 1.85 and 1.5 kb/min, respectively, in JEFF and CHEF cells grown under normal conditions (Técher et al., 2013). To facilitate comparison, fork speed was expressed here as percentage of the control in each experiment. Regardless of cell types and species, we confirmed that Chk1 depletion or inhibition leads to an approximately 30% decrease in fork speed (Figures 1B, 1C, S1A, and S1B). JEFF cells were also depleted of ATR by RNA silencing (Figure 1D). Similar levels of fork slowing were observed in cells depleted of ATR or Chk1.

Fork slowing often results from limiting or imbalanced dNTP availability, which can be corrected by addition of precursors in the culture medium (Bester et al., 2011; Burrell et al., 2013; Chabosseau et al., 2011; Courbet et al., 2008; Wilhelm et al., 2014). We therefore asked whether addition of deoxyribonucleosides (dA, dC, dG, and dT; hereafter referred to as dNs) alleviates fork slowing in cells deficient in ATR or Chk1. In non-deficient cells, we observed that exogenous dNs slow fork progression in JEFF cells, most probably because they induce pool imbalance. In contrast, dNs increase fork speed in CHEF cells, suggesting that dNTPs are limiting in these cells. Strikingly, exogenous dNs fully suppressed fork slowing in both JEFF and CHEF cells depleted of Chk1 as compared to non-depleted cells supplemented with dNs (Figures 1B and 1C). Similar results were obtained in cells treated with a small-molecule inhibitor of Chk1, UCN-01 (Figure S1B). As shown in Figure 1D, dN addition does not rescue fork progression in JEFF cells depleted of ATR. Rather, we observed additive effects of dN addition and ATR depletion on fork slowing. These results strongly suggest that Chk1 deficiency, but not ATR deficiency, alters the concentration or the balance of dNTPs available for the replication machinery and, consequently, that different mechanisms lead to fork slowing in cells deficient in either ATR or Chk1.

DNA Breaks Are Induced in Chk1- Depleted, but Not ATR-Depleted, Cells

It has been reported that Chk1-deficient cells display DNA lesions in the absence of exogenous replication stress. Consistently, in Chk1-depleted JEFF cells compared to control cells, western blot analyses show increased phosphorylation of histone H2AX on serine 139 (γH2AX) and of p53 on serine 15 (p53-s15P) (Figures 2A and S2A). Notably, γH2AX and p53-s15P do not increase in ATR-depleted JEFF cells in the absence of replication stress (Figure 2A). These results were confirmed in cells treated with UCN-01 or VE-822, an inhibitor of ATR (Figure S2B). Focusing on JEFF cells depleted of Chk1, immunofluorescence analysis additionally shows that γH2AX forms foci that co-localize with 53BP1 (Figures S2C and S2D), another marker of DNA damage (reviewed in Ciccia and Elledge, 2010). We then checked directly for the presence of single- and/or double-strand DNA breaks in JEFF cells depleted of either protein, using alkaline and neutral Comet assays (Saldivar et al., 2012). In agreement with the DDR status, the Comet assays showed that tail moment (a general measure of DNA damage) is weakly impacted upon ATR depletion but increases markedly upon depletion of Chk1 (Figures 2B and S2E).

dN Addition Does Not Prevent Damage Formation in Chk1-Depleted Cells

DNA damage formed upon Chk1 deficiency may result from an increased number of stalled forks. However, in agreement with previous reports (Koundrioukoff et al., 2013; Speroni et al., 2012), fork asymmetry (a marker of stalling) does not increase significantly in JEFF and CHEF cells depleted of Chk1 compared to control cells (Figure S2F). In addition, the levels of p53-s15P and γH2AX remain high in Chk1-depleted cells supplemented with exogenous dNs, regardless of the timing of dN addition (Figure 2C). Consistently, dNs do not reduce tail moment in Chk1-depleted cells (Figures 2D and S2G). Therefore, neither DNA damage nor DDR activation is suppressed under conditions that restore fork movement. We conclude that the decrease in fork speed occurring in Chk1-deficient cells does not cause the DNA damage observed in this genetic context.

of relative fork speed distribution. Medians and p values are indicated above the distributions. Data are from a representative experiment. Right: histograms are the means of median relative fork speed ±SEM. The number of independent experiments (n) is indicated above the histogram.
(C) Impact of dNs addition on Chk1-dependent modulation of fork movement in CHEF cells. Data are presented as in (B).
(D) Impact of dNs addition on ATR-dependent modulation of fork movement in JEFF cells. Data are presented as in (B).
See also Figure S1.

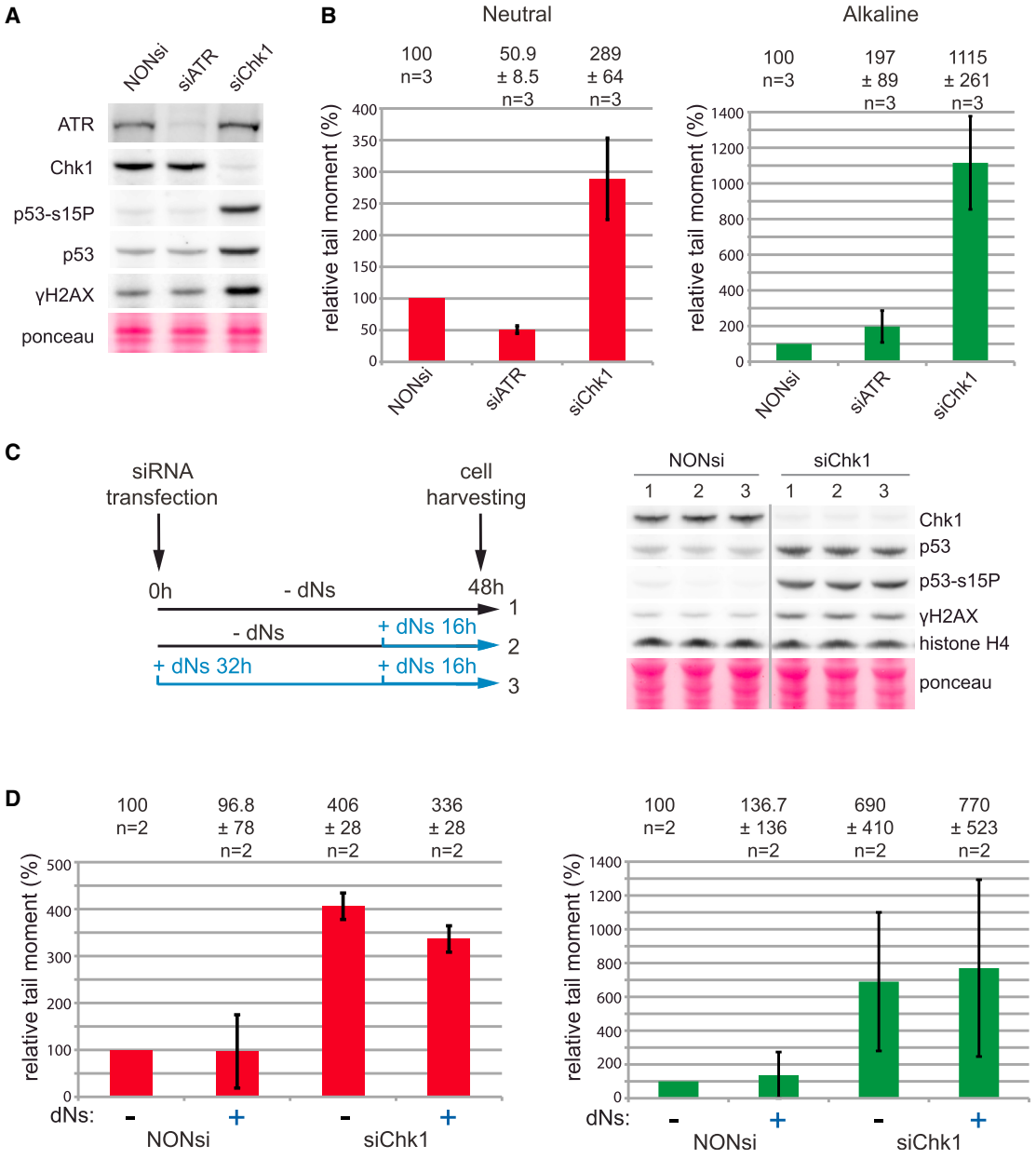


Figure 2. DNA Breaks Are Induced in Chk1- but Not in ATR-Depleted Cells

(A) Depletion of Chk1, but not ATR, activates DDR. Western blot analysis of cell extracts in the indicated transfection conditions. Ponceau staining was used as a loading control.

(B) DNA damages formed upon ATR and Chk1 depletion assessed by neutral (red) and alkaline (green) comet assays. Histograms represent the mean ± SD of relative tail moment. Transfection conditions, means, SD, and the number of independent experiments (n) are indicated.

(C) Addition of dNs does not suppress DDR activation in cells depleted of Chk1. Left: scheme of the protocol. Right: western blot analysis of cell extracts in the indicated conditions of transfection. Conditions of dN supplementation are numbered as in left panel.

(D) Addition of dNs does not suppress DNA damages in cells depleted of Chk1. Neutral (red) and alkaline (green) comet assays are presented as in (B).

See also Figure S2.

DNA Lesions in Chk1-Deficient Cells Rely on Mus81-Eme2 and Mre11 Nucleases

It has been reported that DNA damage arising in the absence of Chk1 depends on the nucleases Mre11 and Mus81 (Forment et al., 2011; Murfuni et al., 2013; Thompson et al., 2012). We found that co-depleting JEFF cells of Chk1 and either Mus81 or Mre11 weakly suppressed γH2AX and p53-s15P accumulation compared to cells depleted of Chk1 alone (Figure S3A). However, Chk1-deficient cells co-depleted of both nucleases display strongly reduced γH2AX and p53-s15P accumulation

(Figures 3A, S3A, and S3B). In good agreement with the DDR status, Comet assays show that depletion of both Mre11 and Mus81 reduces tail moment in Chk1-deficient cells (Figures 3B and S3C). In cells co-depleted of Chk1 and Mus81 and treated with 20 μM of mirin to inhibit Mre11 nuclease action (Shibata et al., 2014), p53-s15P accumulation is suppressed to the same level as in cells co-depleted of Chk1, Mus81, and Mre11 (Figure S3B). These results confirm that Mre11 nuclease action is essential to reach a threshold of damage triggering DDR.

The activity of Mus81 depends on its association with Eme1 in mitosis or Eme2 in S phase (Pepe and West, 2014). We therefore determined whether depletion of either protein affects the DDR status and/or the tail moment in control and Chk1-deficient cells (Figures 3C, 3D, and S3D–S3F). Depletion of Eme1 or Eme2 alone does not markedly impact these phenotypes. In a Chk1-deficient background, depletion of Eme1 reduces neither p53-s15P accumulation nor the tail moment, while depletion of Eme2 significantly rescues both phenotypes. Thus, in Chk1-deficient cells, depletion of Eme2 suppresses damage formation more efficiently than depletion of Mus81, most likely reflecting incomplete depletion of Mus81 (Figure 3A). We conclude that DDR activation arising spontaneously in Chk1-deficient cells relies on Mre11-dependent processing of lesions induced upon unscheduled activation of Mus81-Eme2.

Suppression of DNA Lesions Rescues Fork Speed in Chk1-Deficient Cells

We used molecular combing to determine whether depletion of both Mus81 and Mre11 (or mirin treatment instead of Mre11 depletion), or depletion of either Eme1 or Eme2, impacts replication dynamics in JEFF cells depleted or not of Chk1 (Figures 3E, 3F, and S3G). In Chk1-proficient cells, fork speed is not significantly impacted upon co-depletion of Mus81 and Mre11, while treatment with mirin or depletion of either Eme1 or Eme2 modestly slows fork movement. In Chk1-deficient cells, all conditions that reduce DNA damage significantly suppress fork slowing as compared to cells depleted of Chk1 alone. Conversely, depletion of Eme1, which does not prevent damage formation in Chk1-depleted cells, does not rescue fork movement (Figure 3F). Strikingly, across all genetic backgrounds tested, we found that fork speed shows a significant linear negative correlation with the level of p53-s15P ($R^2 = 0.75$; Figure 3G). Together, these results show that the degree of fork slowing depends on the level of damage present in the cells rather than on the Chk1 status per se.

DDR Signaling Regulates Fork Movement in Chk1-Deficient Cells

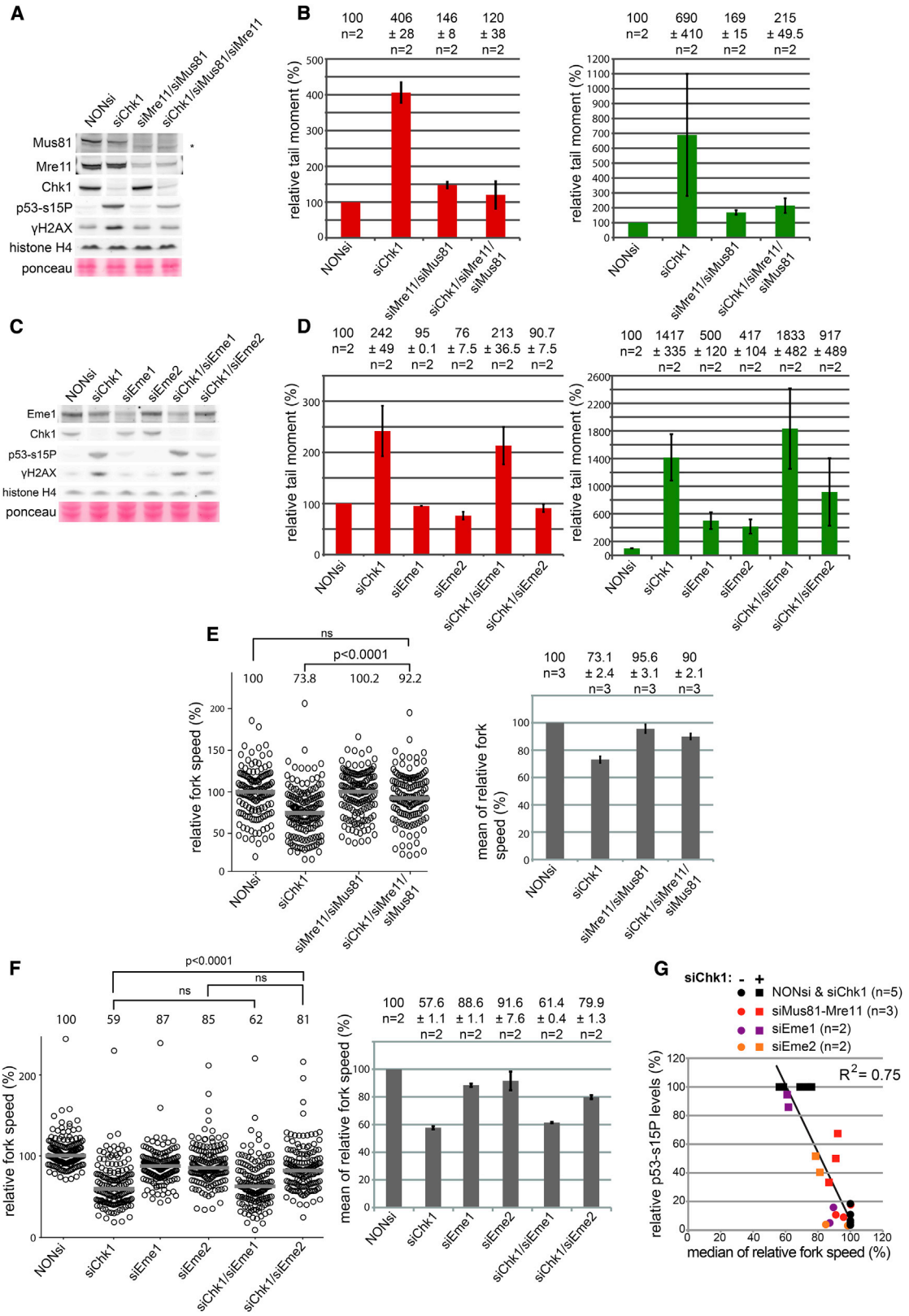
We then asked whether signaling of these DNA lesions by the ATM branch of the DDR is involved in fork speed regulation. As expected, turning off the ATM pathway in Chk1-deficient cells reduces p53-s15P accumulation (Figures 4A–4D and S4A) but not damage formation (exemplified in Figure 4E). Fork speed analyses showed that depletion of ATM, Chk2, or p53 alone does not significantly impact fork movement, but co-depletion of Chk1 and either protein significantly suppresses fork slowing compared to cells depleted of Chk1 alone (Figures 4A–4C). Similar results were obtained in cells treated with Chk2 inhibitor II (Figure S4B). In addition, activation of the ATM pathway has been shown to rely on the histone acetyltranferase Tip60 (also known as KAT5) (Gorrini et al., 2007; Kaidi and Jackson, 2013; Sun et al., 2005). We thus depleted Tip60 in JEFF cells depleted or not of Chk1. Because of a lack of antibodies able to detect the endogenous protein, depletion of Tip60 was assessed at the mRNA level by qRT-PCR (Figure S4C). We found that depletion of Tip60 alone does not affect fork speed but co-depletion of Tip60 and Chk1 significantly suppresses fork slowing compared to depletion of Chk1 alone (Figure 4D). Together, these results show that activation of the ATM branch of the DDR, not the breaks per se, induces fork slowing in Chk1-depleted cells.

JEFF cells co-depleted of Chk1 and either ATM, Chk2, p53, or Tip60 display lower level of p53-s15P than cells depleted of Chk1 alone (Figures 4A–4D). Quantification of residual accumulation of the marker remarkably shows that fork speed is inversely correlated to the level of p53-s15P ($R^2 = 0.9$) (Figure 4F), which strongly supports our previous conclusion.

Fork Slowing Depends on p53R2 in Chk1-Depleted Cells

We reasoned that p53R2, the RNR subunit regulated in a p53-dependent manner (Tanaka et al., 2000), could link DDR to dNTP availability in Chk1-depleted cells. We first determined whether Chk1 depletion impacts the level of p53R2 in JEFF cells. In good agreement with previous reports showing that p53R2 is upregulated in cells treated with DNA-damaging agents (Pontarin et al., 2008; Tanaka et al., 2000), we found that the level of p53R2 mRNA increases by a factor of 2 and the amount of p53R2 protein increases by approximately 60% in Chk1-depleted cells as compared to control cells (Figure S5A). We also studied the sub-cellular localization of the protein. It has been shown that most p53R2 localizes in the cytoplasm in control cells. Upon DNA damage, massive re-localization of RNR subunits has been described in some reports (D'Angiolella et al., 2012; Tanaka et al., 2000; Zhang et al., 2009), but not in others (Hu et al., 2012; Niida et al., 2010; Pontarin et al., 2008), suggesting that this response is cell type specific and/or depends on the type of damage. In JEFF cells, we observed that the protein is not massively imported in the nucleus upon Chk1 depletion (Figure S5B, left). Independently of cytoplasm to nucleus re-localization, previous reports have shown that nuclear RNR is recruited to repair foci in cells treated with DNA-damaging agents (Hu et al., 2012; Niida et al., 2010). We therefore performed immunofluorescence detection of p53R2 and 53BP1 in JEFF cells depleted or not of Chk1 (Figure S5B). We found that approximately 50% of 53BP1 foci display p53R2 signal, both in control and in Chk1-depleted cells, while the number of cells with 53BP1 foci and the number of 53BP1 foci per nucleus increase in Chk1-depleted cells (Figure S2D for quantification). Together, these results show that Chk1 depletion modulates the amount and the localization of p53R2, as do treatments with DNA-damaging agents.

We then depleted JEFF (Figure 5A, left) and CHEF (Figure S5C) cells of p53R2 and determined how this depletion impacts the replication dynamics. We found that depletion of p53R2 alone does not affect fork speed (Figures 5A and 5B). Noticeably, upon co-depletion of Chk1 and p53R2, fork speed increases significantly in JEFF cells and is completely restored in CHEF



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cells, compared to cells depleted of Chk1 alone (Figures 5A and 5B). Therefore, forks slow in a p53R2-dependent manner, suggesting that overexpression, modification and/or cellular localization of this RNR subunit limits precursors available for the replication machinery.

Chk1 Depletion Does Not Impact Measured dNTP Pools

In order to further explore this phenomenon, we asked whether DDR signaling impacts precursor pools. We measured dNTP concentrations in cell extracts using a classical enzymatic assay (Sherman and Fyfe, 1989). Because dNTP pools increase strongly in S phase, we compared cell-cycle distribution of cells depleted or not of Chk1. We found that the proportion of cells in S phase is not significantly affected upon Chk1 depletion (Figure S5D). Pool measurement showed no significant changes in JEFF and CHEF cells depleted of Chk1 relative to their respective controls (Figure 5C), consistent with a previous report showing that treatment of mammalian cells with DNA-damaging agents does not affect global dNTP pools (Håkansson et al., 2006).

To clarify the relationships linking fork movement to global pools, we measured fork speed in parallel to pool size in JEFF cells treated with increasing concentrations of HU. All drug concentrations tested caused significant, dose-dependent reduction of fork speed (Figure 5D), while the pools were altered in a more complex manner (Figures 5E, S5E, and S5F). As previously described (Skoog and Nordenskjöld, 1971), we found that treatment with 0.1–1 mM HU increases the pyrimidine pools and reduces the purine pools, with a major impact on dATP. Treatment with 20 μM HU specifically lowers the dATP pool, and treatment with 5 or 10 μM of HU does not significantly impact any of the pools. Thus, 5–10 μM of HU significantly slow replication forks, while global dNTP pools appear unaffected. Notably, the treatment with 10 μM of HU reduced fork speed to the same extent as Chk1 depletion. Hence, the measured global pools do not accurately reflect the concentrations of dNTPs available for DNA polymerases.

Origin Density Depends on Fork Speed, Not on Chk1 Status

In parallel to fork slowing, we observed that the median of inter-origin distances (IODs) decreases by approximately 30%–40% in JEFF and 25%–30% in CHEF cells upon Chk1 depletion (Figures 6A–6H). We therefore determined whether conditions that restore fork speed also restore IODs. We found, both in human and Chinese hamster cells, that dN supply restores normal IODs in cells depleted of Chk1 (Figure 6B). As IODs take into account mainly initiation events occurring within replicon clusters, we also measured fork densities that offer a more global estimation of origin usage. We found that fork density increases approximately 2.5-fold in JEFF cells depleted of Chk1 compared to control cells, and that this increase is totally abrogated in the presence of dNs (Figure 6C). We then determined whether suppression of damage or of damage signaling restores IOD in Chk1-depleted cells. We found that, compared to cells depleted of Chk1 alone, IODs significantly increase in Chk1-deficient cells co-depleted of Mus81 and Mre11 (Figure 6D), of Eme2 (Figure 6E), of ATM (Figure 6F), or of Tip60 (Figure 6G). IODs also significantly increase in JEFF and CHEF cells co-depleted of Chk1 and p53R2 (Figure 6H). Together, these results therefore link the activation of extra origins to damage signaling, precursor pools, and fork speed.

This conclusion was reinforced by plotting the median IODs against the median of relative fork speed, including the results of HU treatments (Figures S6A and S6B). We found that both replication parameters are linearly correlated (Figure 6I; R^2 = 0.81). Together these results show that the density of initiation events observed in all genetic contexts we studied, and in all conditions of HU treatment or dN supplementation, correlates with fork speed independently of the Chk1 status.

DNA Damage Is Responsible for Growth Alteration in Chk1-Deficient Cells

It has been repeatedly reported that Chk1-deficient cells display reduced growth capability, but whether this phenotype results from DNA damage formation or from abnormal replication dynamics is still debated (reviewed in González Besteiro and Gottifredi, 2015). We confirm here that the doubling time of JEFF cells depleted of Chk1 or treated with UCN-01 increases by a factor of 2 and 3, respectively (Figures 7A and 7B). The fact that it is possible to restore the replication dynamics or to attenuate damage formation in Chk1-deficient cells offered us the possibility to analyze the contribution of each parameter to growth impediment. We found that the doubling time of these cells is not improved upon dN addition, while it is significantly restored upon co-depletion of Chk1 and Eme2. These results suggest that growth rate is affected by DNA damages, not by modulation of the replication dynamics. Consistent with this view, we found that inhibition of Chk2 in cells deficient in Chk1 drastically alters cell growth, although it significantly restores the replication dynamics (Figure S4B).

Figure 3. Chk1-Deficient JEFF Cells Display Nuclease-Dependent DNA Lesions and DDR Activation, Resulting in Perturbed Fork Progression

(A) Co-depletion of Mus81 and Mre11 suppresses DDR activation in Chk1-depleted cells. Western blot analyses are presented as in Figure 2. Asterisk shows unspecific band.

(B) Co-depletion of Mus81 and Mre11 suppresses damages in Chk1-depleted cells. Neutral (red) and alkaline comet assays are presented as in Figure 2B.

(C and D) DDR activation (C) and DNA damages (D) are suppressed in cells co-depleted of Chk1 and Eme2. (D) Results are presented as in (A) and (B).

(E) Impact of Mus81 and Mre11 depletion on Chk1-dependent modulation of fork movement in JEFF cells. Data are presented as in Figure 1B. Left: ns indicates p value >0.05.

(F) Impact of Eme1 or Eme2 depletion on Chk1-dependent modulation of fork speed. Data are presented as in (E).

(G) Graph showing the linear correlation between relative median fork speed and relative p53 s15P levels. The coefficient of determination (R^2) is indicated.

See also Figure S3.

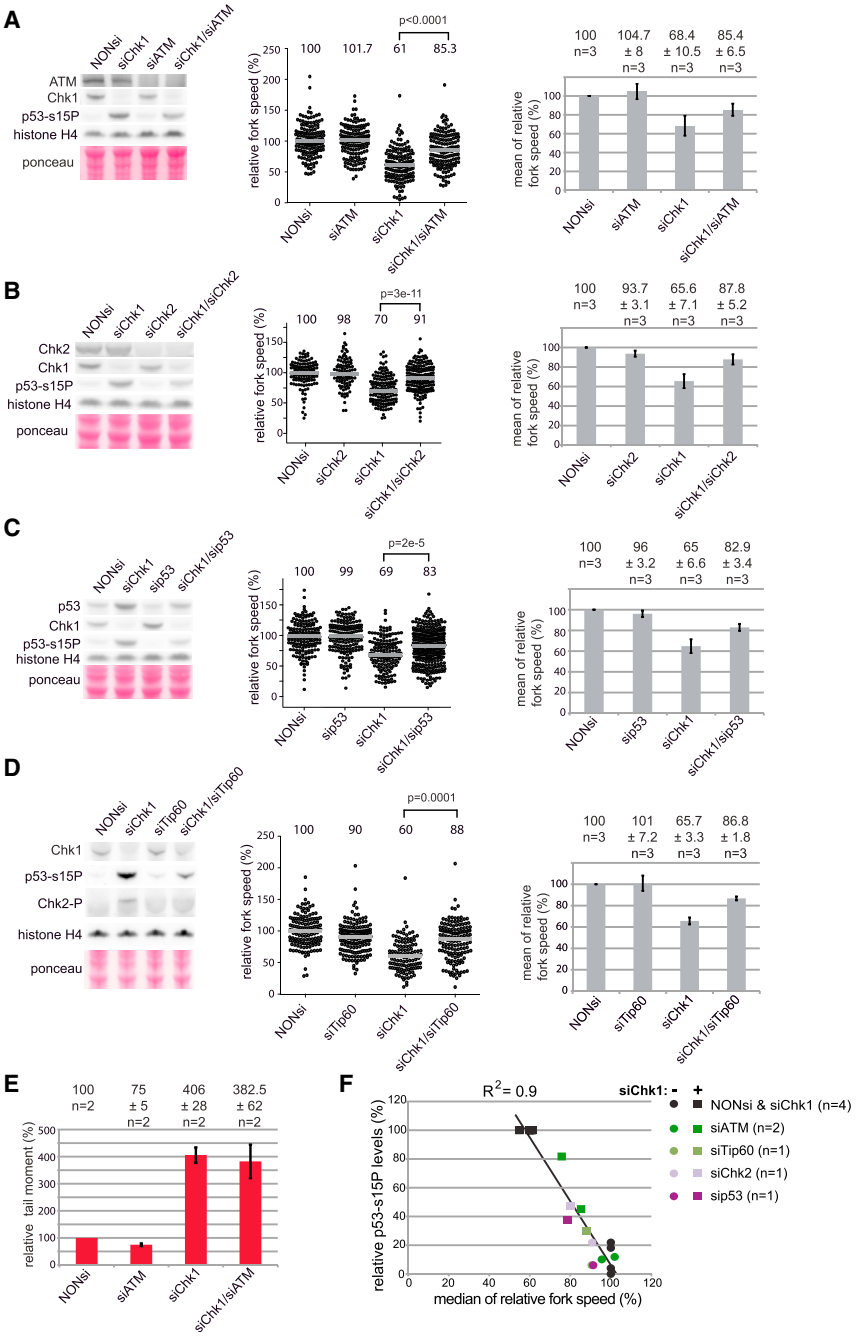

Figure 4. Inactivation of the ATM Branch of DDR Alleviates Fork Slowing in Chk1-Depleted JEFF Cells

(A–D) Impact of ATM (A), or Chk2 (B), or p53 (C), or Tip60 (D) depletion on fork speed in cells depleted or not of Chk1. Data are presented as in Figure 1B. Western blots: Ponceau staining and histone H4 were used as a loading control. The efficiency of Tip60 depletion was assayed by qRT-PCR due to the lack of antibodies recognizing the endogenous protein, see Figure S4C.

(E) Neutral comet assay performed with cells co-depleted of ATM and Chk1. Data are presented as in Figure 2B.

(F) Graph showing the linear correlation between relative median fork speed and relative p53 s15P levels. The coefficient of determination (R^2) is indicated. Data correspond to representative experiments shown in middle panel.

See also Figure S4.

anism. We show here that dN addition suppresses fork slowing in Chk1-deficient cells. By contrast, dNs fail to restore fork movement in ATR-depleted cells. These results show that different mechanisms are responsible for fork slowing in cells depleted of ATR or Chk1. They also exclude the possibility that exogenous dNs, through global facilitation of fork progression, result in non-specific bypass of any type of fork impediment. We therefore conclude that Chk1 deficiency most probably perturbs the replication dynamics by limiting dNTPs available for the replication machinery. We however failed to detect variations in the global dNTP pools of Chk1-depleted cells as compared to non-depleted cells. Strikingly, we also failed to detect pool reduction in non-depleted cells treated with very low doses of HU that nevertheless reduce fork speed to the same levels as Chk1 depletion. This latter result suggests either that the dNTP assay is not sensitive enough to detect small pool variations or that global pool measurement does not correctly reflect the amount of precursors locally available for DNA polymerases (see below).

DISCUSSION

ATR and Chk1 Modulate Fork Speed through Different Mechanisms

As previously reported for different cell types (reviewed in Zeman and Cimprich, 2014), we observed that deficiency in Chk1 or ATR leads to fork slowing and activation of extra-origins. Because the two proteins act sequentially in the ATR branch of the DDR, it was commonly considered that either depletion impacts the replication dynamics via the same mech-

Depletion of Chk1, but Not ATR, Triggers DNA Damages

Chk1 and Wee1, but not ATR, stood out in two high-throughput screens for kinases involved in the maintenance of genome stability in non-stressed cells (Beck et al., 2010; Domínguez-Kelly et al., 2011). Other reports have shown that Chk1 deficiency leads to spontaneous accumulation of DNA damage resulting in activation of the ATM branch of the DDR (Buisson et al.,

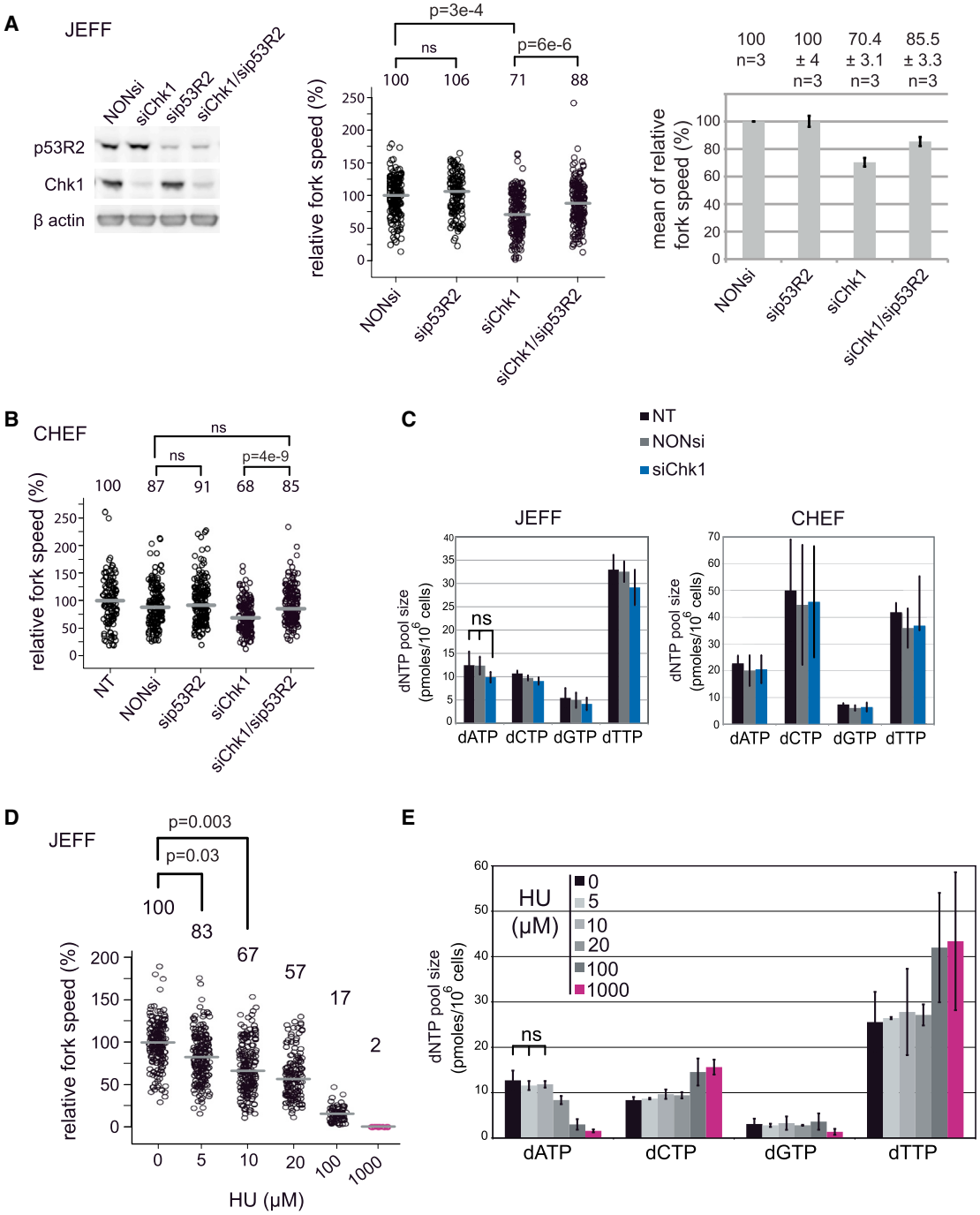

Figure 5. Relationships Linking p53R2 to Fork Speed and Pool Size in Cells Depleted of Chk1, Comparison with Cells Treated with HU

(A and B) Depletion of p53R2 alleviates fork slowing induced upon Chk1depletion. (A) JEFF cells: data presented are as in Figure 1B. (B) CHEF cells: dot plots of relative distributions of fork speed. In these cells, the efficiency of p53R2 depletion was assayed by qRT-PCR due to the lack of antibodies recognizing the hamster protein (see Figure S5C).

(C) Determination of dNTP pool size in the indicated transfection conditions in JEFF (left) and CHEF (right) cells. The mean ± SD of dNTP pools has been calculated from five and four independent experiments in JEFF and CHEF cells, respectively. ns, not significant (p value >0.05). Note that the percentage of cells in S phase is not altered upon Chk1 depletion in JEFF cells (see Figure S5D).

(D and E) Impact of various doses of HU on fork speed and dNTP pools in JEFF cells. (D) Dot plots are as in Figure 1. JEFF cells were treated for 6 hr with the indicated doses HU. (E) Pool size in JEFF cells treated as in (D). Mean of two to four independent experiments is shown. Bars indicate SD. Normalization of measured dATP pools by the percentage of S phase is presented in Figures S5E and S5F.

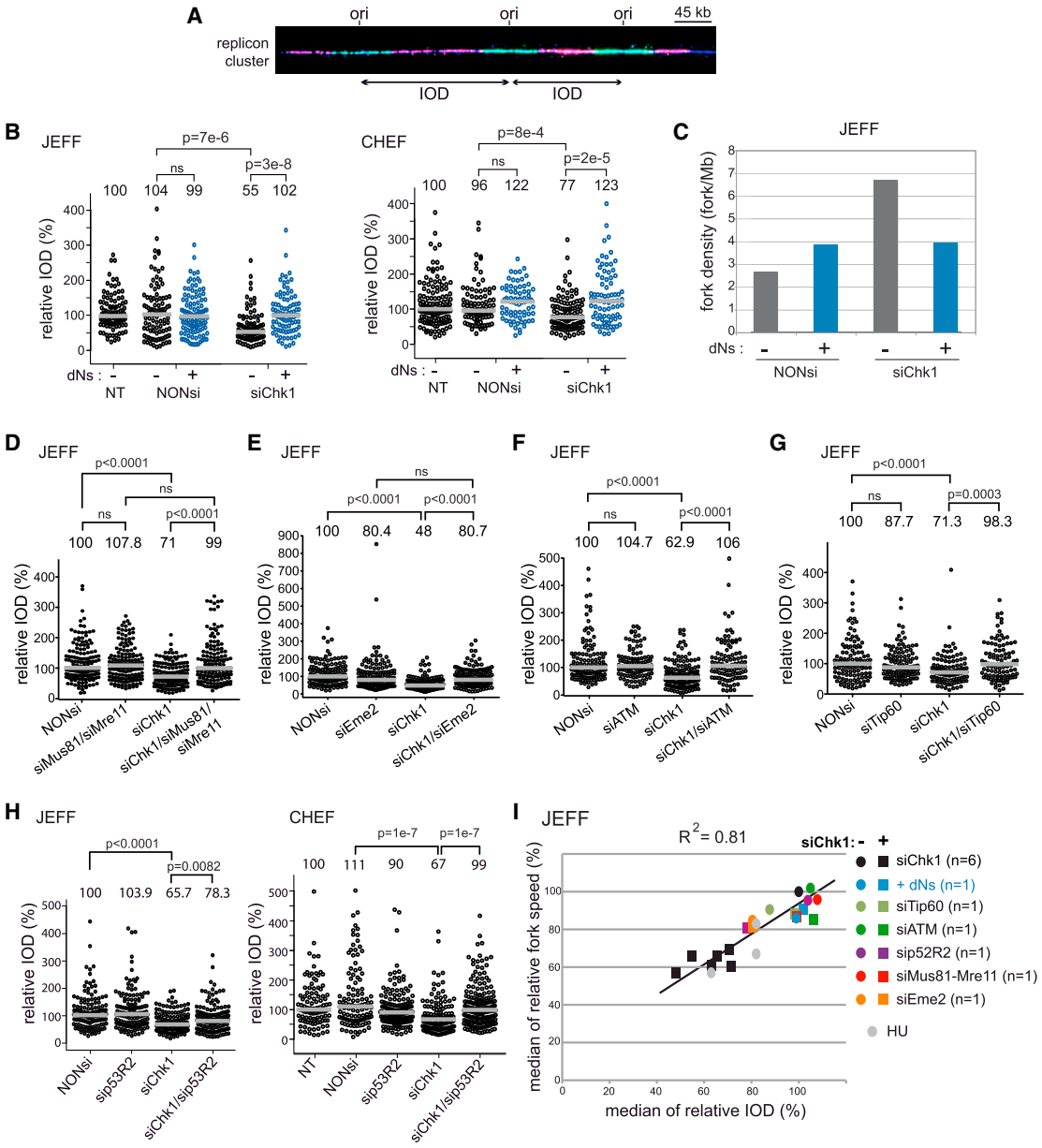

Figure 6. Origin Firing Is Fine-Tuned by Fork Speed Independently of the Chk1 Status

(A) Example of a replicon cluster. IOD, distance separating two adjacent origins (ori).

(B) Distribution of relative IODs in JEFF and CHEF cells (as indicated). Data are presented as in Figure 1. Median IODs in non-transfected or NONsi-transfected cells were used to normalize each set of data.

(C) Fork density was assessed in JEFF cells transfected and supplemented as indicated. Fork density is the number of forks divided by the total length of DNA molecules (Mb) and normalized by the percentage of S phase cells. Corresponding percentages of cells in S phase are shown in Figure S4D.

(D–H) Distribution of relative IODs. Data are presented as in (B).

(I) Graph showing the linear correlation between relative median fork speed and relative median IOD in JEFF cells. The coefficient of determination (R^2) is indicated. Results correspond to experiments showing fork speed in Figure 1B (dNs), Figure 3E (Mus81-Mre11), Figure 3F (Eme2), Figure 4A (ATM), Figure 4D (Tip60), Figure 5A (p53R2), and Figure 5D (HU) are presented. Values of relative median IODs upon HU treatment are shown in Figures S6A and S6B.

2015; Forment et al., 2011; Syljuåsen et al., 2005; Thompson et al., 2012), while ATR deficiency does not, or does so only weakly (Couch et al., 2013; Eykelenboom et al., 2013; Toledo et al., 2013). Here, we confirm the major role of Chk1 and the minor role of ATR in the maintenance of genome integrity in unchallenged lymphoblastoid JEFF cells. These results contrast with the key role of ATR in fork stabilization under replication stress, notably through the control of RPA pool (Toledo et al., 2013) and SMARCAL1 activity (Couch et al., 2013). The apparently ATR-independent role of Chk1 we observed in unstressed cells

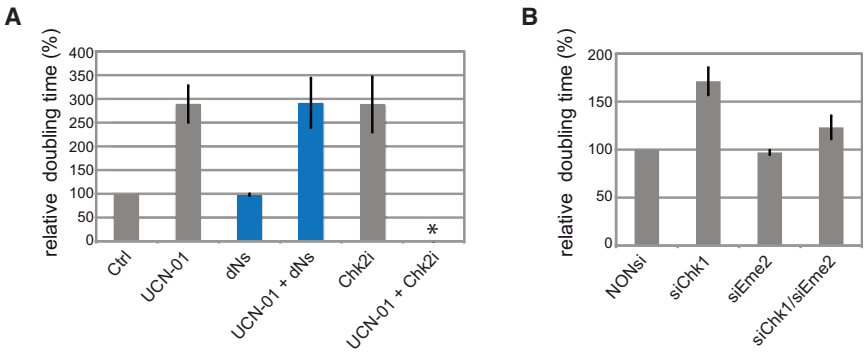

Figure 7. DNA Damage Impact Growth Rate of Cells Deficient in Chk1

(A) Doubling time of JEFF lymphoblastoid cells untreated (Ctrl) or in the indicated conditions of treatment. Cells were counted each 24 hr during 1 week. Growth curves were built and doubling time was calculated. Histograms represent the mean ± SD (n = 2). *Cells treated with both UCN-01 and Chk2i completely fail to grow.

(B) Doubling time of JEFF cells transfected with control siRNA (NONsi), Chk1 siRNAs (siChk1), and/or Eme2 siRNAs (siEme2). Cells were counted each 24 hr during 3 days post transfection, namely, during the period of efficient depletion. Histograms as in (A) (n = 3).

most probably reflects the redundancy of upstream kinases. Indeed, a recent report shows that Chk1 deficiency disrupts not only the canonical ATR-Chk1 cascade, but also the newly described DNA-PK-Chk1 pathway (Buisson et al., 2015).

DNA Damages Accumulated in Chk1-Depleted Cells Depend on Nucleases

The mechanisms involved in DNA damage formation in Chk1-depleted cells have been intensively studied, but the interpretation of the results remains unclear. One model proposes that modulation of the replication dynamics occurring in Chk1-deficient cells triggers the damage (Syljuåsen et al., 2005). However, this hypothesis is inconsistent with several observations: (1) cells treated with low doses of HU or aphidicolin that modulate the replication dynamics in the same proportion as Chk1 depletion do not display DNA lesions (Domínguez-Kelly et al., 2011; Koundrioukoff et al., 2013; Wilhelm et al., 2014), (2) at least in the cells studied here, ATR depletion does not impact genome stability in the absence of exogenous stress while it affects the replication dynamics in the same proportion as Chk1 depletion, (3) restoration of normal replication dynamics upon dN addition does not suppress damage in Chk1-depleted cells.

It has been reported that damage arising in the absence of Chk1 rely on nucleases Mre11 (Thompson et al., 2012) and/or Mus81 (Forment et al., 2011). Indeed, we found that in JEFF cells depleted of Chk1, co-depletion of Mre11 and Mus81 or treatment with mirin, an inhibitor of Mre11 activity, efficiently suppresses accumulation of γH2AX and p53-s15P. Mus81 can associate with Eme1 or Eme2. Mus81-Eme2 promotes cleavage and restart of stalled forks in S phase, while Mus81-Eme1 preferentially cleaves recombination intermediates in G2/M (Pepe and West, 2014). We show here that depletion of Eme2, but not of Eme1, suppresses break formation and DDR activation in Chk1-depleted cells. Together our results strongly suggest that unscheduled activation of Mus81-Eme2 elicits inappropriate cleavage of forks staling at sequences intrinsically difficult to replicate or at DNA lesions arising spontaneously during S phase. Mre11-dependent resection of cleavage products would then trigger the ATM pathway.

Regulation of Mus81 activity by cell-cycle kinases has been reported in budding yeast (Gallo-Fernández et al., 2012; Matos et al., 2013), and a recent report proposes similar regulation in mammalian cells (Choi et al., 2013). Several reports also suggest a functional link between Mre11 and the core cell-cycle kinases, notably Cdk2 (Buis et al., 2012; Huertas and Jackson, 2009; Peterson et al., 2011). In addition, it has been shown that Cdk2 activity increases upon Chk1 depletion (Buisson et al., 2015; Sørensen et al., 2003; Zhao et al., 2002), and that partial inhibition of Cdks by roscovitine or depletion of Cdc25A alleviates DNA damages in Chk1-deficient cells (Syljuåsen et al., 2005; Thompson et al., 2012). We therefore propose that DNA damage elicited in Chk1-deficient cells may be due to Cdk2 upregulation, which results in unscheduled activation of Mus81-Eme2 and Mre11 activities.

Fork Slowing in Chk1-Deficient Cells Relies on Damage Formation and Signaling

Here, we report that fork speed is restored in Chk1-depleted cells in conditions that suppress damage, notably depletion and/or inactivation of Mus81-Eme2 and Mre11. Conversely, restoration of DNA replication dynamics upon dN addition does not suppress damage. These results show that the damage is responsible for fork slowing, not the reverse. In good agreement with this conclusion, it has been previously shown that exogenously induced DNA lesions slow fork progression (Merrick et al., 2004; Shimura et al., 2006).

We then questioned the role of damage signaling in the control of fork movement in Chk1-depleted cells. We analyzed cells co-depleted of Chk1 with either Tip60/KAT5, ATM, Chk2, or p53. We found that p53-s15P accumulation is reduced and fork movement significantly restored in co-depleted cells as compared to cells depleted of Chk1 alone. Fork slowing in Chk1-depleted cells therefore relies on activation of the ATM branch of the DDR.

The accumulation of p53-s15P was incompletely suppressed in cells co-depleted of Chk1 and nucleases or of Chk1 and either intermediate of the ATM signaling pathway. This situation may result from incomplete depletion of the proteins understudy and/or from crosstalk within the DDR network. We took advantage of the situation to compare the level of residual p53-s15P to that of fork speed rescue. We remarkably found that both parameters are inversely correlated, which again supports our conclusion that signaling of damages up to p53 dictates fork speed in Chk1-depleted cells.

P53R2 Modulates Fork Speed in Chk1-Depleted Cells

One target of activated p53 is the gene encoding p53R2 (Tanaka et al., 2000). We thus asked whether p53R2 contributes to fork slowing in Chk1-depleted cells. We observed that depletion of p53R2 alone does not induce DNA damage and does not impact fork speed, which confirms that p53R2 is not strongly involved in the constitution of global pools in undamaged S phase cells (reviewed in Mathews, 2015). Although counterintuitive, we found that depletion of p53R2 significantly rescues fork speed in Chk1-depleted cells, as does silencing of other proteins of the ATM pathway.

In contrast to yeast cells, mammalian cells do not respond to damage formation by a major expansion of dNTP pools (this study and Håkansson et al., 2006). However, the pools may be regulated locally, for example, within sub-nuclear compartments created by the recently described PAR-mediated protein assemblies (Altmeyer et al., 2015). In agreement with this possibility (1) nuclear RNR re-localizes to repair foci in G1 cells treated with DNA-damaging agents, which most probably increases local dNTP concentration and facilitates repair (Hu et al., 2012; Niida et al., 2010), (2) an enrichment of R1 and R2 in newly replicated chromatin has been shown in untreated cells (Alabert et al., 2014), possibly mediated by direct interaction between DNA polymerase α and R1-R2 (Taricani et al., 2014), suggesting that at least part of nuclear RNR is associated with the forks. This association may favor the channeling of precursors to replication forks. Consistently, it was estimated that the effective dNTP concentration at the level of replication forks is 3- to 4-fold higher than the global intracellular concentration (Mathews and Sinha, 1982). Whether localization of part of nuclear RNR to repair foci upon damage disturbs the association of RNR with replication forks and how p53-dependent regulation of p53R2 contributes to this process remain unknown. Alternatively, subtle modulation of the global pools may escape detection with available techniques, as suggested here in the case of cells treated with low doses of HU. Further work will be needed to decipher the regulation of RNR in control cells and in cells containing DNA lesions. Nevertheless, the fact that depletion of a RNR subunit alleviates fork impediment in Chk1-deficient cells points to a molecular link between DNA damage, precursor availability, and fork speed.

Origin Density Is Modulated by Fork Speed Rather Than the Inverse

Previous results, which we confirm here, have shown an increase in the density of initiation events in Chk1-depleted cells (Katsuno et al., 2009; Maya-Mendoza et al., 2007; Petermann et al., 2010). We show in addition that the density of initiation strictly correlates with fork speed in all genetic backgrounds we studied. This tight inter-dependence of the two parameters does not allow us to determine whether DNA damage primarily controls fork speed or origin firing. However, the key role of precursors in the restoration of replication dynamics gives clue to this question. Indeed, in the absence of Chk1, addition of dNs or p53R2 depletion restores both fork speed and IODs. These results strongly suggest that the increase in origin firing observed in Chk1-deficient cells is the consequence, not the cause, of dNTP starvation. In this hypothesis, origin spacing would be defined independently of the Chk1 status by the so-called compensation mechanism. This regulatory process maintains the rate of DNA synthesis through modulation of initiation density according to fork speed variations (reviewed in Yekezare et al., 2013).

It was reported that Cdk2 downregulation restores both origin density and fork speed in Chk1-depleted cells (Petermann et al., 2010; Syljuåsen et al., 2005), which suggested a model in which Chk1 represses latent origins in a Cdk2 dependent-manner in non-perturbed S phases. In Chk1-depleted cells, the increase in active origins would titrate some limiting factor, resulting in fork slowing. This switch in replication dynamics would then cause DNA lesions. As discussed above, in Chk1-depleted cells damage results from unscheduled activation of Mus81-Eme2 and Mre11 and is the cause, not the consequence, of the replication phenotype. In addition, dN supply not only restores fork speed, but it also restores origin density, which is not expected in the model presented above. Together with reports suggesting that Cdk2 stimulates the activity of Mus81 and Mre11 (Choi et al., 2013; Thompson et al., 2012), our work suggests a new interpretation of previous results. Indeed, downregulation of Cdk2 or Cdc25A could alleviate DNA damage by limiting nuclease activity, which would restore fork movement and, in turn, origin density.

Conclusions

A number of endogenous and exogenous conditions may interfere with DNA replication and perturb S phase progression, resulting in DNA damage. Collectively, these conditions are commonly referred as to inducing "replication stress," resulting in genome instability. Notably, Chk1 deficiency leads to modulation of fork movement and initiation density, features that are generally considered as the archetype of replication stress. We show here that such modulation of the replication dynamics is not responsible for damage taking place in these cells. Rather, by slowing fork movement, the pathway we identified may contribute to stabilize fork progressing along damaged templates while activation of latent origins may ensure an efficient backup in case of fork stalling or collapse. In good agreement with this view, we found that DNA damage, not modulation of the replication dynamics, is responsible for growth alteration in Chk1-deficient cells. We therefore propose that such moderate modification of the replication dynamics is not a deleterious process but rather represents a protective mechanism elicited in response to DDR activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Nucleotide Precursors Complementation

JEFF cells were grown in RPMI 1640 + GlutaMAX-I medium. CHEF cells were grown in minimum essential medium (MEM) supplemented with Earle's salts, 1% MEM amino acids, 1 mM sodium pyruvate, and 2 mM L-glutamine. Media contained 10% fetal calf serum and 100 μg/ml of penicillin and streptomycin. UCN-01, mirin, and Chk2 inhibitor II were purchased from Sigma and VE-822 from Active Biochem. Media were complemented with dNs (Sigma), at a concentration of 20 μM each.

Molecular Combing

Combing and immunodetection are described in Supplemental Experimental Procedures.

Statistical Analysis of Molecular Combing Data

The R environment was used for all the analyses (R Development Core Team [2011]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, https://www.r-project.org/). Graphs and R code will

be sent upon request. Statistical significance was set to $p \leq 0.05$, and p values are shown on graphs. Further information on statistical analyses are presented in Supplemental Experimental Procedures.

Measurement of dNTP Pool Size

Briefly, cells were lysed in 10% ice-cold trichloroacetic acid (TCA) and 15 mM $MgCl_2$ (15 min on ice). After centrifugation (10 min at 10,000 × *g*), the supernatant was neutralized by two extractions with a mixture of trifluoromethane (78% v/v) and trioctylamine (22% v/v). The aqueous phase was stored at −80 C° for later analysis. Quantification of dNTPs was carried out as described by Sherman and Fyfe (1989).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.093.

AUTHOR CONTRIBUTIONS

H.T., S.K., S.C., T.W., and O.B. carried out experiments. G.A.M. performed statistical analysis. M.D. planned the project. H.T., S.K., B.S.L., O.B., and M.D. wrote and edited the paper.

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