

REPORT

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## NEK8 regulates DNA damage-induced RAD51 foci formation and replication fork protection

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### ABSTRACT

Proteins essential for homologous recombination play a pivotal role in the repair of DNA double strand breaks, DNA inter-strand crosslinks and replication fork stability. Defects in homologous recombination also play a critical role in the development of cancer and the sensitivity of these cancers to chemotherapy. RAD51, an essential factor for homologous recombination and replication fork protection, accumulates and forms immunocytochemically detectable nuclear foci at sites of DNA damage. To identify kinases that may regulate RAD51 localization to sites of DNA damage, we performed a human kinome siRNA library screen, using DNA damage-induced RAD51 foci formation as readout. We found that NEK8, a NIMA family kinase member, is required for efficient DNA damage-induced RAD51 foci formation. Interestingly, knockout of *Nek8* in murine embryonic fibroblasts led to cellular sensitivity to the replication inhibitor, hydroxyurea, and inhibition of the ATR kinase. Furthermore, NEK8 was required for proper replication fork protection following replication stall with hydroxyurea. Loading of RAD51 to chromatin was decreased in NEK8-depleted cells and *Nek8*-knockout cells. Single-molecule DNA fiber analyses revealed that nascent DNA tracts were degraded in the absence of NEK8 following treatment with hydroxyurea. Consistent with this, *Nek8*-knockout cells showed increased chromosome breaks following treatment with hydroxyurea. Thus, NEK8 plays a critical role in replication fork stability through its regulation of the DNA repair and replication fork protection protein RAD51.

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DNA repair; genome instability; homologous recombination; NEK8; RAD51; replication fork protection

### Introduction

Defects in DNA repair play a critical role in development of cancer and sensitivity of cancer cells to anti-cancer drugs. Homologous recombination (HR) is a mechanism of DNA repair that utilizes the undamaged homologous sequence as a template to repair double strand breaks (DSBs).<sup>1</sup> HR is particularly important for preventing cancer development, as exemplified by the observation that the genes regulating HR, such as *BRCA1* and *BRCA2*, are tumor suppressor genes linked to breast/ovarian cancer.<sup>2,3</sup> Cancer cells that are defective in HR are sensitive to various anti-cancer drugs including interstrand DNA crosslinking agents (cisplatin, carboplatin and mitomycin C (MMC)) and poly (ADP-ribose) polymerase (PARP) inhibitors.<sup>4</sup> Therefore, a better knowledge of HR regulation will further our understanding of both cancer development and cancer therapy.


In higher eukaryotes, the genetic inactivation of many HR genes leads to lethality during the very early stages of development, suggesting that these proteins likely play key roles in DNA replication or in the repair of replication errors.<sup>5</sup> HR is required for the restart of replication forks in fission yeast, but the specific role of HR in replication fork restart in higher

eukaryotes remains elusive.<sup>6,7</sup> One key HR protein involved in DNA replication is the recombinase RAD51, which is also a Fanconi anemia protein, *FANCR*.<sup>8</sup> RAD51 localization to active replication forks is required to prevent the accumulation of single-stranded DNA (ssDNA) regions directly at the fork. It is also hypothesized that this accumulation of RAD51 is required to prevent MRE11-dependent degradation of nascent DNA, which allows for continuous DNA replication.<sup>9</sup> Other proteins involved in the Fanconi anemia and HR pathway also have important roles in replication fork protection. For example, *BRCA2/FANCD1* is directly required for replication fork protection<sup>10</sup> through its recruitment of RAD51 to protect nascent DNA.<sup>11</sup>

NEK8 is a member of the human NIMA-related kinase (NEK) family,<sup>12</sup> which contains 11 serine/threonine protein kinases. NEKs have mainly been studied in relation to their role in cell cycle progression, centrosome regulation, and cilio-genesis. NEK8 contains an N-terminal kinase domain. NEK8 differs from all but one other NEK kinase, NEK9, in that its C-terminal domain contains motifs similar to the regulator of chromatin condensation protein, *RCC1*.<sup>13</sup> A missense mutation in the *RCC1* domain of *Nek8* is reported as phenotypically

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causative in the mouse model of juvenile cystic kidney disease (JCK), where ciliary localization of the mutant Nek8 protein is defective.<sup>14</sup> Similarly, mutations in the conserved RCC1 domain of *Nek8* are causative of the phenotypes observed in the rat model of Lewis polycystic kidney disease.<sup>15</sup> Germline mutations have been identified in human *NEK8* that are implicated in the childhood autosomal recessive kidney disease nephronophthisis (NPHP),<sup>16</sup> in 3 patients with Ivemark syndrome, which is similar to polycystic kidney disease,<sup>17</sup> and in patients initially believed to have Alagille syndrome.<sup>18</sup> Most recently, novel *NEK8* mutations were identified in 5 familial ciliopathy cases, where *NEK8* missense mutations cause increased  $\gamma$ H2AX foci, suggesting defects in DNA repair, which may lead to increased apoptosis during cell proliferation.<sup>19</sup> Furthermore, a missense mutation of *NEK8* is reported as a potential driver mutation in pancreatic cancer<sup>20</sup> and *NEK8* is overexpressed in human breast cancer.<sup>21</sup> These findings suggest a role of *NEK8* in cancer development.

Intriguingly, *NEK8* localizes not only to the centrosomes<sup>16</sup> and primary cilium,<sup>22</sup> but also to the nucleus.<sup>12</sup> Nuclear functions of *NEK8* had not been studied until recently where *NEK8* was linked to the ATR-mediated replication stress response via regulation of the protein kinase CDK2.<sup>23</sup> Cells deficient in *NEK8* are characterized by an increase in histone H2AX phosphorylation, a sign of spontaneous DSBs. These DSBs further accumulate when replication forks stall. *NEK8*-deficient cells also exhibit reduced replication fork rates, unscheduled origin firing, and increased replication fork collapse.<sup>23</sup> As a result, *NEK8*-deficient cells are sensitive to replication inhibition by aphidicolin, a phenotype which is rescued by inhibition of CDK activity. Interestingly, *NEK8* interacts with the checkpoint kinase ATR, CHK1 and the ATR interacting partner, ATRIP.<sup>23</sup> Furthermore, kidneys of *Nek8*-mutant mice accumulate DNA damage, and loss of *Nek8* or replication stress similarly disrupts renal cell architecture in these mice.<sup>23</sup> Therefore, there exists evidence that *NEK8* functions in the DNA damage response and DNA replication.

Phosphorylation plays an important role in the regulation of the DNA damage and replication stress responses. Several kinases have been implicated in the regulation of HR,<sup>24–27</sup> and multiple high-throughput screens have been used to identify factors required for HR and the replication stress response.<sup>28–31</sup> However, a comprehensive analysis of kinases involved in the regulation of HR following damage with an inter-strand cross-linking agent has not been reported leaving our understanding of the regulation of HR by phosphorylation incomplete.

Here we show that *NEK8* is a critical regulator of RAD51 focus formation following DNA damage and genome stability following replication stress.

## Results

### **Kinome siRNA screening identified *NEK8* as a protein important for DNA damage-induced RAD51 foci formation**

To identify novel kinases that regulate DNA damage-induced RAD51 foci formation, initially we performed an siRNA kinome library screen. The siRNA library contained 3 siRNA duplexes per gene targeting 713 human kinases. We performed

the screen using inhibition of MMC-induced RAD51 foci formation in the human osteosarcoma cell line, U-2 OS, as read-out. RAD51 foci were quantified with an automated high-throughput fluorescence microscope equipped with foci counting software (Fig. 1A). The average number of foci per cell was converted to normalized z-scores (Fig. 1B and Table S1), where we focused on kinases with negative z-scores. Negative z-scores are indicative of kinase depletion leading to inhibited RAD51 foci formation following DNA damage. As RAD51 foci form mainly in S and G2 phases of the cell cycle,<sup>32</sup> we evaluated the percentage of cells in S phase following kinase depletion, using cyclin A immunostaining (Fig. S1 and Table S1). A total of 20 kinases were selected as positive hits (inhibition of RAD51 foci without decrease of cyclin A positive cells) in the primary screen (Table S1). To validate the result, the pooled siRNAs were de-convoluted and the 3 independent siRNAs against each candidate kinase were used to confirm the result in multiple cell lines (Fig. S1C and S2A–B).

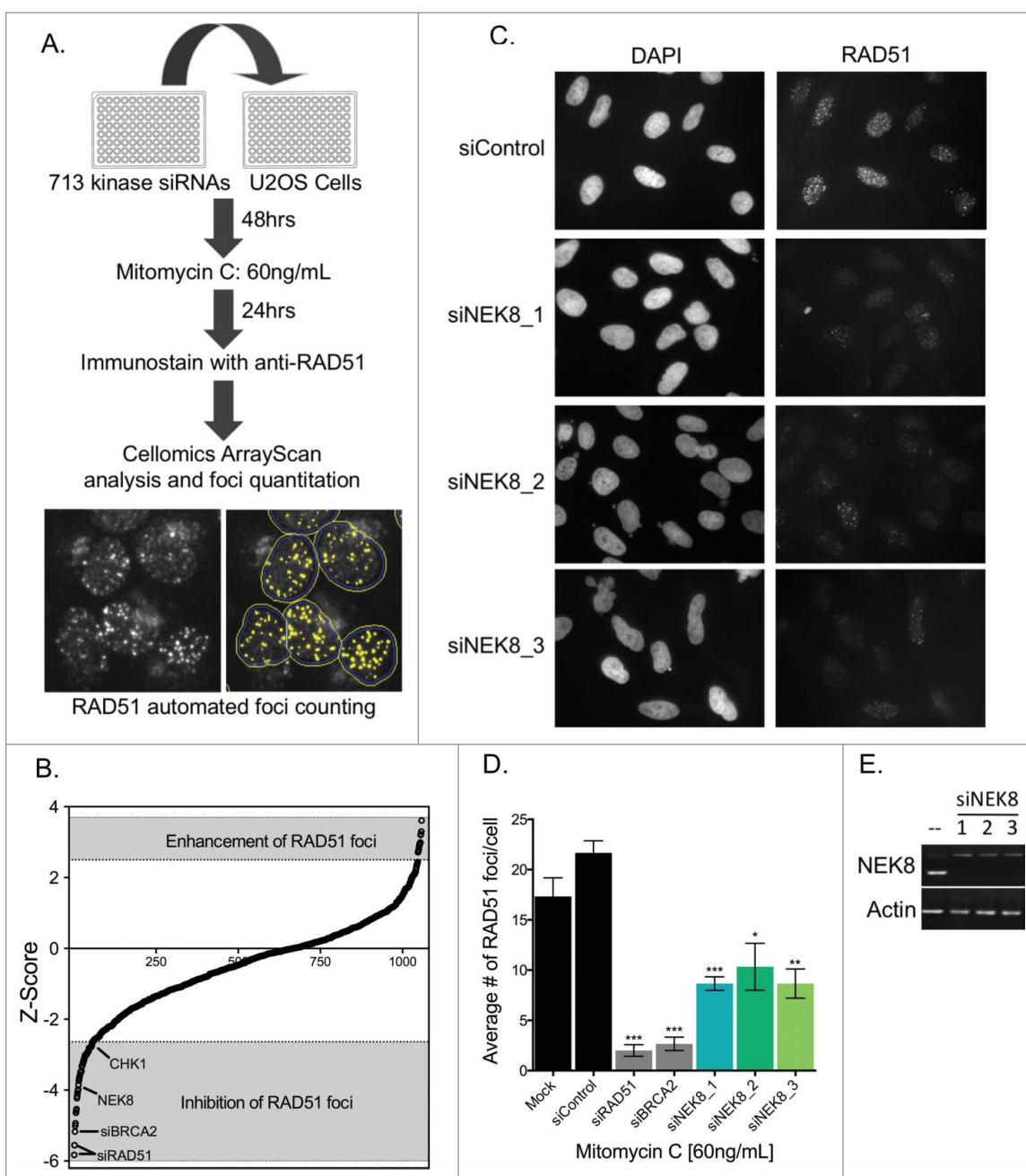
Among those genes whose siRNAs decreased MMC-induced RAD51 foci formation, *NEK8*, *PAK4* and *DGKD* emerged as promising candidates (Fig. 1B and S1C). We chose to focus on *NEK8* as other NIMA-related kinases had been implicated in proper cell cycle progression and the DNA damage response,<sup>33</sup> and *NEK8* depletion by 3 independent siRNAs led to inhibition of MMC-induced RAD51 foci formation, while efficiently reducing *NEK8* mRNA levels (Fig. 1C–E).

### ***NEK8* modulates RAD51 foci formation following DNA damage and replication fork stall**

To determine if the effect *NEK8* depletion had on RAD51 foci formation was specific to interstrand cross-links created by MMC, we next treated *NEK8*-depleted U-2 OS cells with various DNA damaging agents (Fig. S3A–D). RAD51 foci were also reduced in *NEK8*-depleted cells after treatment with ionizing radiation (IR) (Fig. 2A–B) as well as the replication inhibitor, hydroxyurea (HU) (Fig. 2B), suggesting that the effect of *NEK8* depletion on RAD51 foci formation is not limited to inter-strand cross-links. We also tested *Nek8*<sup>−/−</sup> mouse embryonic fibroblasts (MEFs) and control MEFs for Rad51 foci formation following DNA damage. Reduced DNA damage-induced Rad51 foci formation was observed in *Nek8*<sup>−/−</sup> MEFs in all conditions tested (Fig. 2E–F). Moreover, we observed similar phenotypes in the additional human cell line, HeLa, following treatment with MMC and HU (Fig. S4), suggesting that the effect of *NEK8* deficiency on RAD51 foci formation is not cell type or species specific.

As formation of RAD51 foci is contingent on many key upstream factors and events in the HR pathway,<sup>1</sup> we analyzed the effect of *NEK8* depletion on the expression of a few of these factors. Depletion of *NEK8* did not affect the expression of the proteins important for RAD51 foci formation, such as BRCA2, RPA, and CHK1 (Fig. 2C). Similar results were observed in *Nek8*<sup>−/−</sup> MEFs (Fig. 2G). Furthermore, HU induced RPA foci form normally in *NEK8*-depleted U 2-OS cells (Fig. S5A) and in *Nek8*-knockout MEFs compare with control MEFs (Fig. S5B) following treatment with HU.

We noted a mild decrease in RAD51 protein expression following depletion of *NEK8* with one of the *NEK8* siRNAs



**Figure 1.** Kinome siRNA screening identified NEK8 as a protein important for DNA damage-induced RAD51 foci formation. **A.** Screening strategy for identification of kinases required for RAD51 focus formation. U-2 OS cells were transfected with a siRNA kinome library, treated with MMC, immunostained and imaged for RAD51 focus formation. Representative images of RAD51 focus formation via automated acquisition and quantitation are shown. **B.** The mean Z-score of each kinase from the RAD51 foci screen are ranked (n = 2). **C.** Representative image of MMC (60 ng/mL, 24h)-induced RAD51 foci in U-2 OS cells. **D.** Quantification of the average number of RAD51 foci observed per U-2 OS cell following treatment with MMC (60 ng/mL, 24h). **E.** U-2 OS cells were transfected with siRNA (20nM). RNA was collected, converted to cDNA and amplified by PCR to confirm depletion. (n = 3, +/- SEM). \* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001 relative to siControl.

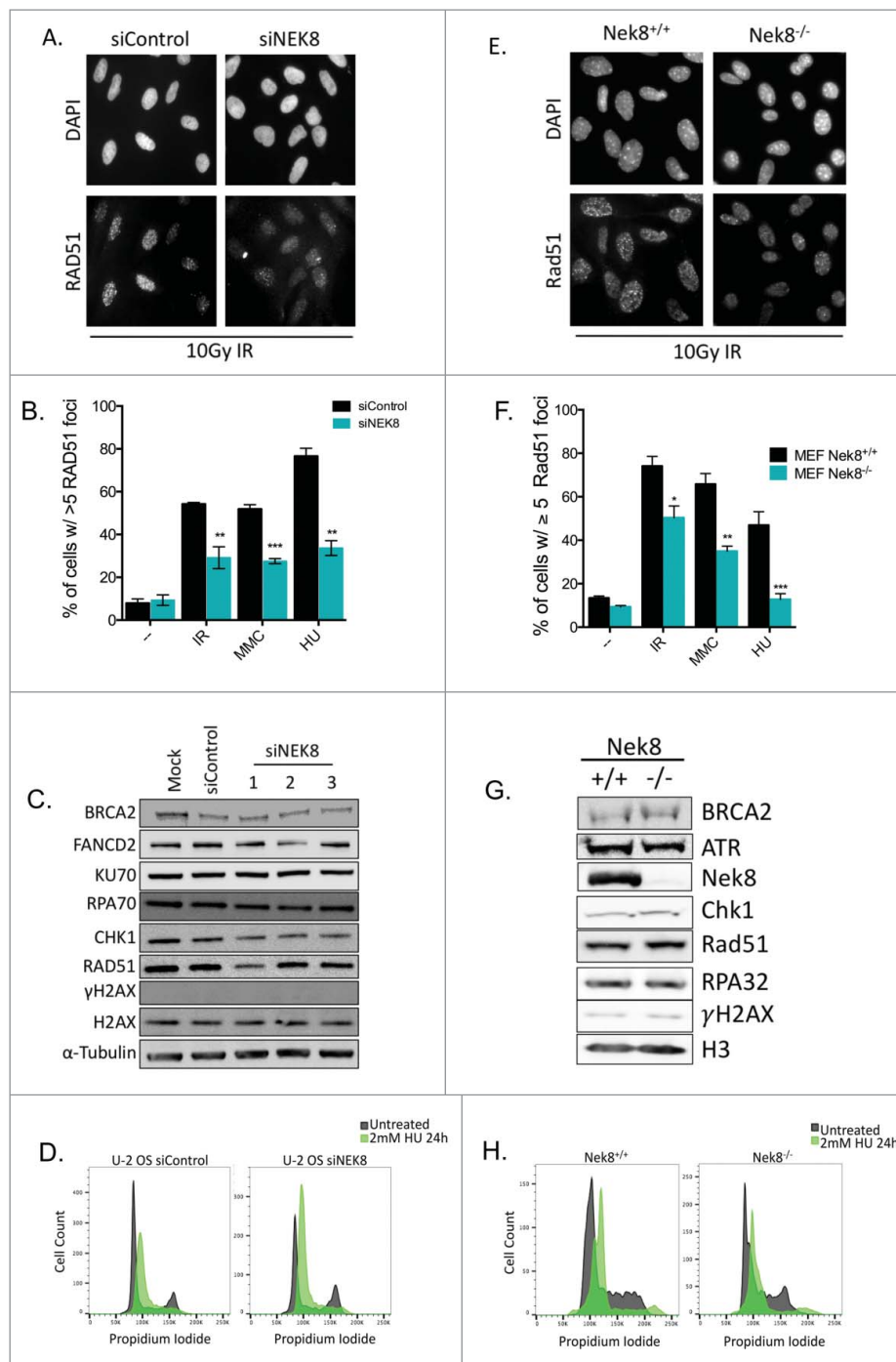
(siNEK8\_1) in U-2 OS cells (Fig. 2C), but other two NEK8 siRNAs did not affect RAD51 protein expression level and Rad51 protein expression was not decreased in *Nek8*-knockout MEFs compare with control MEFs (Fig. 2G).

Depletion or knockout of NEK8 did not significantly alter cell cycle profiles of U-2 OS cells or MEFs in untreated (Fig. 2D and H, gray profiles) and HU-treated (Fig. 2D and H, green profiles) conditions, signifying that the effect of NEK8 depletion on RAD51 foci formation is not mediated by cell cycle change. Together, these data demonstrate that NEK8 is a critical factor for RAD51 foci formation following DNA damage.

The specificity of effect of NEK8 on RAD51 foci formation is not damage type, cell type, or species specific, but appears to be universal in nature.

#### **NEK8 modulates resistance to DNA damaging agents and replication stress**

As a key component of the HR pathway, tightly controlled regulation of RAD51 function mediates the resistance to various anti-cancer drugs including inter-strand DNA crosslinking agents and PARP inhibitors.<sup>4</sup> We tested the effect of loss

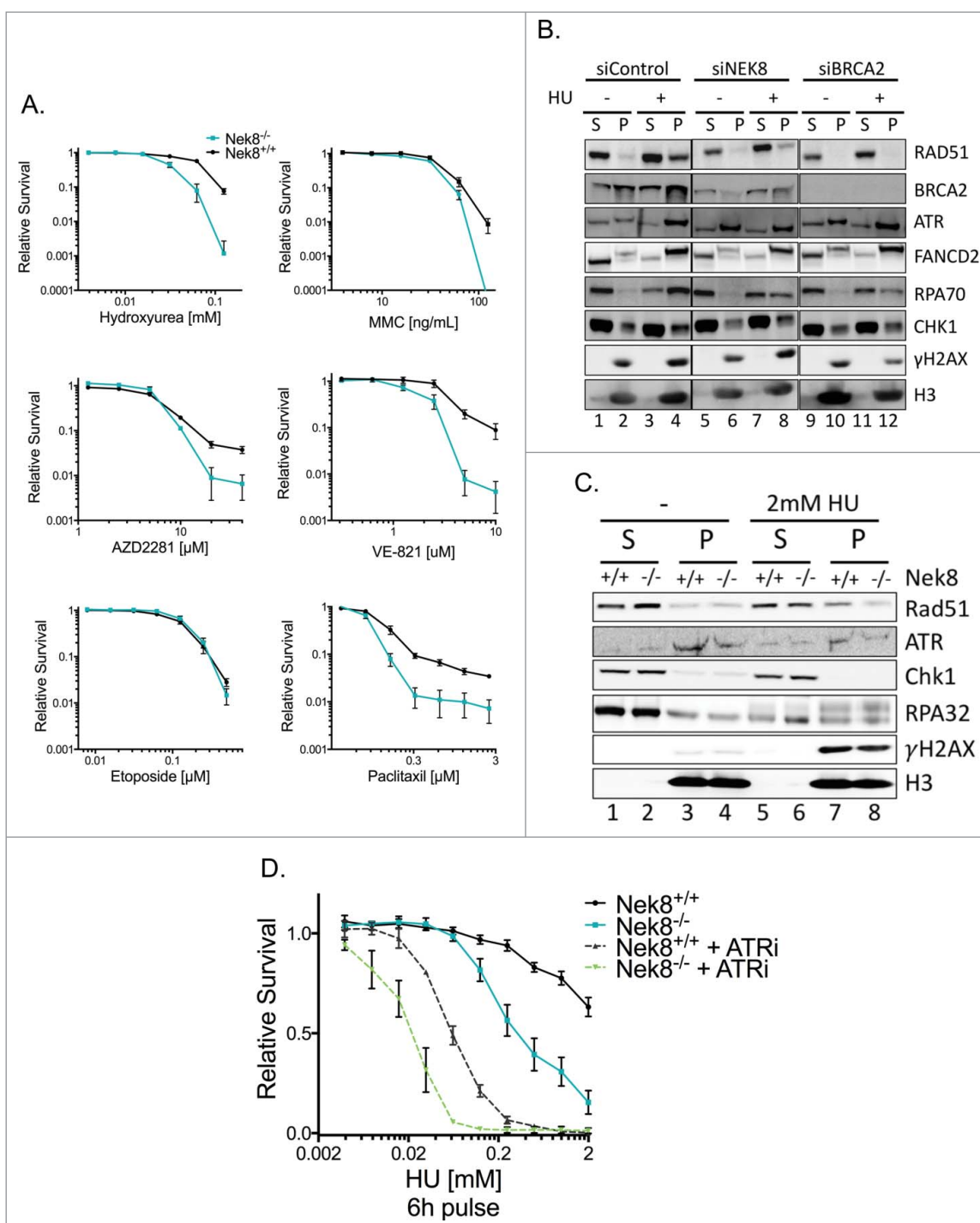


**Figure 2.** NEK8 modulates RAD51 foci formation following DNA damage and replication fork stall. A. Representative image of IR (10Gy, 6h)-induced RAD51 foci in U-2 OS cells treated with indicated siRNA. B. Quantification of the percentage of siRNA depleted U-2 OS cells with greater than 5 RAD51 foci following treatment with IR (10Gy, 6h), MMC (60 ng/mL, 24h) and HU (2 mM, 24 h). C. Western blot of key DNA repair protein expression in U-2 OS cells depleted of NEK8. D. siRNAs targeting NEK8 were transfected into U-2 OS cells (20nM), treated with HU (2 mM, 24 h) or untreated, then fixed and stained with propidium iodide prior to FACS cell cycle analysis. E. Representative image of IR (10Gy, 6 h) induced RAD51 foci in *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs. F. Quantification of the percentage of *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs with greater than 5 RAD51 foci following treatment with IR (10Gy, 6 h), MMC (60 ng/mL, 24 h) and HU (2 mM, 6 h). G. Western blot of key DNA repair protein expression in *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs. H. *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs were treated with HU (2 mM, 24 h) or untreated, harvested, fixed and stained with propidium iodide prior to FACS cell cycle analysis. (n = 3, +/- SEM). \* = p < 0.05, \*\* = p < 0.01, \*\*\* = p < 0.001 relative to siControl or *Nek8*<sup>+/+</sup> MEFs.

of Nek8 in MEFs on resistance to multiple DNA damaging agents (Fig. 3A). *Nek8*<sup>-/-</sup> MEFs were sensitive to the replication inhibitor, HU, the ATR kinase inhibitor, VE-821 and mildly to MMC and the PARP inhibitor, AZD2281 (Fig. 3A). Consistent with this, U-2 OS cells depleted of NEK8 were mildly sensitive to HU (Fig. S6). *Nek8*<sup>-/-</sup> MEFs were not sensitive to a topoisomerase inhibitor, etoposide

(Fig. 3A).<sup>34</sup> Additionally, *Nek8*<sup>-/-</sup> MEFs were sensitive to the microtubule inhibitor, paclitaxel, suggesting a role for Nek8 during mitosis.

As NEK8-deficient cells were sensitive to HU, in line with a previous report showing NEK8 sensitivity to the replication inhibitor aphidicolin,<sup>23</sup> we hypothesized that NEK8 regulates the function of RAD51 in replication fork protection. To test



**Figure 3.** NEK8 is required for resistance to replication stress and acts independently of ATR. **A.** Cell survival in *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs in response to increasing doses of hydroxyurea, MMC, PARP inhibitor (AZD2281), ATR inhibitor (VE-821), etoposide or paclitaxel as indicated. Cell survival was assayed by crystal violet staining and expressed as a fraction of the untreated control ( $n = 3$ , +/- SEM). **B.** U-2 OS cells were transfected with siRNA (20nM) and treated with or without HU (2 mM 24 h). Cells were then subjected to chromatin fractionation (S, soluble fraction; P, insoluble fraction (chromatin fraction)) and Western blotting. **C.** *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs were treated with or without HU (2 mM 6 h) and then subjected to chromatin fractionation (S, soluble fraction; P, insoluble fraction (chromatin fraction)) and Western blotting. **D.** Cell survival in *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs in response to a 6 h pulse of HU, with or without ATR inhibition followed by release into fresh media. Cell survival was assayed by crystal violet staining and expressed as a fraction of the untreated control ( $n = 7$ , +/-SEM).

this hypothesis, we treated NEK8-depleted U-2 OS and control cells with HU and performed cellular fractionation and western blotting to determine the efficiency of chromatin loading of RAD51. As expected, following treatment with HU, RAD51 chromatin loading was observed in control cells, while RAD51 chromatin loading was inefficient in NEK8-depleted U-2 OS cells (Fig. 3B, lanes 4 and 8). Similarly, chromatin loading of

Rad51 was inefficient in *Nek8*<sup>-/-</sup> MEFs compare with control MEFs (Fig. 3C, lanes 7 and 8). The effect on chromatin loading of RAD51 was not limited to treatment with HU as we observed similar effects on loading of RAD51 in U-2 OS cells following treatment with MMC (Fig. S7, lanes 4 and 12). Additionally, we observed a decrease in chromatin loading of BRCA2 in NEK8-depleted U-2 OS cells (Fig. 3B, lanes 4 and 8;

Fig. S7, lanes 4 and 12) suggesting that the role of NEK8 in RAD51 chromatin loading may be due to a direct effect on BRCA2. However, this phenotype is not fully reproducible in the *Nek8*<sup>-/-</sup> MEF background (data not shown), therefore, we are hesitant to make this conclusion with no further evidence to support this hypothesis.

RAD51 has a non-canonical function in the early stages of replication fork protection, where it is loaded onto a reversed replication fork.<sup>9,10,35-37</sup> To test if NEK8 modulates this function of RAD51 at stalled replication forks, we treated *Nek8*<sup>-/-</sup> MEFs with HU for 6 hours, a time point at which DSB formation is minimal,<sup>37</sup> and then released the cells into fresh media. We then allowed the cells to recover and measured their relative survival. *Nek8*<sup>-/-</sup> MEFs were hypersensitive to HU under these conditions compared with *Nek8*<sup>+/+</sup> MEFs (Fig. 3D), suggesting that Nek8 has a function in the early response to replication fork stall.

Similar to NEK8 deficiency leading to hypersensitivity to HU, recent data shows that acute inhibition of ATR causes rapid cell death in cells experiencing replication stress.<sup>38</sup> As NEK8 has been linked to the ATR-regulated replication stress response via its regulation of CDK activity during S-phase of the cell cycle,<sup>23</sup> and our observation that *Nek8*<sup>-/-</sup> MEFs are sensitive to ATR inhibition (Fig. 3A), we set out to further explore the role NEK8 has in the ATR-mediated replication stress response. We first verified that Nek8 is not required for proper checkpoint activation via phosphorylation on S345 of CHK1 (Fig. S8A) as reported by Liu et. al.<sup>39</sup> We next validated that Nek8 is required for resistance to ATR inhibition by rescuing this phenotype through exogenous expression of wild-type *Nek8* in *Nek8*<sup>-/-</sup> MEFs (Fig. S8B-C). Lastly, we tested the effect of ATR inhibition on cellular survival following treatment with HU in *Nek8*<sup>-/-</sup> MEFs. ATR inhibition further sensitized *Nek8*<sup>-/-</sup> MEFs to HU (Fig. 3D), suggesting that NEK8 and ATR are independently important for cellular survival after replication inhibition.

Taken together, these data suggest that NEK8 plays an important role in cellular resistance to replication stress, in the recruitment of the essential replication fork protection factor RAD51 to chromatin and acts independently of ATR. This role of NEK8 may be an essential early response to replication fork stall mediated by RAD51.

### NEK8 preserves genome stability

Cells having defective Fanconi anemia and HR pathways also exhibit increased levels of replication fork degradation following treatment with replication fork inhibitors and increased levels of genomic instability in response to replication stress.<sup>10,11</sup> NEK8 also regulates replication fork speed, stability and origin firing.<sup>23</sup> However, the mechanism by which this process is regulated is not fully understood. On this basis, we hypothesized that NEK8 is maintaining replication fork protection and genomic stability via its role in the proper function of RAD51 at stalled replication forks.

To test this hypothesis, we first used DNA fiber analysis to investigate replication fork dynamics in *Nek8*<sup>-/-</sup> MEFs. Using a dual labeling technique followed by treatment with HU, we measured both normal progression of replication (IdU tract

length) and replication fork stability via degradation (CldU tract length) (Fig. 4A). In our hands, *Nek8*<sup>-/-</sup> MEFs did not exhibit a significant difference in replication tract length compared with *Nek8*<sup>+/+</sup> MEFs as measured by IdU incorporation (Fig. 4B,F). This is in contrast to a previous report showing *Nek8*<sup>-/-</sup> MEFs having slowed rates of fork progression.<sup>23</sup> Our results may differ due to the different types of assays used.

We next measured the length of CldU tracts following treatment with HU. *Nek8*<sup>-/-</sup> MEFs exhibited significantly shorter CldU tract lengths following HU as compared with *Nek8*<sup>+/+</sup> MEFs (Fig. 4C,F). This suggests that resection of DNA at stalled replication forks is increased in the absence of Nek8. The nuclease MRE11 has been implicated in the degradation of stalled replication forks in the absence of proper fork protection by members of the Fanconi anemia and HR pathways.<sup>10,11</sup> To determine if replication tract degradation in *Nek8*<sup>-/-</sup> MEFs is also mediated by MRE11, we measured degradation (CldU tract length) in cells treated with HU and with or without the MRE11 inhibitor, Mirin (Fig. 4D). *Nek8*<sup>-/-</sup> MEFs treated with HU exhibit shorter CldU tract lengths as compared with *Nek8*<sup>+/+</sup> MEFs. This phenotype is incompletely rescued following treatment with Mirin (Fig. 4E), suggesting that MRE11 may play a role in mediating resection of stalled replication forks in *Nek8*<sup>-/-</sup> MEFs, but is not the only factor involved.

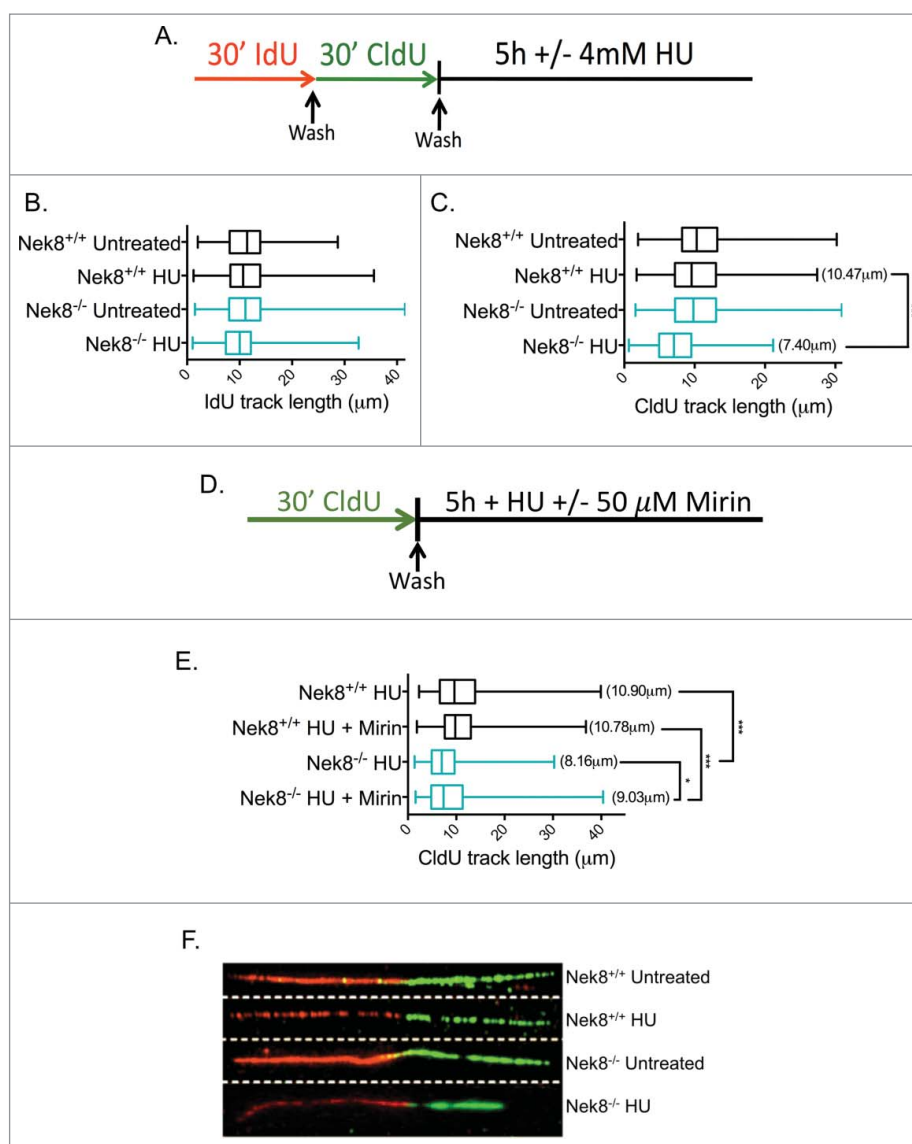
Next, the observation that spontaneous DNA damage accumulates in NEK8-deficient cells,<sup>23</sup> coupled with the assumption that HR is required for the repair of DNA replication-associated spontaneous DNA damage,<sup>40,41</sup> led us to test the efficiency of HR in the absence of NEK8. Using the U-2 OS DR-GFP reporter assay,<sup>42</sup> we found that depletion of NEK8 via siRNA led to a roughly 2-fold decrease in efficient HR when compared with control siRNA-transfected U-2 OS cells (Fig. 5A-B).

Defects in replication fork protection mediated by proteins of the Fanconi anemia and HR pathways increases genomic instability in response to HU.<sup>10,11</sup> Therefore, we next tested if treatment with HU increased levels of genomic instability in *Nek8*<sup>-/-</sup> MEFs via metaphase spread analysis. The difference of basal levels of chromosomal aberrations was not statistically significant between *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs in the absence of replication inhibition by HU (Fig. 5C-D,  $0.06 \pm 0.04$  and  $0.13 \pm 0.06$ ,  $p = 0.398$ ). However, after treatment with increasing doses of HU, *Nek8*<sup>-/-</sup> MEFs showed a drastic increase in the number of chromosomal aberrations per cell compared with *Nek8*<sup>+/+</sup> MEFs (Fig. 5C-D,  $0.2 \pm 0.08$  and  $1.71 \pm 0.55$ ,  $p = 0.011$ ).

Taken together, these data suggest that NEK8 is a critical factor for the maintenance of genomic stability mediated through HR and replication fork protection.

### Discussion

In this study, we conducted a high-throughput siRNA library screen of the human kinome using RAD51 focus formation as a readout and identified the NIMA-related kinase, NEK8, as a regulator of RAD51 foci formation and replication fork stability. NEK8-deficient cells had decreased RAD51 foci formation in response to MMC, IR and HU and showed decreased HR efficiency and an increase in chromosomal aberrations. We found that cells deficient in NEK8 were sensitive to the



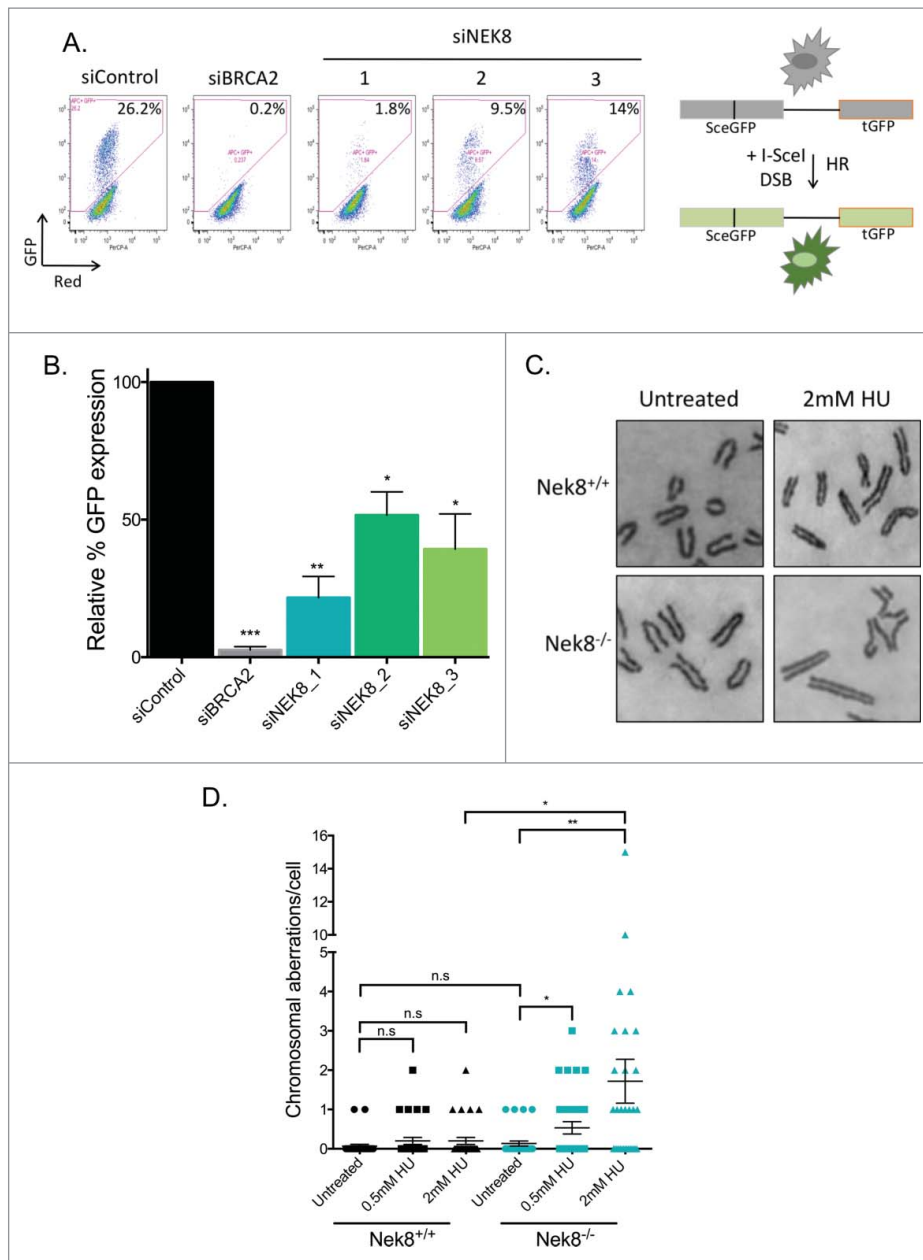
**Figure 4.** *Nek8* prevents replication fork degradation following replication fork stall. A. Schematic of experimental conditions for DNA replication tract assay. Red tracts, IdU; Green tracts, CldU. B. IdU tract length in HU treated or untreated *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs. Median tract length is designated in box and whisker plots. C. CldU tract length in HU treated or untreated *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs. Mean tract length is denoted in parenthesis and median tract length is designated in box and whisker plots. D. Schematic of experimental conditions for DNA replication tract assay treated with Mirin. Green tracts, CldU. E. CldU tract length in HU treated (4mM, 5 h) and Mirin treated or untreated *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs. Mean tract length is denoted in parenthesis and median tract length is designated in box and whisker plots. F. Representative DNA fiber images from *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs either treated or untreated with HU. (n=~300 fibers/sample) \*\*\* = p < .001, \* = p < .05.

replication stress-inducing agent HU (Fig. 3A), consistent with previous reports showing that NEK8 deficiency renders cells sensitive to aphidicolin.<sup>23</sup>

Among the human NEK family kinases, NEK8 along with other family members, NEK1, NEK6, and NEK11, have been implicated in the DNA damage response, though to differing degrees.<sup>23,39-43,49</sup> NEK6 inhibition is required for proper activation of cell cycle arrest following DNA damage,<sup>47</sup> while activation of NEK1 and NEK11 is required for DNA damage checkpoint control, DNA damage repair and the response to damage induced by ultraviolet radiation, IR, cisplatin and other drugs.<sup>44,46,50,51</sup> NEK1 also orchestrates HR and replication fork stability via regulation of RAD54.<sup>43</sup> Similarly, the NIMA-related kinase in *Saccharomyces cerevisiae*, Kin3, is involved in the response to DNA adduct damage.<sup>52</sup> Our findings suggest that NEK8 further links the NIMA-related kinase family to the

DNA damage response, where NEK8 maintains a complex regulatory role in the repair of DNA lesions requiring HR as well as having a significant role in the protection of replication forks.

Interestingly, we observed hypersensitivity to short-term replication stress in *Nek8*<sup>-/-</sup> MEFs (Fig. 3D). This phenotype suggests that *Nek8* has an important function in the early stages of replication fork protection, possibly mediated through RAD51. Previous data suggests that key components of the Fanconi anemia and HR pathways, including RAD51, have important roles in the early stages of replication fork protection and recovery, possibly mediated by the HR-associated protein, RAD18.<sup>10,11,37,53</sup> The cell cycle checkpoint kinase, ATR, is also an important factor in replication fork protection.<sup>54-57</sup> NEK8 interacts with ATR, ATR-interacting protein (ATRIP), and CHK1.<sup>23</sup> Previous data also suggests that proper regulation of



**Figure 5.** *Nek8* is important for homologous recombination efficiency and the maintenance of genome stability. **A.** U-2 OS DR-GFP cells were transfected with siRNA (20nM). 24 h post transfection cells were transfected with pCBASce or control plasmids. GFP expression was detected and quantified via flow cytometry. Representative GFP+ population is denoted as a percentage. **B.** Quantification of U-2 OS DR-GFP assay using relative GFP expression ( $n = 3$ ,  $\pm$  SEM, \* $p < .05$ , \*\* $p < .01$ ). **C.** Representative images of metaphase chromosomes of *Nek8*<sup>+/+</sup> and *Nek8*<sup>-/-</sup> MEFs treated with or without HU for 6 h and then released into fresh media for 16 h. The cells were then treated with colcemid and processed for metaphase analysis. **D.** Quantification of mean chromosome aberrations per metaphase (aberrations include: breaks, gaps, radials and other translocations) ( $n = 30$  metaphases per sample,  $\pm$  SEM).

RAD51 expression modulates DNA replication and replication fork protection in a CHK1-dependent manner.<sup>58</sup> We observed that *Nek8*<sup>-/-</sup> MEFs were sensitive to inhibition of ATR and were further sensitized to HU following ATR inhibition (Fig. 3D). This suggests that NEK8 has a separate and distinct function from ATR in the early stages of replication fork protection and possibly in the maintenance of chromosomal stability. Precise molecular mechanisms by which NEK8 cooperates with these factors (ATR, CHK1, RAD18, RAD51 and other Fanconi anemia proteins) in the replication fork protection remain to be elucidated.

We observed an increase in degradation of stalled replication forks in the *Nek8*<sup>-/-</sup> MEFs (Fig. 4). This phenotype is not

completely unexpected as previous reports show that protection of nascent DNA at stalled replication forks requires key factors in the Fanconi anemia and HR repair pathways.<sup>10,11</sup> We found that the nuclease MRE11 may play a role in degradation of stalled replication forks in the absence of *Nek8* (Fig. 4E). However, MRE11 is likely not the only factor responsible, since the MRE11 inhibitor, mirin, only partially restored fork protection in HU-treated *Nek8*<sup>-/-</sup> MEFs. Other nucleases, including DNA2 and MUS81, as well as other factors required for replication fork protection, such as BOD1,<sup>57</sup> could also be involved in regulating replication fork degradation in *Nek8*<sup>-/-</sup> MEFs. *Nek8*<sup>-/-</sup> MEFs also exhibited an increase in genomic instability following treatment with HU (Fig. 5C–D). This phenotype is



likely explained by a decrease in HR (Fig. 5A–B) coupled with the inability to efficiently restart replication after replication inhibition,<sup>23</sup> ultimately manifesting as genome instability and cellular sensitivity to replication inhibiting drugs. It would be worthwhile to explore the functional domains of NEK8 which are required for the observed phenotypes in our experimental system. Previous reports suggest the importance of both the kinase domain and RCC1 domains of NEK8 as being important for its localization to the ciliary axoneme, and as such, its function in NPHP, as well as for its role in the ATR-mediated replication stress response.<sup>12,23</sup> Understanding the functional significance of these domains in replication fork protection and genome stability would provide mechanistic insight and a foundation for the future development of therapeutic strategies targeting NEK8.

Lastly, NEK8 localizes to the centrosomes,<sup>16</sup> primary cilium<sup>22</sup> and nucleus.<sup>12</sup> Interestingly, the functional interaction between DNA repair proteins and centrosome proteins is an emerging concept.<sup>59</sup> Several DNA repair proteins including BRCA1, BRCA2 and RAD51, localize to the centrosome, in addition to the nucleus, and are involved in the regulation of multiple processes, such as centrosome functionality.<sup>59,60</sup> Mutations in genes which cause ciliopathies, including the centrosomal protein CEP164,<sup>61,62</sup> ZNF423, MRE11 and FAN1<sup>61–63</sup> also have important functions in the DNA damage response, suggesting a link between centrosomes, ciliopathies and DNA repair pathways. The observation that *Nek8*<sup>-/-</sup> MEFs were sensitive to the microtubule poison drug paclitaxel (Fig. 3A), suggests that similar to the other NEK family kinases, NEK2, NEK5 and NEK6,<sup>64</sup> NEK8 may have a functional role in proper mitotic chromosome segregation, whose errors may drive cancer progression. This observation also further links the DNA damage response, the replication stress response and ciliopathies.

In summary, we describe multiple phenotypes associated with the loss of NEK8 in mammalian cells. Our data highlight important functions of NEK8 in the replication stress response. The identification of NEK8 as a regulator of HR and replication stress response provides further insight into the intricate regulatory mechanisms behind an already complex cellular machine responsible for the response to DNA damage and replication stress. However, these data also raise questions about the role of NEK8 in fine tuning the response to DNA damage, an area which remains cloudy. Furthermore, these findings provide further evidence to the interplay between centrosomal proteins and the DNA repair machinery, highlighting the importance for future research in this area.

## Materials and methods

### Cell lines

U-2 OS and HeLa were purchased from the American Type Culture Collections. *Nek8*<sup>-/-</sup> and *Nek8*<sup>+/+</sup> MEFs<sup>23,65</sup> were a gift of the Cimprich lab (Stanford) with permission from the Beier lab (Seattle Children's). U-2 OS DR-GFP cells were a gift of the Jasin Lab (Memorial Sloan Kettering).<sup>66</sup> Cell lines were cultured in DMEM containing 10% FBS, 2 mM L-glutamine

and 1X Pen/Strep in a humidified 5% CO<sub>2</sub> containing atmosphere at 37°C.

### siRNA kinome library screening

U-2 OS cells were transfected (20nM, Lipofectamine RNAi-MAX) with the MISSION siRNA human kinase panel library (Sigma) in 384 well glass bottom cell culture plates (Thermo Scientific, #4331). Control siRNAs were spiked-in and included BRCA2,<sup>67</sup> RAD51 (5'-AACTAATCAGGTGGTAGCTCA-3'), ATR (5'-AACCTCCGTGATGTTGCTTGA-3'), and Chk1 (5'-AAGGGATAACCTCAAATCTC-3'). Two days post-transfection, cells were treated with MMC (60 ng/mL) for 24 h. Cells were then simultaneously fixed and permeabilized (2% PFA and 0.5% Triton X-100 in PBS for 20 minutes) followed by immunostaining for RAD51 or cyclin A. Images were acquired with the Cellomics Arrayscan microscope (Thermo Scientific) and processed as described.<sup>68</sup> Using DAPI to define nuclei, the average number of foci per cell (RAD51) and nuclear intensity (cyclin A) was quantitated using the automated counting software. The Z-score was calculated based on the formula  $Z = (X - \mu_{nc}) / \sigma$ , where X was the individual sample average,  $\mu_{nc}$  was the mean of the negative control population in each plate and  $\sigma$  was the standard deviation of the whole population. Screening was independently replicated 2 times.

### Immunofluorescence microscopy

Immunofluorescence microscopy was conducted as previously described.<sup>68</sup> Briefly, transfected cells were grown on coverslips, treated with MMC (60 ng/mL, 24 h), IR (10 Gy, 6 h) or HU (2 mM, 24 h) and then simultaneously fixed and permeabilized (2% PFA and 0.5% Triton X-100 in PBS for 20 minutes). Cells were immunostained for RAD51 and RPA. Images were acquired with an inverted fluorescent microscope (TE2000, Nikon) and analyzed using ImageJ (National Institute of Health). At least 300 cells per experimental point were scored for the presence of foci. Each experiment was repeated 3 times independently.

### RT-PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and reverse-transcribed using the SuperScript III First-Strand Synthesis system (ThermoFisher). cDNA of target was amplified via PCR with the primers: TEX14 (For 5'-TAGACTCCCCGCAGCGGCTT-3', Rev 5'-TGAAACCAAGGCATAGCCTTCCC-3'), NEK8 (For 5'-GCAAGCCCTACAACCAGAAG-3', Rev 5'-ACTCATGATCTTCAGCACCAG-3'), PAK4 (For 5'-CTCCTCGTTCATCC TGGTGT-3', Rev 5'-GAGCTGCTCTTCAACGAGGT-3'), DGKD (For 5'-GCTTGTGCAAGAAGGAGGAC-3', Rev 5'-ACTGTGTGCGACAAGACCTG-3'), RPS6KL1 (For 5'-AAGGGGTCAC TGTGAGGATG-3', Rev 5'-AGGCTCCCCTGTAGAAGCTC-3'), FUK (For 5'-CTGGAGCCATGAGCTTCTTC-3', Rev 5'-TGTGCTGAGGAGCTGGTATG-3'), PTK6 (For 5'-CTGCAGACAGACAGCCAGAG-3', Rev 5'-CCTGGGGTTTACTGAGGTGA-3'). Amplified targets were resolved on a 2% agarose gel. Images of gels were obtained with a UV imager (BioRad Gel Doc XR+).

### Western blotting

Whole-cell extracts were prepared and resolved by polyacrylamide gel electrophoresis as described.<sup>68</sup> Proteins were transferred onto nitrocellulose membranes. Antibodies against  $\alpha$ -Tubulin (CST), Actin (sc-1616-R, Santa Cruz), ATR (N-19, Santa Cruz), BRCA1 (D-9, Santa Cruz), BRCA2 (Ab-2, Calbiochem), CHK1 (G-4, Santa Cruz), FANCD2 (Abcam), FLAG (M-2, Santa Cruz),  $\gamma$ H2AX (JBW301, Millipore), GFP (Life Technologies), H2AX (Millipore), H3 (Abcam), Ku70 (Abcam), mNEK8 (gift from David Beier),<sup>14</sup> hNEK8 (N-17, Santa Cruz), RAD51 (H-92, Santa Cruz and BAM-10-001, CosmoBio) and RPA70 (CST) were probed with horseradish peroxidase-conjugated anti-mouse, anti-rabbit (GE Biosciences) or anti-goat IgG (sc-2020, Santa Cruz). Chemiluminescence was used for detection and membranes were digitally scanned with an Imagequant LAS 4000 (GE Biosciences). Images were processed using Photoshop CS (Adobe Systems, Inc.) and PowerPoint (Microsoft, Inc.).

### Cell cycle analysis

siRNA-transfected U-2 OS cells or Nek8-knockout MEFs were treated with HU (2 mM, 24 h) or not treated. Cells were then fixed and stained with propidium iodide for DNA content. Flow cytometry was performed to determine the cell cycle phase distribution (Canto 2 or LSR-2) and cell cycle profiles were obtained with FloJo software.

### siRNAs and plasmids

siRNAs targeting BRCA2,<sup>67</sup> RAD51 (target sequence: 5'- AAC-TAATCAGGTGGTAGCTCA-3'), and NEK8 (#1 target sequence: 5'- TCACTCTTCTGGTTGTAGG-3', #2 target sequence: 5'- TCAGAGGAGAAGCAATATC-3', #3 target sequence: 5'- AGA-GATAGGTGCAAAGGTG-3') were transfected at 20nM using Lipofectamine RNAiMAX (ThermoFisher). AllStars siRNA (Qiagen) was used as a negative control. I-SceI expression vector, pCBASce, was a gift from the Jasin lab (Memorial Sloan Kettering). mNek8 plasmid was a gift from the Beier lab (Seattle Children's). mNek8 coding sequence was PCR amplified from the mNek8 plasmid and cloned into pMMP-IRES-puro. Retroviruses were produced as previously described.<sup>69</sup> Nek8<sup>+/+</sup> and Nek8<sup>-/-</sup> MEFs were transduced with pMMP-IRES-puro (empty vector) or pMMP-IRES-puro-mNek8 retroviruses were selected with puromycin at 3  $\mu$ g/mL for 3 d.

### Homologous recombination assay

U2OS DR-GFP cells were transfected with siRNAs and then transfected 24 hours later with pCBASce (I-SceI expression vector). Two days after plasmid transfection, cells were harvested and analyzed using a FACSCalibur analyzer or an LSRII analyzer to determine the percentages of GFP- positive cells.

### Cell fractionation

Cell fractionations were prepared as described.<sup>70</sup> Briefly, cells were resuspended in buffer CSK (10mM PIPES, pH = 6.8,

100mM NaCl, 1mM EGTA, 1mM EDTA, 300mM Sucrose, 1.5mM MgCl<sub>2</sub>, 0.1% Triton-X-100 and protease inhibitors) and incubated in ice for 5 min. Samples were centrifuged at 1500 g for 5 min. Supernatant was collected and stored (soluble fraction). Pellets (pellet fraction) were washed once in CSK buffer and then re-suspended in sample buffer (0.05 M Tris-HCl (pH 6.8), 2% SDS, 6%  $\beta$ -mercaptoethanol) and boiled for 5 min.

### Survival assay

Cell survival was measured by a crystal violet absorbance-based assay. Cells were seeded onto 12-well plates at a density of 6–9  $\times 10^6$  cells/well. The next day, cells were treated with increasing concentrations of drug and incubated for 5 to 8 more days. After that, cellular monolayers were fixed, stained with crystal violet and re-solubilized as previously described.<sup>68</sup>

### DNA fiber assay

DNA fiber assay was conducted as previously described with some changes.<sup>10</sup> Briefly, cells were labeled with IdU (50  $\mu$ M), washed, and labeled with CldU (50  $\mu$ M). Cells were then exposed to HU (4 mM), Mirin (50  $\mu$ M) or untreated media. DNA fibers were essentially spread as described<sup>71</sup> before standard detection of IdU and CldU tracts (primary antibodies:  $\alpha$ -IdU,  $\alpha$ -BrdU from BD Biosciences;  $\alpha$ -CldU,  $\alpha$ -BrdU from Novus Biologicals and secondary antibodies: Alexa Fluors 488 and 594, respectively, from Invitrogen). Fibers were imaged on an inverted fluorescent microscope (TE2000, Nikon) and analyzed using ImageJ software. Statistics were calculated using Prism6 (GraphPad Inc.).

### Metaphase spread analysis

Seven  $\times 10^5$  cells were seeded 24 h prior to treatment with HU (4 mM) and treated with colcemid (0.1  $\mu$ g/ml, GIBCO). Cells were swollen with 0.075M KCL (15min, 37°C), fixed with methanol/acetic acid (3:1), dropped onto a microscope slide, stained with 5% Giemsa, and mounted with ENTELLAN NEW (Electron Microscopy Sciences) before imaging with an inverted fluorescent microscope (TE2000, Nikon).

### Statistical analysis

A Student's *t*-test was used to evaluate significance of differences in all experiments (Excel, Microsoft, Inc. and Prism6, GraphPad, Inc.). All experiments were expressed as mean  $\pm$  SEM. A *P* value < 0.05 was considered significant.

### Abbreviations

DSB	double strand break
HR	homologous recombination
HU	hydroxyurea
IR	ionizing radiation
JCK	juvenile cystic kidney disease
MEFs	mouse embryonic fibroblasts
MMC	mitomycin C

NEK NIMA-related kinase  
 NPHP nephronophthisis  
 PARP poly (ADP-ribose) polymerase  
 ssDNA single-stranded DNA

## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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