A High Throughput RNAi Screen Reveals the Protein Kinase NEK8 Regulates Replication Fork Protection via the DNA Repair Protein RAD51

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Abstract

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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 previously unknown kinases that may regulate RAD51 localization to sites of DNA damage, we performed a human kinome siRNA library screen, using DNA damage-induced RAD51 focus formation as readout. We found that NEK8, a NIMA family kinase member, is required for efficient DNA damage-induced RAD51 focus formation. Interestingly, we found that knockout of Nek8 in murine embryonic fibroblasts leads to cellular

sensitivity to the replication inhibitor, hydroxyurea (HU), but not to other DNA damaging agents. Furthermore, we show that NEK8 is required for proper replication fork protection following replication stall with HU. Loading of the essential DNA repair and replication fork protection factors, RAD51 and BRCA2, was decreased in NEK8 depleted and deficient cells lines. Using single-molecule DNA fiber analysis, we show that nascent DNA tracts are degraded in the absence of NEK8 following treatment with HU. We show an increase in genomic instability in Nek8 null murine cells following treatment with HU. Thus, NEK8 plays a critical role in replication fork stability through its regulation of the DNA repair and replication fork protection protein RAD51. Lastly, the discovery that NEK8 functions in resistance to replication inhibiting agents raises the possibility that NEK8 may have clinical utility as a therapeutic target for tumor chemosensitization.

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DEDICATION

To my late grandparents: Onofre and Maria Baros, Manuel Abeyta

To my grandmother: Patricia Abeyta

To my parents: Elmer and Virginia Abeyta

To my sister and brother-in-law: Valerie and Carlos Mondragon

To my niece and nephew: Jamie and Andrew Mondragon

Chapter 1. INTRODUCTION

DNA Repair and Maintenance of Genome Stability

The maintenance of genome stability is of utmost importance for organismal survival. This task is especially daunting as the typical diploid human genome contains roughly $6x10^9$ base pairs, which must be maintained with correct chromosomal organization from one somatic generation to the next. This feat can be especially difficult for an organism which is faced with upwards of ten thousand DNA damaging events per day [1]. These DNA damaging events arise from a multitude of endogenous as well as exogenous sources. For example, exogenous sources of radiation (ultraviolet (UV) or ionizing radiation (IR)), and chemicals such as chemotherapeutics can lead to base damage or DNA strand breaks [2]. Endogenous sources of damage include metabolic processes which generate reactive oxygen species also leading to damaged bases or DNA strand breaks, or the creation of abasic nucleotides arising from spontaneous hydrolysis [2, 3]. The lesions created by these types of DNA damage can hinder essential cellular processes such as DNA replication and transcription. As such, the immediate sensing and repair of these lesions is of utmost importance for survival of the organism.

The importance of genomic stability is highlighted by the multitude of cancer susceptibility syndromes which are caused by defective factors that mediate DNA repair [4]. For example, inheritance of defective alleles of important factors in the DNA repair pathway homologous recombination (HR), such as FANCS/BRCA1, FANCD1/BRCA2, XRCC2, FANCO/RAD51C, RAD51D, FANCN/PALB2 and others can lead to the cancer susceptibility syndromes, Fanconi anemia (FA) and familial breast and ovarian cancer [4-8]. Also, mutations in genes whose products are important in coordinating nucleotide excision repair can confer

hypersensitivity to UV radiation and lead to UV-induced tumor formation in Xeroderma pigmentosum (XP) patients [4].

The most dangerous lesion created by both endogenous and exogenous forms of DNA damage is widely considered to be the DNA double strand break (DSB). These types of lesions, if misrepaired or left unrepaired can lead to gross chromosome rearrangements or loss of whole chromosomes [1, 9]. DSBs are not tolerated well by eukaryotes, where a single DSB, if left unrepaired, can lead to cell cycle arrest [10] or cell death [11]. DSBs arise from multiple sources including exposure to certain DNA damaging agents including IR, DNA inter-strand crosslinking agents, and radiomimetics as well as replication fork stalling and collapse [9, 12]. There are multiple methods for the repair of DSBs including non-homologous end joining (NHEJ), alternative-NHEJ (alt-NHEJ), single strand annealing (SSA) and HR (Figure 1.1) [12]. NHEJ occurs through all phases of the cell cycle and requires minimal DNA end processing. NHEJ however, is considered to be an error-prone method of DNA repair as the broken ends of DNA are re-ligated without the use of a template [13]. HR primarily occurs during S- and G2-phase of the cell cycle when a sister chromatid is present [12]. As such, HR is considered an error-free method of DNA repair due to its use of a homologous template for the restoration of information following DNA damage [12].

HOMOLOGOUS RECOMBINATION

The current model of HR was first proposed in 1983, opening the door to what is now an extensive field of molecular biology [14]. Conceptually, HR can be broken into three distinct phases, presynapsis, synapsis and post synapsis. Presynapsis is characterized by the damaged DNA being processed to form a single-stranded DNA (ssDNA) overhang (Figure 1.1A) [12]. Upon the formation of a DNA DSB, the damage is sensed by the MRN (MRE11/RAD50/NBS1)

complex which localizes to the broken DNA ends and promotes the autophosphorylation and subsequent activation of the PI3K-like kinase, ATM [15, 16]. Once activated, ATM then initiates the DNA damage response by phosphorylating up to 700 of its downstream targets [17]. One important ATM target is the histone variant, H2AX [17]. Phosphorylated H2AX (γH2AX) serves as both a signaling mechanism and docking point for the recruitment of additional proteins required for proper DNA damage response. This process is mediated by γH2AX being recognized by the protein MDC1, which then recruits more ATM molecules to the site of DNA damage, which serves to amplify the ATM signal [18, 19].

Along with perpetuating the DNA damage signal, the MRN complex plays an important role along with the other nucleases, CtIP, EXO1 and DNA2, in the initial and extended resection of the damaged DNA [20]. This resection results in the formation of 3' single stranded DNA overhangs, which are estimated to be up to several kilobases in length [21]. These sections of ssDNA are bound by the heterotrimeric replication protein A (RPA) [22]. RPA serves multiple roles in HR including the elimination of secondary structures in ssDNA which allows for downstream loading of RAD51 filaments [23, 24], protection and stabilization of ssDNA from degradation [25], and the activation of the ATR kinase through its interacting partner, ATRIP [26]. CHK1, the downstream effector kinase of ATR, then phosphorylates the recombinase protein RAD51 at Thr-309. This phosphorylation event is required for localization of RAD51 to chromatin [27].

RPA bound to ssDNA forms a kinetic barrier against the formation of RAD51 filament assembly. As such, mediator proteins are required to facilitate the replacement of RPA with RAD51 filaments [12]. These mediator proteins include the breast and ovarian cancer predisposition gene products, BRCA1, BRCA2 and PALB2 as well as the RAD51 paralogs,

RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3 [2, 12]. These proteins interact with RAD51, promote the displacement of RPA and/or its assembly onto 3'-tailed ends, and stabilize the RAD51 protein filament so that it can perform homology search and strand invasion efficiently [27].

The synaptic phase is characterized by the RAD51 filament performing homology search and DNA-strand invasion, generating a displacement loop (D-loop), where the invading strand primes for DNA synthesis within the heteroduplex (Figure 1.1B). D-loop integrity is maintained by coating of the ssDNA in the loop by RPA [28] which prevents the reannealing of complementary strands. After formation of the stable heteroduplex, the RAD51 filament is disassembled by the chromatin remodeler, RAD54, which allows for DNA synthesis [29].

During post synapsis the three distinct pathways of HR are distinguished (Figure 1.1C). The D-loop is the branching point for these multiple subpathways which include break-induced replication (BIR), synthesis-dependent strand annealing (SDSA) and the double holiday junction subpathway (dHJ). BIR, which is essentially a full fledged replication fork, restores integrity of the chromosome but may lead to loss of heterozygosity. SDSA inherently leads to non-crossover events, which reduce potential genomic rearrangements. Formation of dHJs has the possibility of leading to crossover events, the main source of genetic variation in meiosis, or to non crossover events. In all, HR occurring via any of these three sub pathways typically leads to error-free repair of DNA DSBs [29].

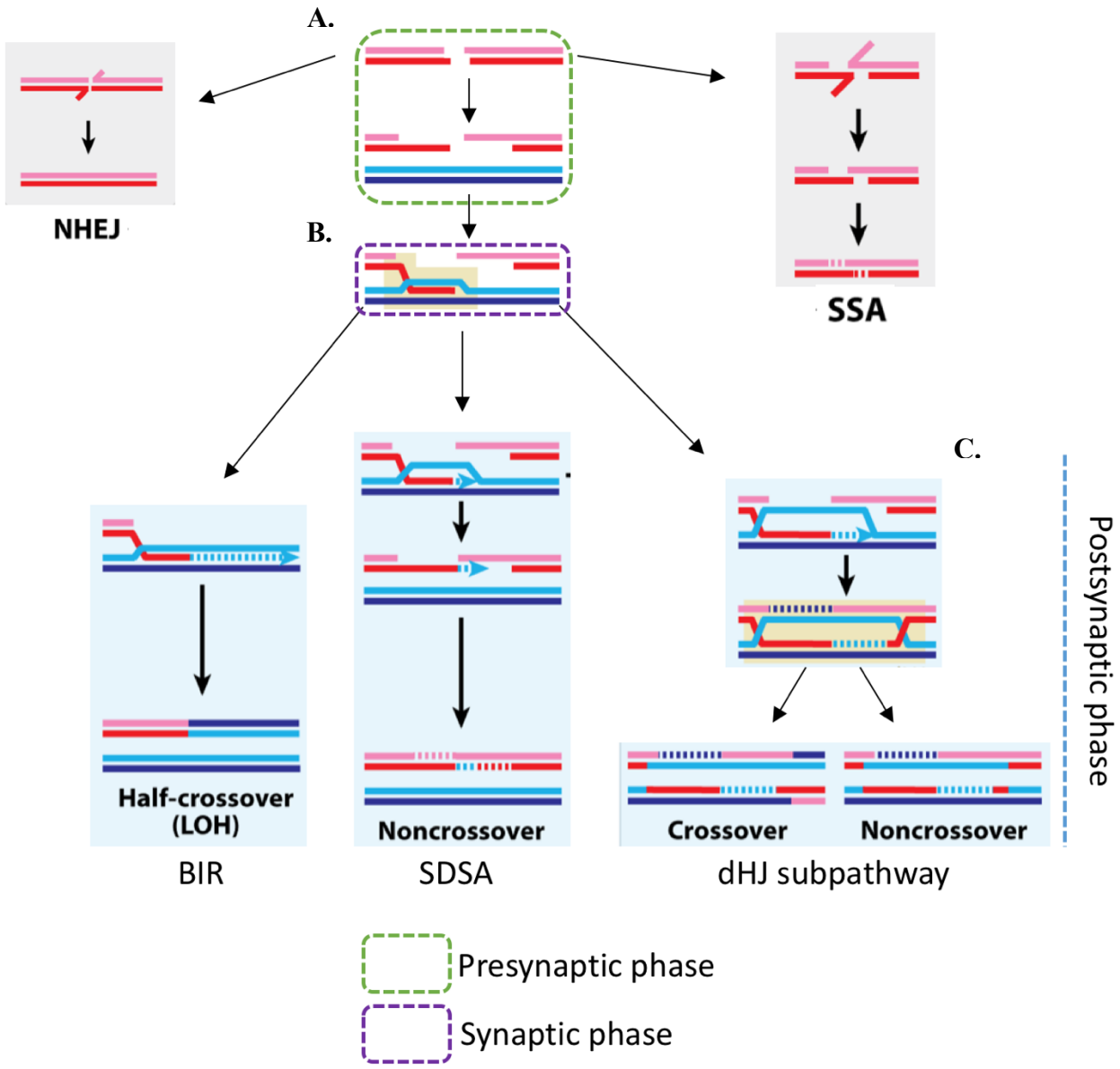


Figure 1.1. **Pathways of repair of DNA double strand breaks**. Two RAD51 independent pathways of DSB repair are presented, non-homologous end joining and single strand annealing. **A**. The presynaptic phase of HR involves sensing of the DSB followed by resection of the DNA leading to a 3' single stranded DNA overhang. **B**. The synaptic phase of HR is characterized by the formation of a D-loop, which is mediated by the RAD51 recombinase. **C**. The post synaptic phase of HR is divided into three subpathways, break-induced replication, synthesis-dependent strand annealing, and the double holiday junction subpathway. Adapted from [12].

RAD51 FOCUS FORMATION

The RAD51 recombinase forms punctate sub-nuclear foci that can be visualized immunocytochemically following DNA damage with DSB inducing agents such as IR, mitomycin C, and the replication inhibitor hydroxyurea (HU) [21, 30]. These DNA damage-induced RAD51 foci co-localize with BrdU under non-denaturing conditions, which supports the notion that RAD51 foci are bound to ssDNA, suggesting that these foci represent RAD51 filament formation *in vivo* [21]. As mentioned previously, the estimated length of ssDNA at which repair foci are bound is in the kilobase range, suggesting RAD51 foci potentially represent RAD51 filaments containing thousands of RAD51 monomers [21]. Though, it remains unclear if RAD51 foci represent a RAD51 filament at a single break site, or are representative of multiple break sites in close proximity to one another [21]. Lastly, it is widely regarded that overly bright and large foci represent excessively long RAD51 filaments bound to ssDNA. These filaments form when homology search is still ongoing or has failed [21].

While there remains some uncertainty to what RAD51 foci are representing, it is clear that their formation in response to DNA damage is a thorough indicator of RAD51 loading proficiency on ssDNA as well as dedication and efficiency of HR. This process is reliant on several key upstream factors in the HR pathway as well as many proteins having roles in a multitude of cellular processes [12]. The main mediator of RAD51 loading on chromatin in higher eukaryotes is the breast and ovarian cancer susceptibility gene product, BRCA2 [2, 31]. BRCA2 contains multiple motifs which allow BRCA2 to interact directly with RAD51 and DNA [32]. The BRCA2 protein contains eight BRC repeats, with the ability to bind RAD51 monomers via six of these repeats. Purified BRCA2 has the ability to load RAD51 on ssDNA coated with RPA *in vitro*, where it allows for promotion of strand invasion [32]. One key aspect of BRCA2

activity is to downregulate the ATP hydrolysis of RAD51, which serves to stabilize the RAD51 nucleofilament on ssDNA [32, 33].

RAD51 focus formation mediated by BRCA2 is also dependent on two other breast and ovarian cancer susceptibility gene products, BRCA1 and PALB2. Multiple mechanisms have been described showing BRCA1 localization to chromatin as an important modulator in the recruitment of BRCA2-RAD51 complexes through PALB2 [34]. PALB2 is an important factor as it insures that HR only occurs during S- and G2-phase of the cell cycle [35] as well as interacts with and stabilizes RAD51 filaments, therefore modulating strand invasion *in vitro* [34].

Other mediators of RAD51 focus formation include the RAD51 paralogs. In humans, there are five identified paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) [36-40]. Evidence for the RAD51 paralogs as mediators of RAD51 focus formation are in that purified RAD51B and RAD51C heterodimers are required for RAD51-mediated strand exchange *in vitro* [41]. As further evidence, mutations in these paralogs in mammalian and chicken cells decrease DNA damage-induced RAD51 focus formation [42-45].

POST-TRANSLATIONAL MODIFICATIONS THAT REGULATE HOMOLOGOUS RECOMBINATION

The regulation of HR involves not only the aforementioned proteins, but also an intricate network of post-translational modifications (PTMs). Genetic studies have provided extensive insight into the role of PTMs, including phosphorylation, in the regulation of HR, [46]. One key HR and DNA replication protein, RPA, is phosphorylated both by the checkpoint kinases ATM and ATR, as well as the CDK cell cycle kinases. Phosphorylation of RPA by these kinases is essential for recruitment of RAD51 to sites of DSBs as well as HR as a mechanism for replication fork restart during replication stress [26, 47]. RAD51 has also been shown to be

phosphorylated by multiple kinases. Phosphorylation of Tyr-315 on RAD51 is mediated by the BCR/ABL kinase and promotes RAD51 recombinase-mediated unfaithful HR repair [48] and phosphorylation of Tyr-54 by c-Abl inhibits RAD51 binding to DNA as well as its strand exchange capabilities [49]. The kinase CHK1 phosphorylates RAD51 on Thr-309, which is required for efficient RAD51 focus formation and resistance to the replication inhibitor HU [27]. Moreover, RAD51 is also phosphorylated in a Mec1 (ATR)-dependent manner on Ser-192, by PLK1 on Ser-14 and by CK2 on Tyr-13, all in a cell cycle and DNA damage dependent manner [50, 51], suggesting that modification of RAD51 can affect RAD51 activity[50].

As mentioned previously, the protein kinase ATM is required for efficient HR as the key upstream signaling mechanism of DSBs [15, 16]. Active ATM is believed to have up to 700 downstream phosphorylation targets [17], many of which have roles in HR. Phosphorylation of the key HR proteins CtIP and BRCA2 by the cyclin-dependent kinases (CDKs) help to promote an elegant mechanism by which HR is regulated by cell cycle progression [52-55].

In brief, this short overview of some of the PTMs involved in regulating HR show the complexity and intricate roles these PTMs have in regulating HR at multiple levels. However, a complete picture of all phosphorylation events regulating HR does not exist, therefore a better understanding of how these modifications affect and regulate HR is required.

EUKARYOTIC DNA REPLICATION BASICS

Duplication of the genome is a key event in the life cycle of all proliferating organisms. As such, the maintenance of the physical integrity of chromosomes is essential for preserving overall genomic integrity [56]. In eukaryotes, this process is regulated by the coordinated firing of thousands of individual replication origins used to form bi-directional replication forks [57]. The essential player in DNA replication is known as the replisome, which is an assembly of

macromolecular machines that serve two essential functions, which include, coupling of parental duplex-DNA unwinding with daughter strand synthesis [58], and integrating DNA damage response signals to modulate fork progression, pausing, and restart [59].

The intricate coordination of replication begins prior to S-phase, where each origin is "licensed" by a combination of replication initiation proteins which work to prepare the chromatin for replication to commence during S-phase [57]. Upon origin firing, and commencement of replication, a cell has the difficult task of balancing multiple aspects, such as accuracy, speed and the consumption and distribution of resources which include nucleotides and replication factors, in order to complete replication in an efficient manner [60]. As such, eukaryotic cells have developed an elegant mechanism by which origins are fired in a coordinated fashion and are split into early and late firing origins [57]. Interestingly, a majority of "licensed" origins will never fire during an unperturbed S-phase cycle, but instead appear to be primed for firing as a response mechanism to replication stalling, where they serve to ensure completion of DNA replication through replication fork restart [57].

REPLICATION FORK PROTECTION

During DNA replication, many agents pose serious threats to chromosomal integrity by interfering with fork progression and stability, ultimately leading to replication fork arrest [59]. Such agents include secondary DNA structures, covalent adducts and DNA lesions which can be caused by endogenous side-products of cellular metabolism, such as reactive oxygen species (ROS), exogenous agents which can interfere with DNA replication, as well as intrinsic structural features of specific genomic regions [56]. Cells which ultimately fail to properly respond to replication stress and damaged DNA typically have an accumulation of genetic instability, which is a major driving force in the development of cancer [59, 60].

While replication stress is widely recognized as a significant problem for cellular proliferation and survival, a cohesive mechanism or cellular markers that describe the state of replication stall are not well defined or understood [60]. However, similar to DNA damage signaling, the cell cycle kinase ATR provides many of the markers used to measure replication fork stall. Such markers include phosphorylation of the histone variant H2AX, formation of phosphorylated Ser-33 RPA34 foci, and the phosphorylation of CHK1 on Ser-345 [60]. As such, ATR itself serves an important function in the direct protection of stalled replication forks [61, 62].

Replication forks that are under protection of the ATR pathway can be typically restarted once the source for replication stress has been removed. However, in the presence of a DNA damaging lesions, multiple pathways have been described for the restart of stalled forks [60] (Figure 1.2). First, dormant replication origins licensed prior to S-phase of the cell cycle by the replisome components MCM2-7, fire to restart replication near forks stalled due to DNA lesions [63-65] (Figure 1.2A). Secondly, the replication machinery has been observed to ‘reprime’ replication downstream of a UV induced physical lesion or to undergo template switching, which allows for replication restart. However, ‘repriming’ of the replication fork also leaves a ssDNA segment in its wake [66, 67] (Figure 1.2B and C). These ssDNA segments are believed to be repaired via an error-prone repair mechanism known as translesion synthesis, which is mediated by ubiquitinated PCNA and a translesion synthesis polymerase or through template switching [68-70] (Figure 1.2C). Lastly, a stalled replication fork may undergo a process known as reversal. In the case of replication fork reversal, pairing of the newly synthesized strands form a Holliday junction in a structure called a 'chicken foot'. Restart after Holliday junction formation can be difficult as it typically requires the removal and subsequent re-loading of the replication

machinery [71] (Figure 1.2D). Importantly, the formation of these 'chicken foot' structures occurs roughly one third of the time in response to replication fork stall and requires the HR recombinase RAD51, suggesting that RAD51 mediated replication fork regression is an important mode of replication fork restart [72].

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 [56]. While the role of key HR proteins are broadly characterized for their roles in DNA repair, the roles that they are playing in DNA replication are not fully understood. Interestingly, HR has been shown to be required for the restart of replication forks in fission yeast, and is a prescribed model in higher eukaryotes [73, 74]. One key HR protein involved in DNA replication is the recombinase RAD51. RAD51 has been observed to localize to an active replication fork and the lack of RAD51 at an active fork leads to the accumulation of 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 (Figure 1.2 E) [75]. Recently, other members of the Fanconi anemia and HR pathway have been shown to have important roles in replication fork protection. Specifically, the breast and ovarian cancer predisposition gene and RAD51 mediator protein, BRCA2, is directly required for replication fork protection [76] through its recruitment of RAD51 to protect nascent DNA [77] (Figure 1.2E). However, exactly where RAD51 is loaded onto stalled replication forks is not fully understood, but postulated that it is loaded on to the 'chicken foot' regressed structures formed after replication stall (Figure 1.2F) or alternatively RAD51 may be loaded by BRCA2 onto the ssDNA fragments that form frequently during DNA replication [56].

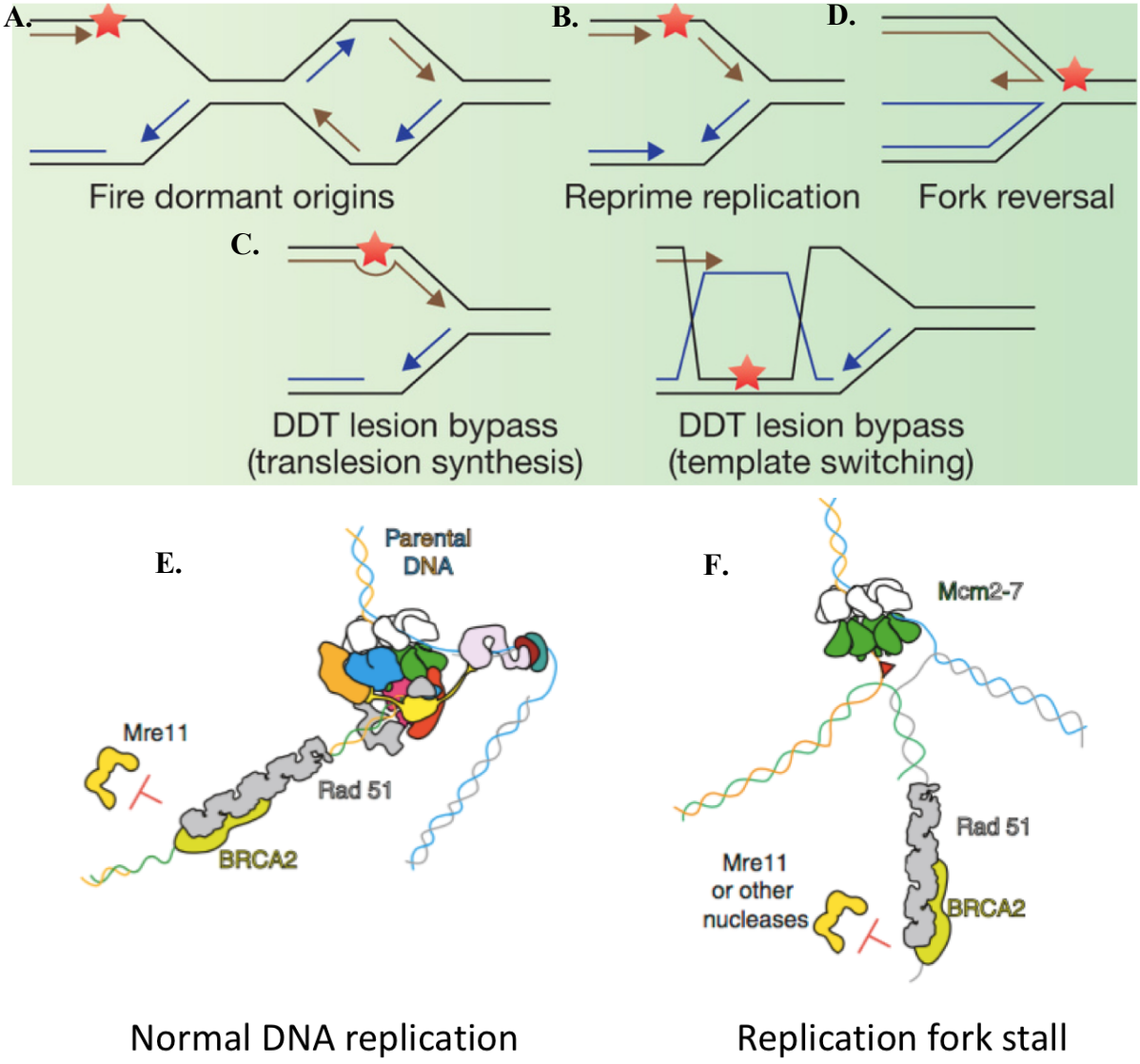


Figure 1.2. **Mechanisms of replication fork restart and protection**. A-D Replication forks stalled at DNA lesions (shown on the leading strand and indicated by a red star) and stabilized by the ATR pathway can restart replication by **A.** firing dormant origins, **B.** repriming replication, **C.** activating the DNA damage tolerance (DDT) pathways and by **D.** reversing the stalled fork. **E**. Nascent DNA degradation by MRE11 during normal replication is protected by the recombinase RAD51. **F.** RAD51 and BRCA2 protect the 'chicken foot' structure from degradation by MRE11 following replication fork stall caused by a DNA lesion denoted by a red triangle. Adapted from [60] and [56]

NEK8

NEK8 belongs to a larger family of kinases known as the Never In Mitosis A (NIMA)-related kinase family (NEKs). NIMA is a serine/threonine protein kinase present in the multicellular filamentous fungus, *Aspergillus nidulans,* whose activation and degradation are essential for mitotic entry and exit [78, 79]. NIMA was first identified as a temperature-sensitive mutant that was never in mitosis (nim) when *Aspergillus* cells were incubated at the restrictive temperature. At the restrictive temperature, *nimA* mutants arrest in G2 with uncondensed chromosomes, cytoplasmic microtubule structures and duplicated, but not separated, spindle poles. When upshifted to the permissive temperature, cells rapidly enter mitosis undergoing chromosome condensation and spindle formation. This suggests that the *nimA* mutation blocks cells at a point immediately before initiation of chromosome condensation and spindle assembly [80].

In mice and humans, there are eleven orthologous members of the NEK family [81, 82]. The NEK proteins have a well-conserved N-terminal kinase domain but have divergent C-terminal tails of varying length [83]. Primary functional characterization of the NEK family kinases has centered on their role in regulation of cell cycle control and ciliogenesis [84, 85]. However, emerging research is beginning to provide insight into the multitude of functional mechanisms in which the NEKs are involved.

Typical of the NEKs, NEK8 contains an N-terminal kinase domain. However, it 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 [83]. It has not yet been shown if NEK8 has a similar mechanistic function to the RCC1 protein, but this function remains possible [84]. NEK8 is shown to primarily localize to the centrosomes [86] and primary cilium [87], as well, it has been observed to localize to the nucleus [85]. NEK8 was first identified when a

missense mutation in the highly conserved RCC1 domain of Nek8 were reported as phenotypically causative in the mouse model of juvenile polycystic kidney disease (JCK), where ciliary localization of the mutant Nek8 protein is defective [88]. Similarly, mutations in the conserved RCC1 domain of Nek8 are causative of the phenotypes observed in the rat model of Lewis polycystic kidney disease [89].

Moreover, three mutations have been identified in NEK8 that are implicated in the childhood autosomal recessive kidney disease nephronophthisis (NPHP), with NEK8 proposed as the candidate NPHP9 gene (Otto et al., 2008). Of the three mutations, two are heterozygous (L330F and A497P) and one is homozygous (H425Y) [86]. Interestingly, all three mutated amino acids (L330, H425 and A497) are found in the RCC1 domain and show evolutionary conservation being identical in humans, mouse, *Xenopus* and zebrafish, with H425 also being conserved in the chordate *Ciona intestinalis* [86]. Similar to the JCK mouse model mutation, the NPHP associated mutants show reduction in ciliary localization in IMCD-3 mouse kidney epithelial cells, supporting the hypothesis that mislocalization of NEK8 results in the formation of renal cysts [86, 90]. Also, three fetuses from a single family with a form of Ivemark syndrome, which is similar to polycystic kidney disease, were reported to contain C-terminal truncation mutations leading to nonsense mediated decay of NEK8, essentially rendering them null for the gene [91]. Most recently, two newborns exhibiting Alagille syndrome like phenotypes were discovered to have mutations in the RCC1 domain of NEK8 which are believed to be causative for their NPHP like presentation [92].

Importantly, NEK8 has also been linked to the ATR mediated replication stress response via regulation of the protein kinase CDK2 [93]. Cells deficient in NEK8 are characterized by an increase in endogenous 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 [93]. 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 was observed to interact with the checkpoint kinase ATR, CHK1 and the ATR interacting partner, ATRIP [93]. It has also been observed that kidneys of Nek8 mutant mice accumulate DNA damage, and loss of Nek8 or replication stress similarly disrupts renal cell architecture in these mice [93]. Therefore, there exists evidence that NEK8 functions in the DNA damage response and DNA replication, providing support for the basis of this dissertation.

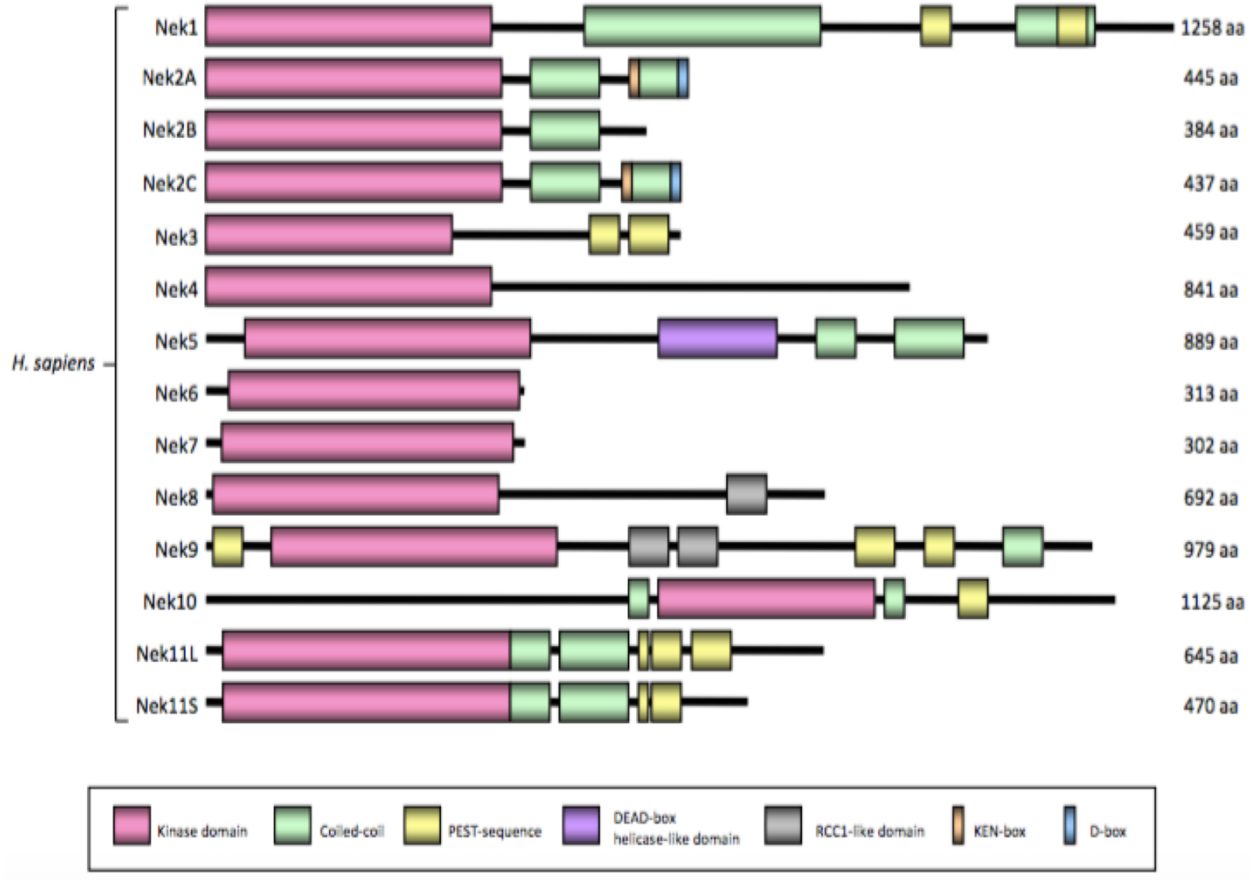


Figure 1.3. **Human NIMA-related kinase family members (NEKs)**. Schematic representation of the human NIMA-related kinases with their sizes indicated. The NIMA kinase family is defined by sequence homology to the *A. nidulans* NIMA kinase domain. The NIMA kinases share a conserved N-terminal catalytic domain (pink) but have divergent C-termini. The C-termini facilitate protein-protein interactions (coiled-coil, RCC1-like domain), or promote proteolytic degradation (PEST-sequence, KEN-box, D-box). There are 11 mammalian NEKs, although these are likely to exist in multiple splice variants. Adapted from [83].

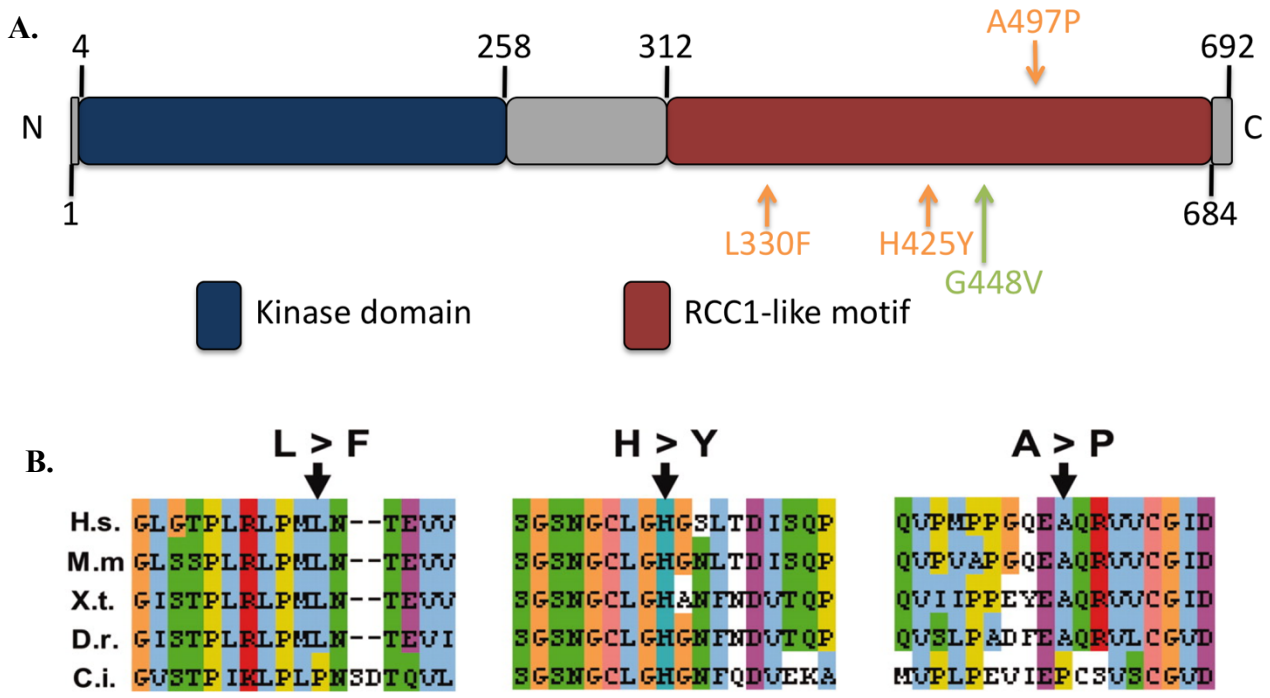


Figure 1.4. **Sites mutated in human NEK8 in NPHP patients are evolutionarily conserved**. **A**. Schematic diagram of NEK8 showing the position of the NPHP mutations in orange. JCK mutation in mouse Nek8 is represented in green. Note all mutations are found in the RCC1 domain. **B.** Alignment of the Nek8 protein sequence mutated in NPHP patients from various species including Homo sapiens (H.s.), Musculus musculus (M.m.), Xenopus tropicalis (X.t.), Danio rerio (D.r.), and Ciona intestinalis (C.i.). Amino acid residues that are within the same group are coded in the same color. Mutated amino acids are indicated with arrowheads. Adapted from [86].

AIMS OF DISSERTATION

The overall aims of this dissertation were to characterize a novel role of human kinases in the response to DNA DSBs. From the results of a high-throughput human kinome siRNA screen, I focused on characterizing the role of the kinase NEK8 as a putative positive regulator of homologous recombination. The results of the screen are presented in Chapter 2. I demonstrated that NEK8 modulates the formation of RAD51 focus formation in response to a multitude of DNA damaging agents and the importance of this process across multiple species. These results are presented in Chapter 3. Potential mechanistic context of NEK8 in the regulation of genome stability and replication fork protection are explored in Chapter 4. The major conclusions and future directions of my project are described in Chapter 5.

Chapter 2. KINOME SIRNA SCREEN IDENTIFIES NEK8 AS A PROTEIN IMPORTANT FOR DNA DAMAGE-INDUCED RAD51 FOCUS FORMATION

ABSTRACT

Homologous recombination (HR) is an important mediator of error-free repair of DNA double strand breaks. Multiple proteins ensure the efficient progression of HR, including the key recombination factor, RAD51. Formation of distinct nuclear foci of RAD51 in response to DNA damage is a critical step in a cells' decision to dedicate itself to HR. RAD51 activity is regulated by many factors including the breast and ovarian cancer susceptibility gene products, BRCA1 and BRCA2, the RAD51 paralogs and by key DNA damage and cell cycle kinases such as ATM, ATR, CHK1 and the CDKs. Deregulation of these proteins can lead to impaired HR, genomic instability and cellular sensitivity to agents requiring homologous recombination for their repair, such as the inter-strand crosslinking agent mitomycin C (MMC) and PARP inhibitors. While many factors, including protein kinases, have been implicated in the intricate regulation of HR, there are likely still yet-to-be identified regulators of the pathway. To address this, we designed and conducted a high throughput siRNA screen of the human kinome to identify kinases with a possible novel role in regulating RAD51 focus formation and HR. Using MMC-induced RAD51 focus formation as a readout, we identified multiple candidate kinases, of which we decided to focus on NEK8. siRNA pools targeting NEK8 efficiently reduced RAD51 focus formation in the kinome screen without effecting cell cycle distribution. These siRNAs efficiently deplete NEK8 mRNA and reproduce the RAD51 phenotype in multiple cell lines. Our findings suggest a potential role for NEK8 in the regulation of RAD51 focus formation and HR.

INTRODUCTION

With the discovery of siRNA in plants [94] and shortly after, the ability to synthesize siRNAs that could induce RNA interference (RNAi) in mammalian cells [95, 96], a new age of molecular and cellular biology arose allowing for the high throughput interrogation of biological systems. RNAi has proven to be a powerful tool in the identification of novel genes in the DNA damage response and other cellular pathways [97-100].

In the maintenance of the human genome, homologous recombination (HR) plays an essential role in the error free repair of DNA double strand breaks (DSBs) [46]. The central component of HR, the recombinase RAD51 is recruited to sites of DSBs to mediate error-free repair. RAD51 bound to single stranded DNA can be visualized as distinct nuclear foci [30]. The recruitment and loading of RAD51 onto DNA requires many factors, including but not limited to, the breast and ovarian cancer susceptibility genes (BRCA1/FANCS, BRCA2/FANCD1, PALB2/FANCN) as well as the RAD51 paralogs (RAD51B, RAD51C/FANCO, RAD51D, XRCC2 and XRCC3) [101, 102].

Post-translational modifications of key HR proteins such as phosphorylation, ubiquitylation, and sumoylation have been shown to function as important regulatory mechanisms of HR [103, 104]. As previously mentioned, regulation of HR via phosphorylation can occur in both a positive and negative manner. This is mediated by key kinases which have been identified as being required for HR via phosphorylation of RAD51 itself or other important HR proteins [46]. However, a comprehensive and complete understanding of how these post-translational modifications are regulating both RAD51 focus formation and HR does not exist [46]. A more complete understanding of how post-translational modifications from both known,

and novel kinases regulate HR, can have large implications in both basic cellular biology as well as in cancer pathobiology.

To address this issue, we proposed to identify kinases that may be playing a novel role in both RAD51 focus formation and HR. To systematically identify kinases that regulate RAD51 and hence HR, we devised a cell-based screen where depletion of kinases by RNAi altered RAD51 nuclear focus formation following treatment with the DNA inter-strand crosslinking agent mitomycin C. In this chapter we present our screen and the validation of the top twenty positive hits. We found that the NIMA-related kinase NEK8 is a strong candidate for a novel regulator of RAD51 focus formation and HR.

RESULTS

Primary identification of kinases that regulate RAD51 focus formation

Initially, we devised a screen using DNA damage-induced RAD51 focus formation as a readout to systematically identify kinases that regulate RAD51 focus formation itself and as a consequence, HR. Our primary step was to determine the proper type of damage to induce RAD51 focus formation under conditions suitable for high throughput screening. We tested multiple forms of damage including DNA interstrand cross-linkers (mitomycin C (MMC, 60ng/mL), cisplatin (CDDP, 5nM), 24h), Topoisomerase inhibitors (camptothecin (CPT), 25nM, 24h) and ionizing radiation (IR, 10Gy, 6h) (Figure 2.1A). For multiple reasons, including A. efficiency of induction of RAD51 focus formation, B. requirement of HR in repair of DNA damage lesion type and C. ease of treatment regimen amenable to high throughput screening, we concluded that a dose of 60ng/mL of MMC was suitable for our high throughput siRNA kinome library screen. The optimal time-point of 24h for RAD51 focus formation was determined via RAD51 focus formation kinetics following treatment with MMC (Figure 2.1B).

A library containing three pooled siRNAs targeting 713 human kinases (Sigma-Aldrich), was expressed in the human osteosarcoma cell line U-2 OS by transfection for 48 hours. Transfected U-2 OS cells were then treated with MMC (60ng/mL) for twenty-four hours, fixed and immunostained for RAD51 and RPA focus formation (Figure 2.1C). Images of foci were acquired by automated robotic microscopy (Cellomics) and the average number of foci per cell was determined by automated foci quantitation (Figure 2.1D). The average number of foci per cell was converted to normalized Z-scores and ranked (Figure 2.2A). We chose to focus on kinases with negative z-scores, where a negative Z-score is indicative of kinase depletion leading to inhibited RAD51 focus formation following DNA damage and hence considered positive hits in the screen.

As RAD51 foci form mainly in S and G2 phase of the cell cycle [105], we evaluated the percentage of cells in S phase following kinase depletion, using cyclin A expression as detected by immunofluorescent nuclear intensity staining. Cyclin A was chosen as it is a nuclear marker of cells that are in S and G2 phase of the cell cycle [106]. Data from an identical screen where MMC was used to investigate kinases required for the formation of FANCD2 focus formation, provided us with the correlative expression of cyclin A. Expression of cyclin A was also converted to a normalized Z-Score (Figure 2.2B). Negative Z-scores are indicative of kinase depletion leading to decreased expression of cyclin A following DNA damage. A Z-score near zero or positive Z-scores are indicative of unaffected cyclin A expression.

To identify kinases required for RAD51 focus formation and HR, we selected as positive hits, kinases with a RAD51 focus formation negative Z-score ($Z < -2.92$) and unaffected (near-zero) or positive cyclin A Z-score (Figure 2.2C). A total of twenty kinases were selected as positive hits in the primary screen (Figure 2.2C, Table 2.1).

For RPA staining, we similarly converted the average number of foci per cell to normalized Z-scores and plotted them as ranked scores (Figure 2.3A). As with the RAD51 screen, negative Z-scores are indicative of kinase depletion leading to inhibition of RPA focus formation following DNA damage and hence considered positive hits in the screen. The range of average foci per cell was plotted as a histogram with a mean of 14.98 foci per cell and a standard deviation of 2.20 (Figure 2.3B). The top twenty kinases from the RPA screen were selected as positive hits in the primary screen (Figure 2.3A, Table 2.2).

We used this data to compare kinases that scored as double positives, both decreasing RAD51 focus formation and RPA focus formation, signifying that these kinases may play an early upstream role in the regulation of homologous recombination, for example prior to DNA end resection or following DNA resection but prior to loading of RPA on single stranded DNA. Of note, depletion of only three kinases decreased both RAD51 and RPA focus formation following MMC treatment in the screen (Table 2.3). One kinase, ERBB2, raised interest, as it was considered a top positive (negative Z-score) hit in the RAD51 foci screen but was also a top negative (positive Z-score) hit in the RPA screen (Table 2.3). This signifies that depletion of ERBB2 lead to a large decrease in RAD51 focus formation, but an increase in RPA focus formation following DNA damage. Hence, ERBB2 could be considered an interesting kinase that may serve a role in RAD51 focus formation downstream of RPA focus formation, or more simply, ERBB2 may be playing a role in the later stages of homologous recombination. As such, we decided to not limit our future validation of the screen hits to kinases that when depleted inhibited both RAD51 and RPA focus formation following MMC induced DNA damage, but instead chose to validate all twenty kinases that scored positive in the RAD51 focus formation screen.

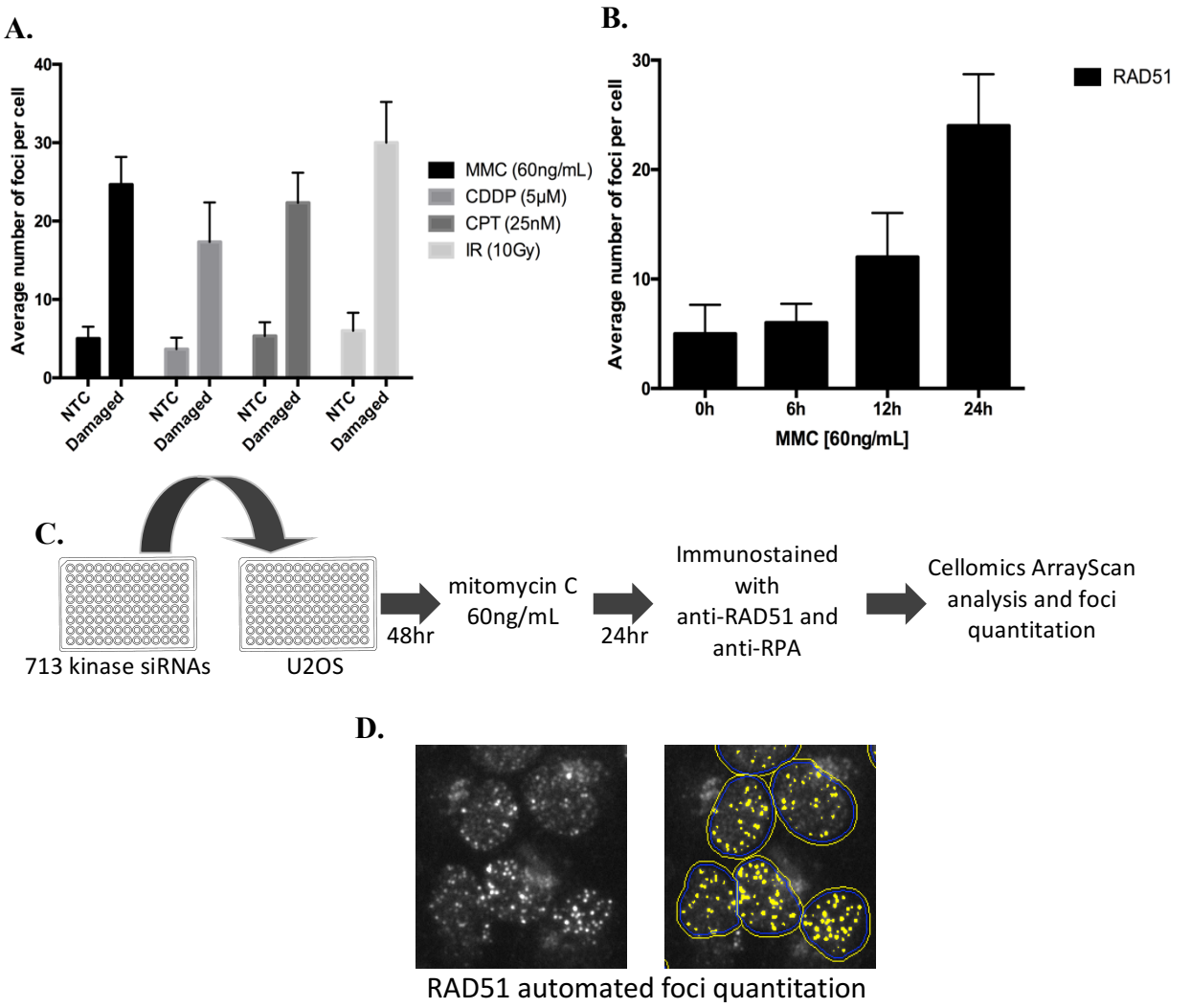


Figure 2.1. **Conditions and schematic of high throughput siRNA kinome screen**. **A.** U-2 OS cells were treated with indicated DNA damaging agent for respective times (MMC, CDDP, CPT: 24h; IR: 6h) and immunostained for RAD51. **B.** U-2 OS cells were treated with MMC [60ng/mL] for increasing time frames and immunostained for RAD51. **C.** Screening strategy for identification of kinases that may be required for RAD51 focus formation and homologous recombination in U-2 OS cells. **D**. Representative images of RAD51 focus formation via automated acquisition and quantitation.

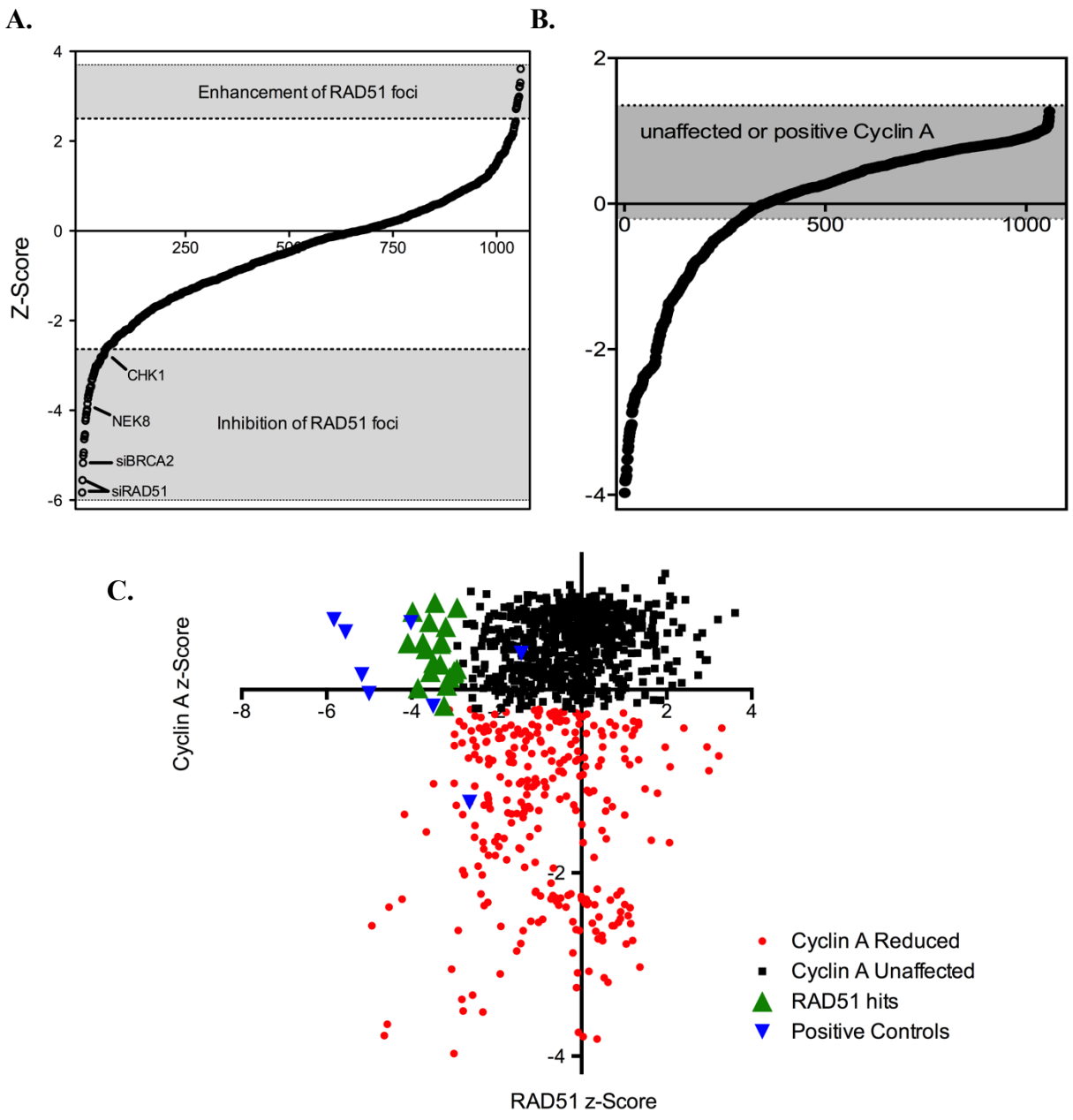


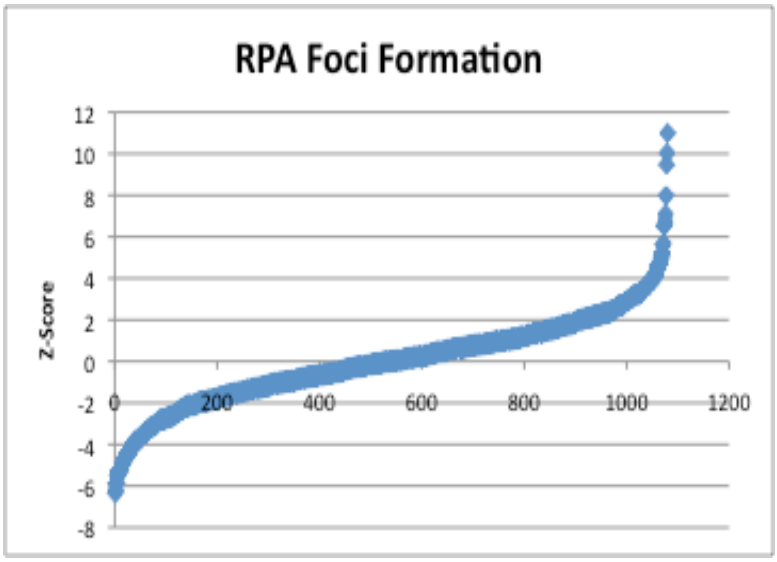
Figure 2.2. **Identification of kinases that regulate MMC-induced RAD51 focus formation in U-2 OS Cells**. **A.** The mean Z-score of each kinase from the RAD51 foci screen are ranked (n=2). **B.** The mean Z-score of each kinase from the FANCD2/cyclin A screen are ranked. **C.** The mean Z-scores from RAD51 and cyclin A screens are plotted to determine RAD51 (+) hits.

Table 2.1. **Summary of library screening to identify kinases that may regulate RAD51 focus formation and homologous recombination.**

The mean Z-scores of two independent experiments (RAD51) and one independent experiment (Cyclin A) are ranked in ascending order. * denotes siRNA (+) controls required for RAD51 focus formation and are not annotated.

Kinase/Control	***Gene Annotation***	***RAD51 Z-Score***	***cyclin A Z-Score***
*siRAD51		-5.82	0.73
*siBRCA2		-5.17	-0.18
*siATR		-2.63	0.39
*siCHK1		-1.93	-1.23
TEX14	Homo sapiens testis expressed sequence 14 (TEX14), transcript variant 2, mRNA	-4.08	0.50
DCAMKL3	PREDICTED: Homo sapiens doublecortin and CaM kinase-like 3 (DCAMKL3), mRNA	-3.98	0.85
SPHK1	Homo sapiens sphingosine kinase 1 (SPHK1), transcript variant 1, mRNA	-3.85	0.01
NEK8	Homo sapiens NIMA (never in mitosis gene a)- related kinase 8 (NEK8), mRNA	-3.73	0.51
PAPSS2	Homo sapiens 3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2), transcript variant 2, mRNA	-3.66	0.44
ERBB2	Homo sapiens v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (ERBB2), transcript variant 2, mRNA	-3.58	0.73
SCYL2	Homo sapiens SCY1-like 2 (S. cerevisiae) (SCYL2), mRNA	-3.56	0.19
RAPGEF3	Homo sapiens Rap guanine nucleotide exchange factor (GEF) 3 (RAPGEF3), mRNA	-3.48	0.29
PAK4	Homo sapiens p21(CDKN1A)-activated kinase 4 (PAK4), transcript variant 1, mRNA	-3.45	0.94
DGKD	Homo sapiens diacylglycerol kinase, delta 130kDa (DGKD), transcript variant 2, mRNA	-3.32	0.27
NEK1	Homo sapiens NIMA (never in mitosis gene a)-related kinase 1 (NEK1), mRNA	-3.31	0.50
TGFBR1	Homo sapiens transforming growth factor, beta receptor I (activin A receptor type II-like kinase, 53kDa) (TGFBR1), mRNA	-3.29	0.52
RPS6KL1	Homo sapiens ribosomal protein S6 kinase-like 1 (RPS6KL1), mRNA	-3.23	-0.17
FUK	Homo sapiens fucokinase (FUK), mRNA	-3.19	0.68
MAP2K3	Homo sapiens mitogen-activated protein kinase kinase 3 (MAP2K3), transcript variant B, mRNA	-3.17	0.04
TAF1	Homo sapiens TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250kDa (TAF1), transcript variant 1, mRNA	-3.10	0.13
RPS6KB2	Homo sapiens ribosomal protein S6 kinase, 70kDa, polypeptide 2 (RPS6KB2), transcript variant 1, mRNA	-3.01	0.17
IRAK2	Homo sapiens interleukin-1 receptor-associated kinase 2 (IRAK2), mRNA	-3.00	0.19
PTK6	Homo sapiens PTK6 protein tyrosine kinase 6 (PTK6), mRNA	-2.93	0.22
IRAK1	Homo sapiens interleukin-1 receptor-associated kinase 1 (IRAK1), transcript variant 1, mRNA	-2.92	0.89

A.



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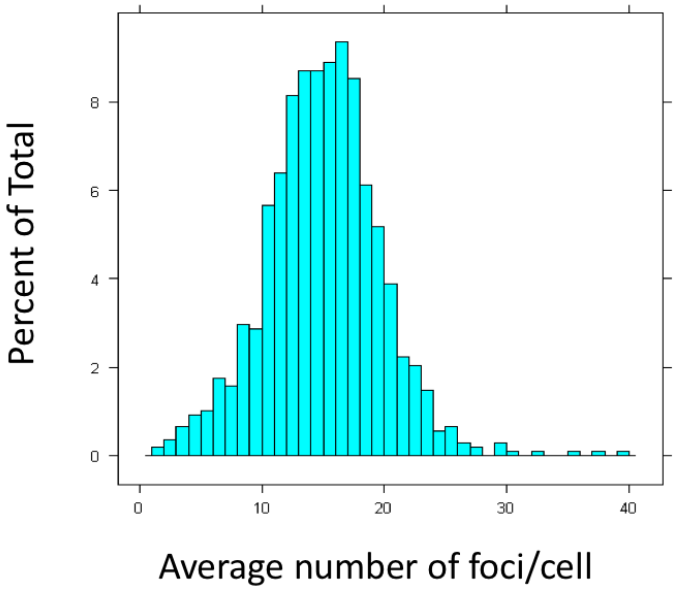


Figure 2.3. **Identification of kinases that regulate MMC-induced RPA focus formation in U-2 OS Cells**. **A.** The mean Z-score of each kinase from the RPA foci screen are ranked (n=2). **B.** The average number of foci per cell from the RPA screen are binned in a histogram to determine the population mean (14.98 +/- 2.20 SD)

Table 2.2. **Summary of library screening to identify kinases that may regulate RPA focus formation and homologous recombination.**

The mean Z-scores of two independent experiments (RPA) are ranked in ascending order. * denotes siRNA (+) controls required for RPA focus formation and are not annotated.

Kinase/Control	***Gene Annotation***	***RPA Z-Score***
*siRPA1		-6.34
*siCtIP		-3.66
NME3	non-metastatic cells 3, protein expressed in (NME3), mRNA	-3.86
PIP5K2A	phosphatidylinositol-4-phosphate 5-kinase, type II, alpha (PIP5K2A), mRNA	-3.86
ITK	IL2-inducible T-cell kinase (ITK), mRNA	-3.84
TNK2	tyrosine kinase, non-receptor, 2 (TNK2), transcript variant 1, mRNA	-3.82
PAK4	p21(CDKN1A)-activated kinase 4 (PAK4), transcript variant 1, mRNA	-3.70
CHUK	conserved helix-loop-helix ubiquitous kinase (CHUK), mRNA	-3.67
WNK4	WNK lysine deficient protein kinase 4 (WNK4), mRNA	-3.57
TNIK	TRAF2 and NCK interacting kinase (TNIK), mRNA	-3.48
CHEK1	CHK1 checkpoint homolog (S. pombe) (CHEK1), mRNA	-3.45
PDGFRB	platelet-derived growth factor receptor, beta polypeptide (PDGFRB), mRNA	-3.42
CDK8	cyclin-dependent kinase 8 (CDK8), mRNA	-3.41
NTRK2	neurotrophic tyrosine kinase, receptor, type 2 (NTRK2), transcript variant c, mRNA	-3.41
KIAA1639	PREDICTED:KIAA1639 protein (KIAA1639), mRNA	-3.26
MYO33	myosin IIIB (MYO3B), mRNA	-3.17
MPP5	membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5) (MPP5), mRNA	-3.11
MUSK	muscle, skeletal, receptor tyrosine kinase (MUSK), mRNA	-3.11
MAP4K1	mitogen-activated protein kinase kinase kinase kinase 1 (MAP4K1), transcript variant 2, mRNA	-3.10
EPHB2	EPH receptor B2 (EPHB2), transcript variant 2, mRNA	-3.08
IRAK2	interleukin-1 receptor-associated kinase 2 (IRAK2), mRNA	-3.04

Table 2.3. **Summary of kinases from library screening that score positive for both RAD51 and RPA focus formation inhibition.**

The mean Z-scores of two independent experiments (RAD51 and RPA) are ranked in ascending order. * denotes a kinase that had a negative RAD51 Z-Score but a positive RPA Z-Score

Kinase	***Gene Annotation***	***RAD51 Z-Score***	***RPA Z-Score***
*ERBB2	Homo sapiens v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian) (ERBB2), transcript variant 2, mRNA	-3.58	4.55
PAK4	p21(CDKN1A)-activated kinase 4 (PAK4), transcript variant 1, mRNA	-3.45	-3.70
FUK	fucokinase (FUK), mRNA	-3.19	-3.04
IRAK2	interleukin-1 receptor-associated kinase 2 (IRAK2), mRNA	-3.00	-3.04

Validation of initial kinases identified as being required for RAD51 focus formation

A caveat to the use of RNAi, especially siRNA, is the risk of off-target effects due to cross-reaction of targets with little sequence similarity to the intended target [107]. This process occurs at high rates due to sequence complementarity in the 3' UTR of the unintended transcript with the seed sequence of the siRNA, similar to the mechanism of mRNA depletion by microRNAs [108]. Multiple reports suggest that this is common occurrence, particularly for the homologous recombination protein, RAD51 [97] [109]. As such, stringent efforts must be made in order to ensure that screen hits are validated. Many criteria have been suggested to aid in increasing the robustness of high throughput siRNA screens [110-116]. To ensure that the kinases identified in our RNAi screen are not off target effects, we chose to validate via the following criteria: 1. Multiple siRNAs must reproduce the phenotype, 2. A bioinformatics algorithm, GESS analysis, designed to identify possible siRNA off-target effects via seed sequence homology in the 3'UTR of protein encoding genes in the human genome [109], 3. siRNAs that produce a phenotype must correlate with depletion of target mRNA or protein, where applicable, and 4. The phenotypes must reproduce with the same siRNAs in multiple cell lines.

First, to validate the results of the kinome siRNA screen, the pooled siRNAs were de-convoluted and the three independent siRNAs against each candidate kinase were used to confirm the result in U-2 OS cells. Among those genes we found to decrease MMC-induced RAD51 focus formation, TEX14, NEK8, PAK4, DGKD (Figure 2.4.1), RPS6KL1, FUK, and PTK6 (Figure 2.4.2) emerged as promising candidates where multiple individual siRNAs targeting those kinases led to a decrease in RAD51 focus formation following damage with MMC. Although we could not fully rule out candidate kinases that had only one siRNA replicate the phenotype, we ultimately chose to focus on the seven candidate kinases that had multiple

siRNAs validated via the first criteria. Of note, only one candidate kinase, NEK8, had all three siRNAs that replicate the RAD51 focus formation phenotype.

Secondly, to identify possible non-specific targeting of 3'UTRs in the human genome by the twenty identified kinase siRNAs, we performed GESS analysis (Figure 2.5A) [109]. To perform the analysis, we used the pool of twenty kinase siRNAs that have been de-convoluted equating to sixty total siRNAs. Based on the previous validation data (Figure 2.4.1 and 2.4.2), siRNAs were divided into two families, those showing a RAD51 phenotype and those not showing a RAD51 phenotype (24 and 36 respectively). This division must be done for purposes of GESS analysis. The siRNAs were then subjected to GESS analysis against the 3'UTR of annotated protein coding genes in the human genome (27,534 total genes). This analysis revealed that none of the sixty total siRNAs tested showed a significant enrichment towards potential non-specific or off-targeting of 3'UTR sequences of protein coding genes in the human genome (Figure 2.5B). Therefore, GESS analysis did not aid in identifying or removing any candidate kinase siRNAs that may be exhibiting off target effects on MMC-induced RAD51 focus formation.

Next, for a candidate kinase siRNA to be considered a validated positive hit, we tested the efficiency of depletion of mRNA targeted by the siRNA. As the candidate kinase pool was already narrowed to seven kinases (Figure 2.4.1 and Figure 2.4.2), we focused on only these kinases. To determine the efficiency of depletion of the kinase siRNAs, we conducted RealTime-PCR (RT-PCR) with primers specific to each target mRNA. For the ease of analysis, we limited this first validation of mRNA depletion to simple RT-PCR and DNA agarose gel analysis. RT-PCR analysis of the candidate kinase siRNAs revealed complete and correlative mRNA depletion by nine total siRNAs targeting three candidate kinases, NEK8, PAK4 and DGKD

(Figure 2.6A). RT-PCR on three of the candidate kinases, RPS6KL1, FUK and PTK6 revealed that none of the siRNAs targeting these genes efficiently depleted their respective mRNAs (Figure 2.6B). This signifies that these candidate kinase siRNAs were displaying off-target effects on MMC induced RAD51 focus formation and were effectively eliminated from our list of candidate kinases. We also note that after multiple failed attempts in U-2 OS and other human cell lines that we were not able to amplify mRNA from the candidate kinase gene TEX14. As such, we chose to also eliminate TEX14 from the list of candidate kinases.

Lastly, to validate that the effect of siRNA depletion of the three remaining candidate kinases was not cell type specific, we tested MMC-induced RAD51 focus formation in one other human cell line. We began by testing the de-convoluted pools of siRNA for the candidate kinases NEK8, PAK4 and DGKD in the human cell line HeLa. Interestingly, as in the human cell line U-2 OS (Figure 2.4.1), all siRNAs targeting the candidate kinase NEK8 significantly decrease efficient MMC-induced RAD51 focus formation (Figure 2.6C). Of the two other remaining candidate kinases, PAK4 had two siRNAs significantly reduce RAD51 focus formation following MMC treatment, while DGKD only had one siRNA validate in HeLa cells (Figure 2.6C).

Based on all of the above data, we chose to focus on the candidate kinase NEK8. This decision was made in part, due to the nature of other NEK family of kinases being implicated in both cell cycle regulation and the DNA damage response [84, 117-119]. Also of note, during the completion of this dissertation, NEK8 was implicated in the ATR-regulated replication stress response and in regulating S-phase specific CDK activity [93], however, the mechanism by which this stress response is regulated by NEK8 remains a mystery. As the candidate kinase PAK4 also appeared to be an interesting target, we did not completely rule this out of future

validation. However, we chose to fully move forward with characterizing the role of NEK8 in the regulation of MMC-induced RAD51 focus formation.

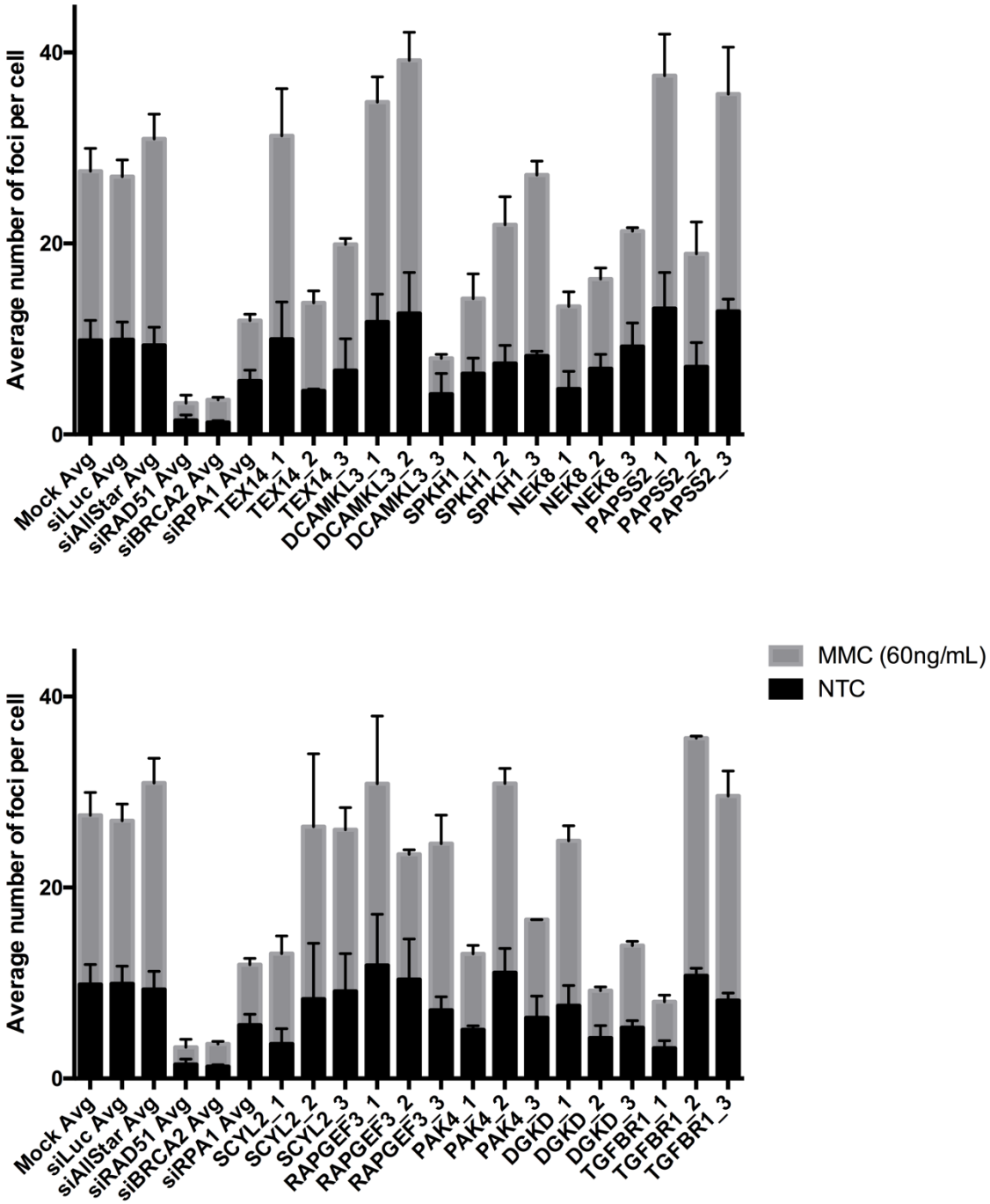


Figure 2.4.1. **Validation of kinases putatively required for MMC-induced RAD51 focus formation.** siRNA pools of kinases scoring positive in the primary kinome screen were de-convoluted and transfected individually into U-2 OS cells (20nM). Cells were then treated with/without 60ng/mL of MMC for 24h, immunostained for RAD51 and quantitated for average number of foci per cell (n=3, +/- SEM).

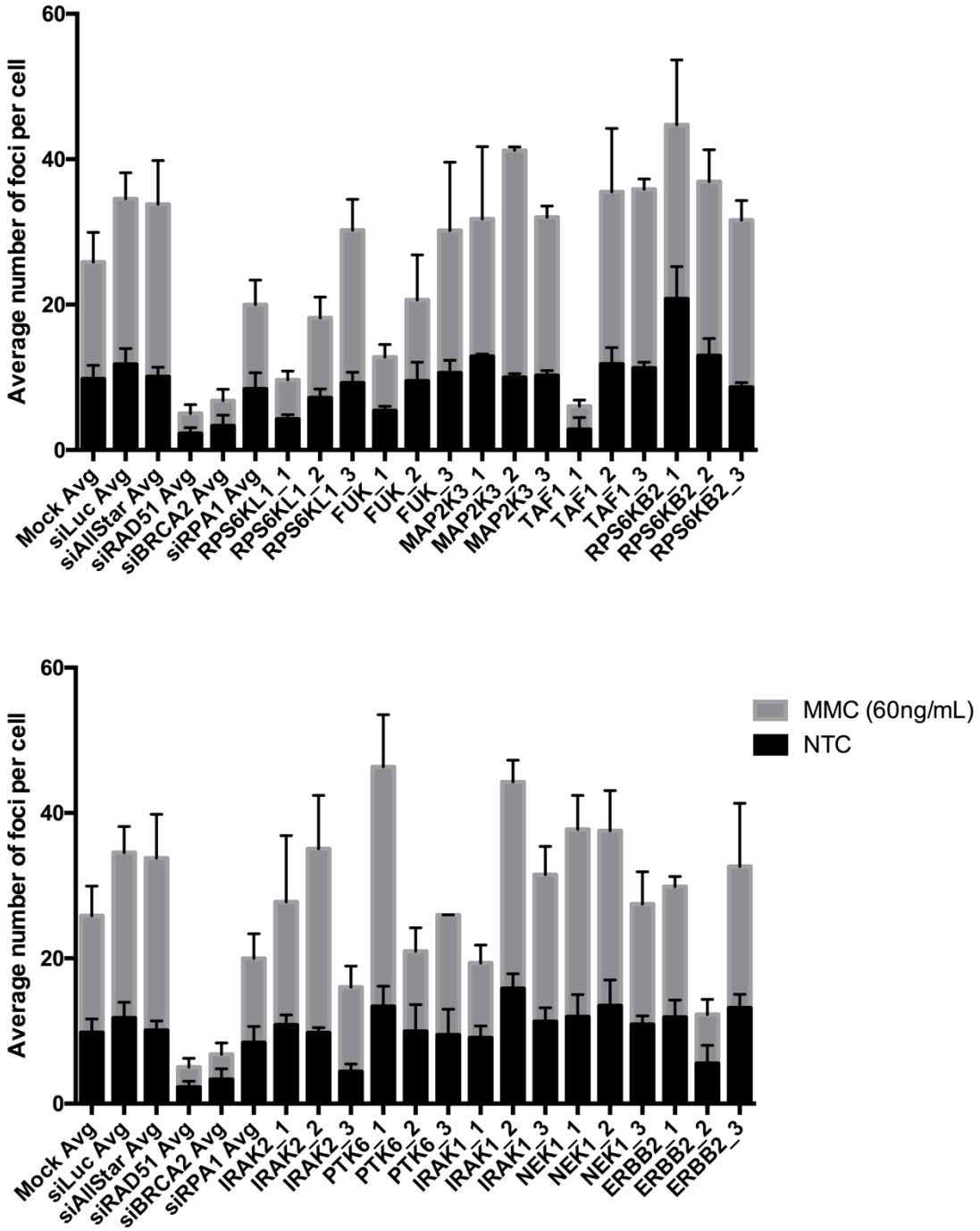


Figure 2.4.2. **Validation of kinases putatively required for MMC-induced RAD51 focus formation.** siRNA pools of kinases scoring positive in the primary kinome screen were de-convoluted and transfected individually into U-2 OS cells (20nM). Cells were then treated with/without 60ng/mL of MMC for 24h, immunostained for RAD51 and quantitated for average number of foci per cell (n=3, +/- SEM).

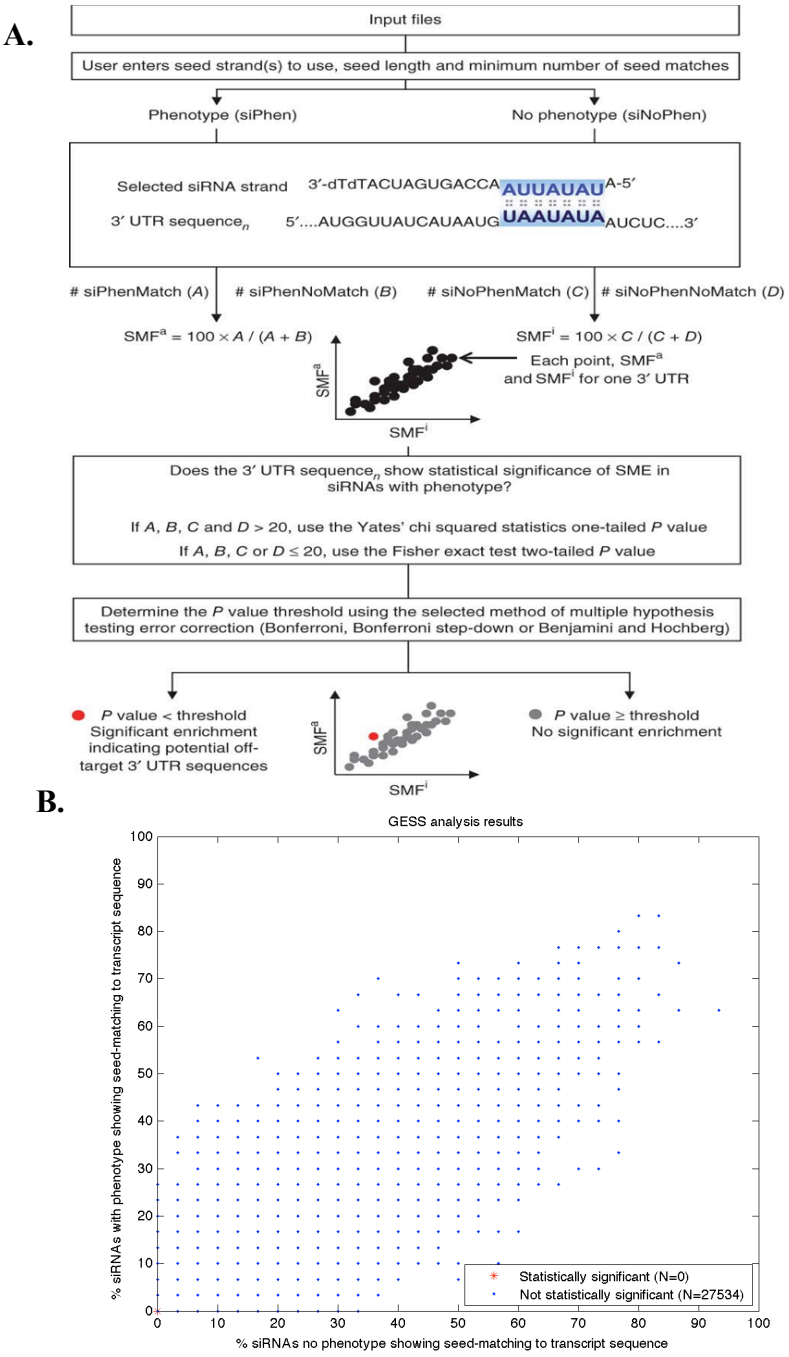


Figure 2.5. **GESS bioinformatics analysis of candidate kinases required for MMC-induced RAD51 focus formation.** **A**. Schematic of GESS bioinformatics analysis, adapted from [109]. **B**. GESS analysis results of siRNAs against candidate kinase genes. Scatter plot represent the percentage of siRNAs in 2 groups that have at least one 7-nucleotide antisense seed sequence match to 27,534 human 3′UTRs. The plot compares siRNAs that individually rescored with a strong phenotype (y axis) with those that did not (x axis).

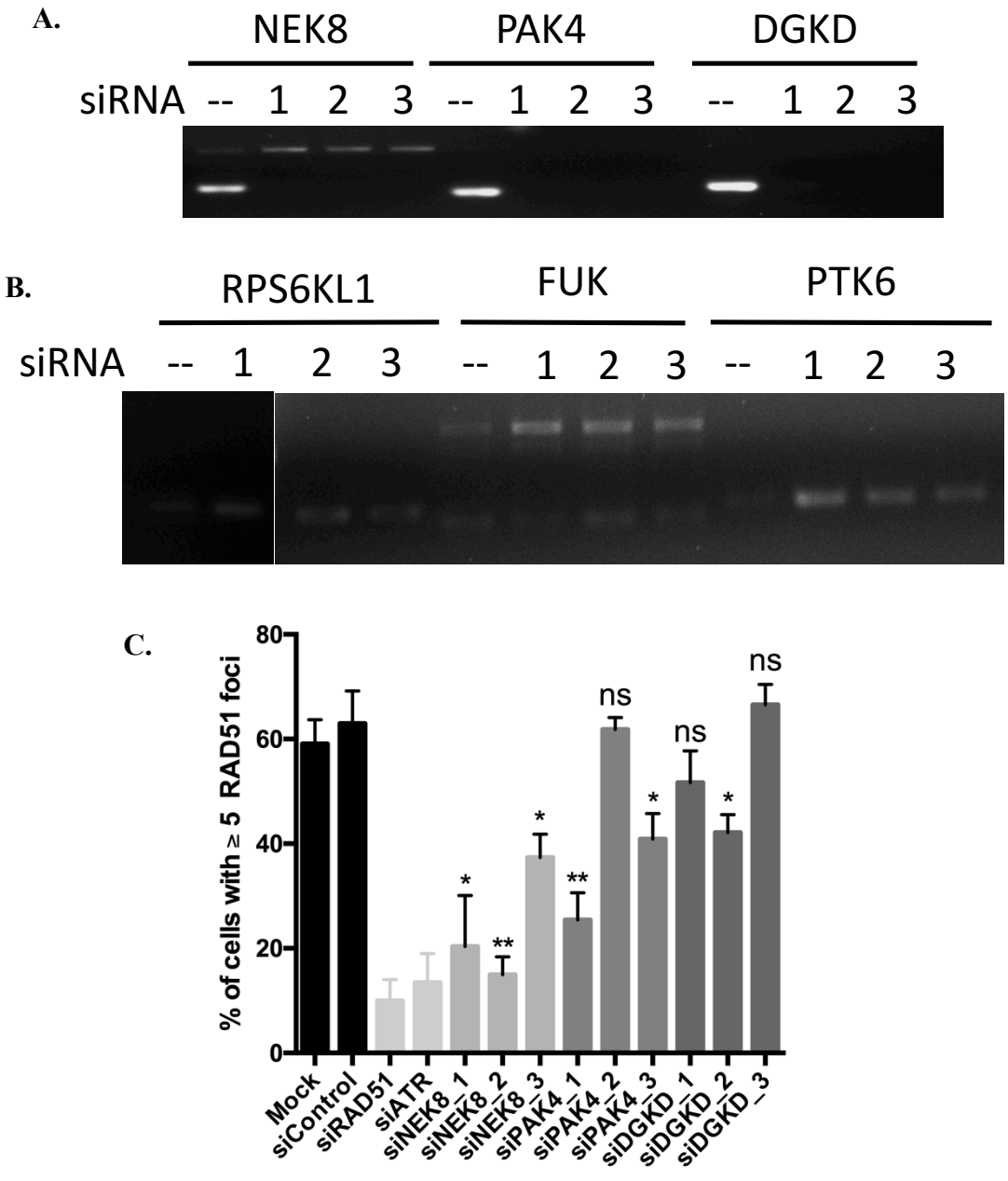


Figure 2.6 **Validation of siRNA specificity in targeting of candidate kinases. A-B**. U-2 OS cells were transfected with siRNA (20nM). RNA was collected, converted to cDNA and amplified by PCR for the gene of interest. **C.** siRNA pools were de-convoluted and transfected individually into HeLa cells (20nM). Cells were then treated with/without 60ng/mL of MMC for 24h, immunostained for RAD51 and quantitated for percentage of cells with greater than 5 foci per cell (n=3, +/- SEM) * = $p<0.05$, ** = $p<0.01$.

DISCUSSION

In this study, we conducted a comprehensive high throughput siRNA screen of a human protein kinase library in the human cell line U-2 OS to systematically identify kinases that potentially regulate RAD51 and RPA focus formation and hence HR. Using the DNA inter-strand crosslinking agent, MMC, we identified twenty candidate kinases which could potentially regulate both RAD51 and RPA focus formation and possibly HR. Using cyclin A expression, we limited the number of kinases in the RAD51 screen to the top twenty hits which did not alter cyclin A expression. This validation point was key as cyclin A is predominantly expressed in S and G2 phases of the cell cycle [106], which is also the time to which formation of RAD51 foci is limited [105]. Of the identified kinases, three potential hits overlapped in both the RAD51 and RPA focus formation screens. However, due to the fact that we also identified kinases that reduced RAD51 focus formation and increase RPA focus formation, we chose to validate all the top RAD51 candidate kinases. Of these, we identified the protein kinase NEK8 as a potential kinase that regulates RAD51 focus formation and HR.

Surprisingly, the highest scoring known kinase required for RAD51 focus formation in our screen was CHK1 (Z-score -2.63), which ranked it as the twenty-fifth strongest hit. As such, we chose to use this Z-score value as the point of our arbitrary cutoff for the top twenty kinases effecting RAD51 focus formation. As for the other kinases known to be required for RAD51 focus formation, ATM (Z-Score 0.29) [120], ATR (Z-Score -1.42) [121], CK2 (Z-Score -0.28) [51], CDK1 (Z-Score -1.32) [122] and CDK2 (Z-Score -.70) [123], all scored below our CHK1 cutoff. This could signify that CHK1 itself exerts the strongest effect on the RAD51 focus formation phenotype of the kinases known to regulate this process. Also, it is possible that the degree of mRNA depletion of these other kinases by siRNA was not sufficient to impact RAD51

focus formation in our experimental system. This, however, remains to be investigated. Also, depletion of a few of these kinases also decreased cyclin A expression (a verification cutoff criteria in our screen) which lead us to exclude them from the list of candidate kinases. Lastly, prior to the identification of PLK1 as a kinase required for RAD51 focus formation [51], our screen identified PLK1 (Z-score -3.15) as a strong positive hit. Unfortunately, it was ruled out prior to any validation as depletion of PLK1 is used in the screening process as a positive control for cell proliferation and apoptosis [124].

Of the twenty positive hits in the RAD51 screen, two kinases, NEK8 and PAK4, passed all steps of the validation process. PAK4 is an interesting target as it decreased both RAD51 and RPA focus formation under our experimental screening conditions (Table 2.3) suggesting that the kinase may have a role in RAD51 focus formation upstream of RPA focus formation. PAK4 is one member of the p21 activated kinase family [125], where it has been reported to be required for the oncogenic transformation of breast cancer cells [126], likely through its role in regulating proper cell cycle transitions [127-129]. As a p21 activated kinase, PAK4 may play an interesting role in the regulation of RAD51 focus formation, as depletion of p21 via RNAi leads to decreased rates of RAD51 foci [130]. This, however, remains to be investigated, as we proceeded in characterizing the novel role NEK8 has in regulating RAD51 focus formation and HR.

Interestingly, during the completion of this dissertation work, our candidate kinase, NEK8, was linked to the ATR-mediated replication stress response via regulation of the protein kinase CDK2 [93]. Prior to this publication, there appeared to be no link between NEK8 and DNA repair or replication processes other than the presence of a highly conserved region similar to that of Regulator of Chromosome Condensation (RCC1) protein on the c-terminus of NEK8

[88]. However, it remains unclear if NEK8 plays a similar role to that of the RCC1 protein [131]. Instead, NEK8 has been predominantly studied for its role as a ciliary kinase and the progression of the polycystic-like kidney disease, nephronophthisis [85, 86, 89, 90, 132-135]. The identification of NEK8 as a possible DNA replication protein was very exciting for us and raised the possibility that NEK8 may be regulating replication fork dynamics via its function in regulating RAD51 focus formation (addressed in Chapter 4). As all three siRNAs targeting NEK8 passed our validation experiments, we decided to move forward with characterizing the function of NEK8 in RAD51 focus formation and HR.

While the approach that we took to identify kinases with novel roles in the regulation of RAD51 focus formation and HR was designed to be as unbiased as possible, there were inherent biases built into the experimental system which may have prevented us from identifying true positive hits. First, the choice of DNA damaging agent, in this case MMC, may have selected for kinases specifically required for the repair of DNA inter-strand crosslinks, but not other types of DNA damage which require RAD51 and HR for their repair. In fact, a recent publication focused on a genome wide screen identifying possible mediators of ionizing radiation-induced RAD51 focus formation, and neither NEK8 or PAK4 were identified as positive hits [98]. Secondly, our validation criteria may have led to discarding true positive hits. One such example is the use of cyclin A nuclear expression status. We chose to limit positive hits in the screen to kinases whose depletion did not alter cyclin A expression, likely eliminating kinases that could play a role in RAD51 focus formation but are also important for key cell cycle transitions. One such kinase is CHK1, which is known to directly phosphorylate RAD51 and is a key mediator of HR [27] Also, the criteria that a candidate kinase must have multiple siRNAs that replicate all validation parameters limited the possible identification of kinases where a single siRNA consistently

passed the mRNA depletion and multiple cell validation points. It is possible that a strong candidate was ruled out because of only one siRNA passing the validation.

In summary, we employed a high throughput screen of the human kinome to systematically identify kinases that regulate MMC-induced RAD51 focus formation and HR. From this screen, we identified two kinases, NEK8 and PAK4, as strong candidates for kinases which may regulate the response to DNA damaging agents which require HR for repair of their lesions. The identification of these kinases adds further complexity and understanding to the cellular regulation of DNA repair. In the next chapters, we attempt to elucidate the mechanism through which the kinase NEK8 regulates RAD51 focus formation.

MATERIALS AND METHODS

Cell Lines

U-2 OS and HeLa were purchased from the American Type Culture Collections. Cell lines were cultured in DMEM containing 10% FBS, 2mM L-glutamine and 1X Pen/Strep in a humidified 5% CO_2 containing atmosphere at 37^{o}C.

siRNA kinome library screening

U-2 OS cells were transfected (20nm, Lipofectamine RNAiMAX) 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 [136], RAD51 (5'-AACTAATCAGGTGGTAGCTCA-3'), ATR (5'-AACCTCCGTGATGTTGCTTGA-3'), and Chk1 (5' AAGGGATAACCTCAAAATCTC-3'). Two days post-transfection, cells were treated with MMC (60ng/mL) for 24h. 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 (Santa Cruz, H-92) and RPA. Images were acquired with the Cellomics Arrayscan microscope

(Thermo Scientific) and processed as described [137]. Using DAPI to define nuclei, the average number of foci per cell 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, μnc was the mean of the negative control population in each plate and σ was the standard deviation of the whole population. Screening was independently replicated two times.

Immunofluorescence microscopy

Immunofluorescence microscopy was conducted as previously described [137]. Briefly, transfected cells were grown on coverslips, treated with MMC (60ng/mL, 24h) 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 three times independently

GESS Analysis

GESS analysis was conducted as described [109, 138].

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'-CTCCTCGTTCATCCTGGTGT-3', Rev 5'-GAGCTGCTCTTCAACGAGGT-3'), DGKD (For 5'-GCTTGTGCAAGAAGGAGGAC-3', Rev 5'-ACTGTGTGCGACAAGACCTG-3'),

RPS6KL1 (For 5’-AAGGGGTCACTGTGAGGATG-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+).

CHAPTER 3. NEK8 MODULATES FORMATION OF RAD51 FOCI FOLLOWING DNA DAMAGE AND REPLICATION STRESS

ABSTRACT

In a high-throughput siRNA library screen targeting the human kinome, we identified the kinase, NEK8, as a novel candidate for the regulator of DNA damage-induced RAD51 focus formation. To begin to elucidate a possible mechanism by which NEK8 regulates RAD51 focus formation, we set out to determine if the effect of NEK8 depletion on RAD51 focus formation is a universal response to DNA damage. We demonstrate that NEK8 depletion decreases RAD51 focus formation following treatment with multiple types of DNA damaging agents including MMC, IR, CDDP and the replication inhibitor HU. We also characterized the role NEK8 plays in expression of DNA repair proteins and in cell-cycle distribution, where we describe a mild decrease in RAD51and CHK1 proteins following NEK8 depletion. Furthermore, we validate these results in murine embryonic fibroblast in which Nek8 has been genetically knocked-out (*Nek8*$^{-/-}$ MEFs). We attempted to exogenously express NEK8 in both U-2 OS and *Nek8*$^{-/-}$ MEFs to rescue the RAD51 phenotype, but we were unsuccessful. We also demonstrate that NEK8 depletion leads to a mild increase in γH2AX as detected by foci and Western blot. Lastly, exogenously expressed NEK8 does not form nuclear foci following DNA damage. Taken together, our findings show that NEK8 regulates RAD51 focus formation in response to DNA damage and replication stress.

INTRODUCTION

The human kinase, NEK8, is a member of the NIMA-related kinases (NEKs) family, which contains 11 serine/threonine protein kinases. NEKs have mainly been studied in relation to their

role in cell cycle progression (centrosome regulation) and ciliogenesis [84, 85]. Missense mutations in the highly conserved RCC1 domain of Nek8 are reported in a mouse model of autosomal recessive polycystic kidney disease [88], a rat model Lewis polycystic kidney disease [89], three human nephronophthisis families [86], three fetuses from a single family with a form of Ivemark syndrome [91] and most recently in two newborns exhibiting Alagille syndrome like phenotypes [92]. Furthermore, a missense mutation of NEK8 is reported as a potential driver mutation in pancreatic cancer [139] and NEK8 is overexpressed in human breast cancer [140]. These findings suggest a role of NEK8 in cancer development.

NEK8 localizes not only to the centrosomes [86] and primary cilium [87], but also to the nucleus [85]. Recently, NEK8 was linked to the ATR mediated replication stress response via regulation of the protein kinase CDK2 [93]. Interestingly, the functional interaction between DNA repair proteins and centrosome proteins is an emerging concept [141]. Several DNA repair proteins (BRCA1, BRCA2 and RAD51) localize to the centrosomes in addition to the nucleus and are involved in the regulation of functional centrosomes [141, 142]. Similarly, NEK1 and NEK11, other members of the NIMA-related kinase family, have been implicated in the DNA damage response [117-119, 143]

We identified NEK8 as a candidate kinase required for RAD51 focus formation following treatment with the inter-strand crosslinking agent MMC (Chapter 2). Three independent siRNAs targeting human NEK8 reproducibly decreased the efficiency of RAD51 focus formation following treatment with MMC in multiple cell lines while efficiently depleting NEK8 mRNA from cells. Since proportion of cells in S phase (cyclin A positive cells) was not reduced in NEK8-depleted cells, this phenomenon is unlikely to be due to cell cycle alteration. In this study we describe the effect of NEK8 depletion on RAD51 foci and foci of other

important DNA repair factors. We also describe the universality of these phenotypes in a murine Nek8 knockout model system.

RESULTS

NEK8 modulates RAD51 focus formation following multiple types of DNA damage and replication stress

Our initial screening results were obtained using MMC as the choice of a DNA damaging agent to induce RAD51 focus formation. As RAD51 foci form following DNA damage leading to DNA double strand breaks [9, 12, 46] and also following long term replications stress [144], we chose to determine if the effect NEK8 depletion had on RAD51 focus formation is specific to interstrand cross-links created by MMC or if it is a universal effect following DNA damage. To test this, we treated NEK8-depleted U-2 OS cells with multiple forms of DNA damage. Consistent with our observations following treatment with MMC (Figure 3.1B), RAD51 foci were also reduced in NEK8-depleted cells after treatment with ionizing radiation (IR) which causes direct DNA double strand breaks (Figure 3.1A and C), another inter-strand crosslinking agent, cisplatin (CDDP) (Figure 3.1D), as well as the replication inhibitor, hydroxyurea (HU) (Figure 3.1E), suggesting that the effect of NEK8 depletion on RAD51 focus formation is not exclusive to interstrand cross-links caused by MMC.

To determine if the effect of NEK8 depletion on RAD51 focus formation was not an off-target effect of the siRNAs, we tested *Nek8*$^{+/+}$ and *Nek8*$^{-/-}$ mouse embryonic fibroblasts (MEFs) [93, 134] for RAD51 focus formation. The *Nek8*$^{-/-}$ MEFs provide for a cleaner system than depletion of NEK8 via siRNA as they are genetic knockouts and do not express NEK8 [134]. Similar to cells depleted of NEK8 by siRNA, *Nek8*$^{-/-}$ MEFs also had decreased RAD51 focus formation following DNA damage with MMC, IR, and HU when compared with *Nek8*$^{+/+}$ MEFs

(Figure 3.2A and B). Taken together this data suggests that NEK8 has a role in positively regulating RAD51 focus formation following DNA damage. Also the effect NEK8 has on RAD51 focus formation appears to be common to inter-strand crosslinking agents, IR, and replication stress. It is also important to indicate that the effect of NEK8 on RAD51 focus formation is not cell type or species specific as we detect a defect in RAD51 focus formation in NEK8-depleted human cells as well as *Nek8*$^{-/-}$ MEFs.

As formation of RAD51 foci is contingent on many key upstream factors in the homologous recombination pathway [9, 12, 46], we analyzed the effect of NEK8 depletion on the expression of these factors. Depletion of NEK8 with three individual siRNAs mildly decreases the total protein expression of RAD51. Of the three individual siRNAs, #1 has the strongest effect on RAD51 protein expression (Figure 3.3A, row 3). Of the other proteins known to be required for RAD51 focus formation that we investigated, all three siRNAs targeting NEK8 mildly decrease the expression of CHK1 (Figure 3.3A, row 3-5). We also observed that following depletion of NEK8 with all three siRNAs, there is a mild decrease in BRCA2 protein expression (Figure 3.3A, rows 3-5), however, this effect appears to be due to siRNA depletion in general in U-2 OS cells as our siRNA control sample also showed a similar mild decrease in BRCA2 protein expression (Figure 3.3A, row 2). Other proteins we tested were ATR, FANCD2, KU70, RPA70 and H2AX, whose protein levels were all unaffected by siRNA depletion of NEK8 (Figure 3.3A, rows 3-5).

Similar to depletion of NEK8 in human cell lines, when compared to *Nek8*$^{+/+}$ MEFs, *Nek8*$^{-/-}$ MEFs have very mildly decreased levels of RAD51 protein expression (Figure 3.3B). Interestingly, we observed a mild decrease of total ATR protein in the *Nek8*$^{-/-}$ MEFs (Figure 3.3B). *Nek8*$^{-/-}$ MEFs have normal expression of BRCA2 when compared to *Nek8*$^{+/+}$ MEFs

(Figure 3.3B While we observed mild decreases in total RAD51 protein levels in NEK8 depleted U-2 OS cells and *Nek8*$^{-/-}$ MEFs, we do not believe that this level of decrease is sufficient to account for the reduction in RAD51 focus formation.

We next tested the cell cycle distribution of NEK8 depleted U-2 OS cells and *Nek8*$^{-/-}$ MEFs. As mentioned previously, RAD51 foci form mainly in S- and G2-phase of the cell cycle [105]. NEK8 depletion did not decrease cyclin A positive cells in our initial screen (Table 2.1). Consistent with this, depletion of NEK8 with three independent siRNAs did not significantly alter cell cycle profiles in undamaged U-2 OS cells (Figure 3.3C). Similarly, we did not detect a decrease in percentage of cells in S phase in the *Nek8*$^{-/-}$ MEFs compared to *Nek8*$^{+/+}$ MEFs in undamaged conditions (Figure 3.3D). The percentage of cells in S phase in the *Nek8*$^{-/-}$ MEFs was even slightly elevated compared to *Nek8*$^{+/+}$ MEFs.

We next investigated the affect of DNA damage on cell cycle distribution of both NEK8-depleted U-2 OS cells and *Nek8*$^{-/-}$ MEFs. Following treatment with MMC, NEK8-depleted U-2 OS cells primarily accumulate in S- and G2-phase (Figure 3.4A). Similarly, in *Nek8*$^{-/-}$ MEFs, we observe a large accumulation of cells in S- and G2-phases of the cell cycle following treatment with both IR and MMC (Figure 3.4B) which is comparable to the cell cycle profiles of DNA damaged *Nek8*$^{+/+}$MEFs. These data indicate that reduced RAD51 focus formation in NEK8 deficient cells is not due to reduced percentage of cells in S-phase.

Taken together these data show that NEK8 modulates RAD51 focus formation following DNA damage induced with inter-strand crosslinking agents (MMC, CDDP), IR, and replication stress (HU). This modulation of RAD51 focus formation is common in the human cell line U-2 OS using siRNA depletion of NEK8. This effect is also replicated in *Nek8*$^{-/-}$ MEFs, further strengthening the notion that the effect NEK8 has on RAD51 focus formation is not an off-target

effect of siRNA in human cell lines. Importantly, depletion or loss of NEK8 expression does not significantly reduce percentage of cells in S phase. Lastly, we also note that this effect does not appear to be cell type or species specific, but appears to be universal in nature.

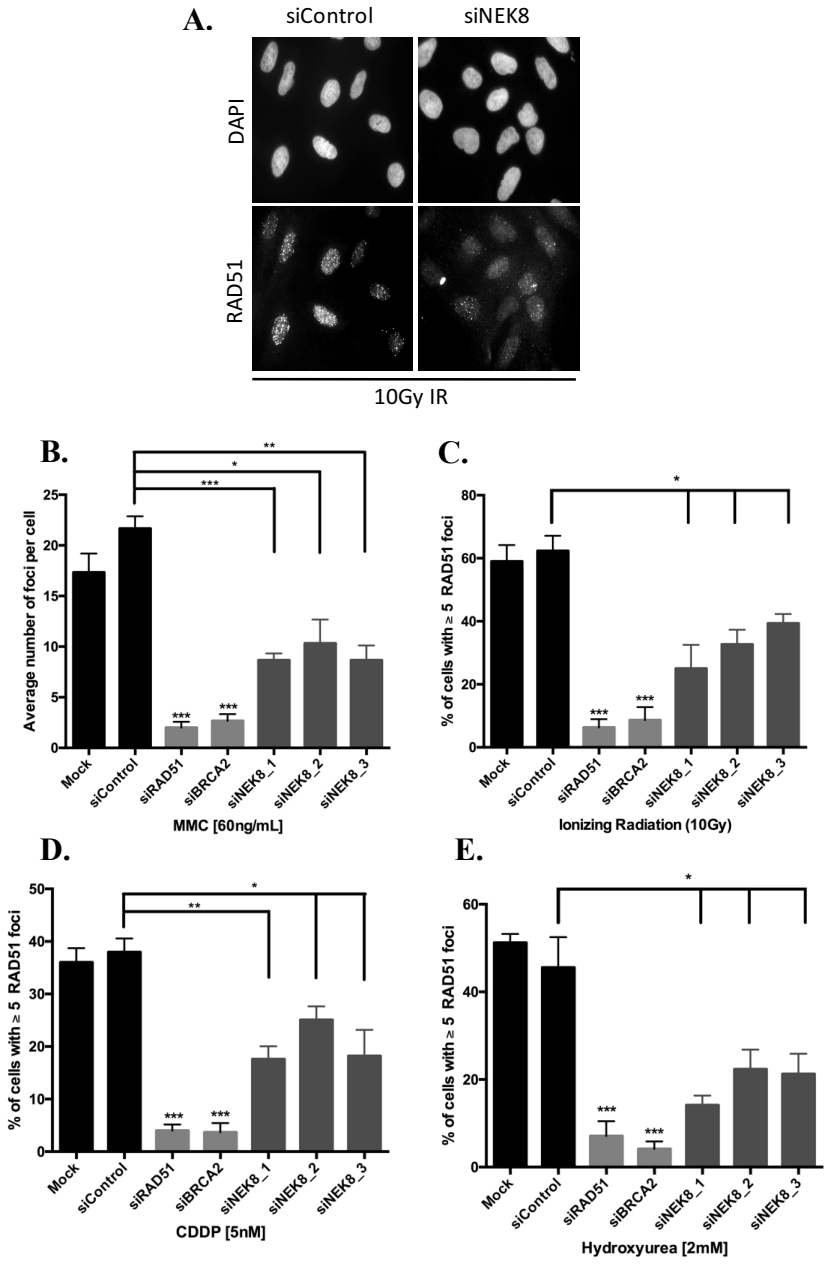


Figure 3.1. **NEK8 depletion leads to decreased RAD51 focus formation in response to multiple types of DNA damage. A.** Representative image of IR (10Gy) induced RAD51 foci. **B-E.** Three independent siRNAs targeting NEK8 were transfected into U-2 OS cells (20nM), 48h later treated with **B**. 60ng/mL MMC, 24h, **C**. 10Gy IR, 6h, **D**. 5nM CDDP, 24h, **E**. 2nM HU, 24h and then fixed and immunostained for RAD51. Quantification is **B**. avg # of foci/cell and **C-E**. % of cells with ≥ 5 foci per cell (n=3, +/- SEM). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

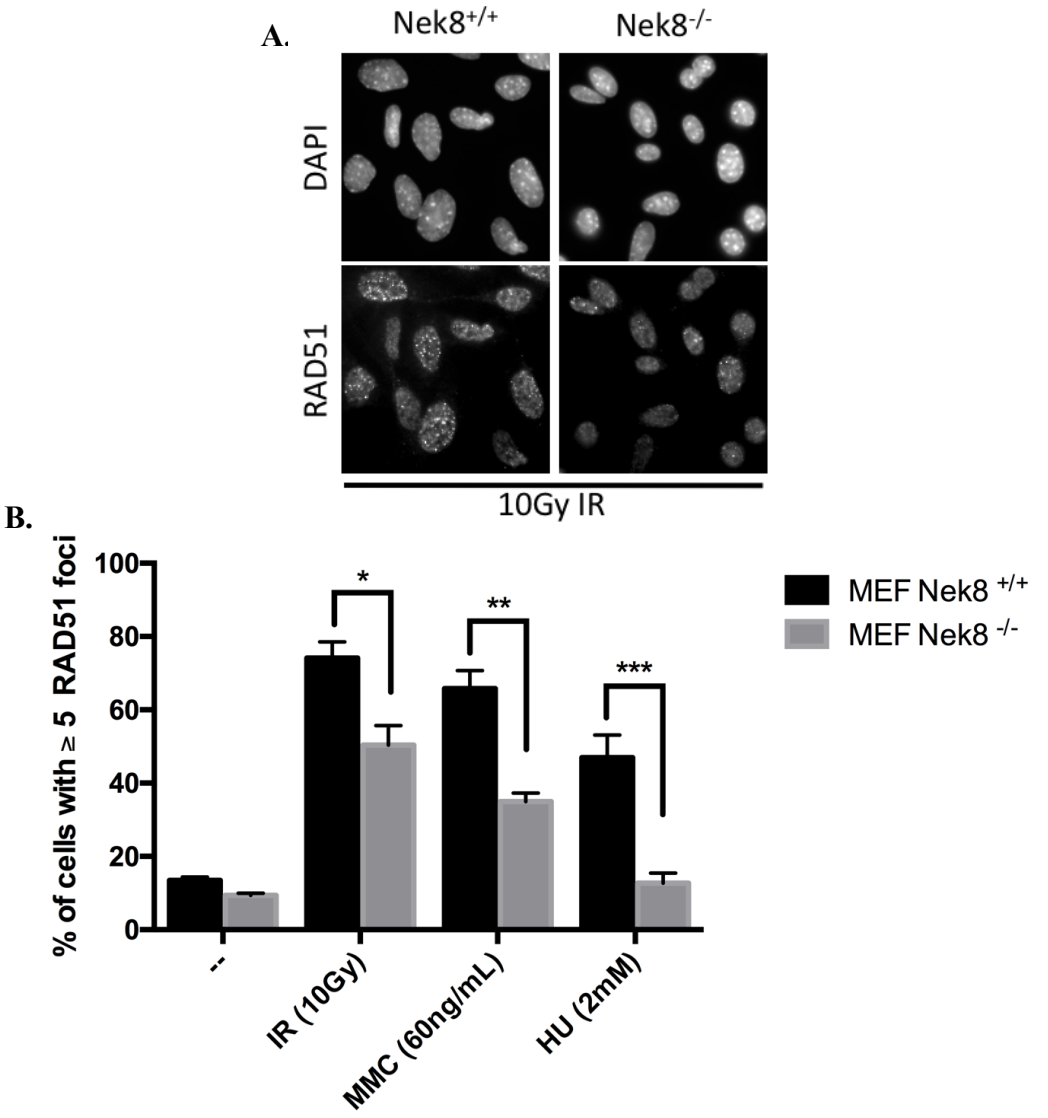


Figure 3.2. **Nek8$^{-/-}$ MEFs have decreased RAD51 focus formation following multiple types of DNA damage. A.** Representative image of IR (10Gy) induced RAD51 foci. **B.** Nek8 MEFs were treated with 10Gy IR, 6h, 60ng/mL MMC, 24h and 2nM HU, 24h, and then fixed and immunostained for RAD51. Quantification is % of cells with ≥ 5 foci per cell (n=3, +/- SEM). * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$

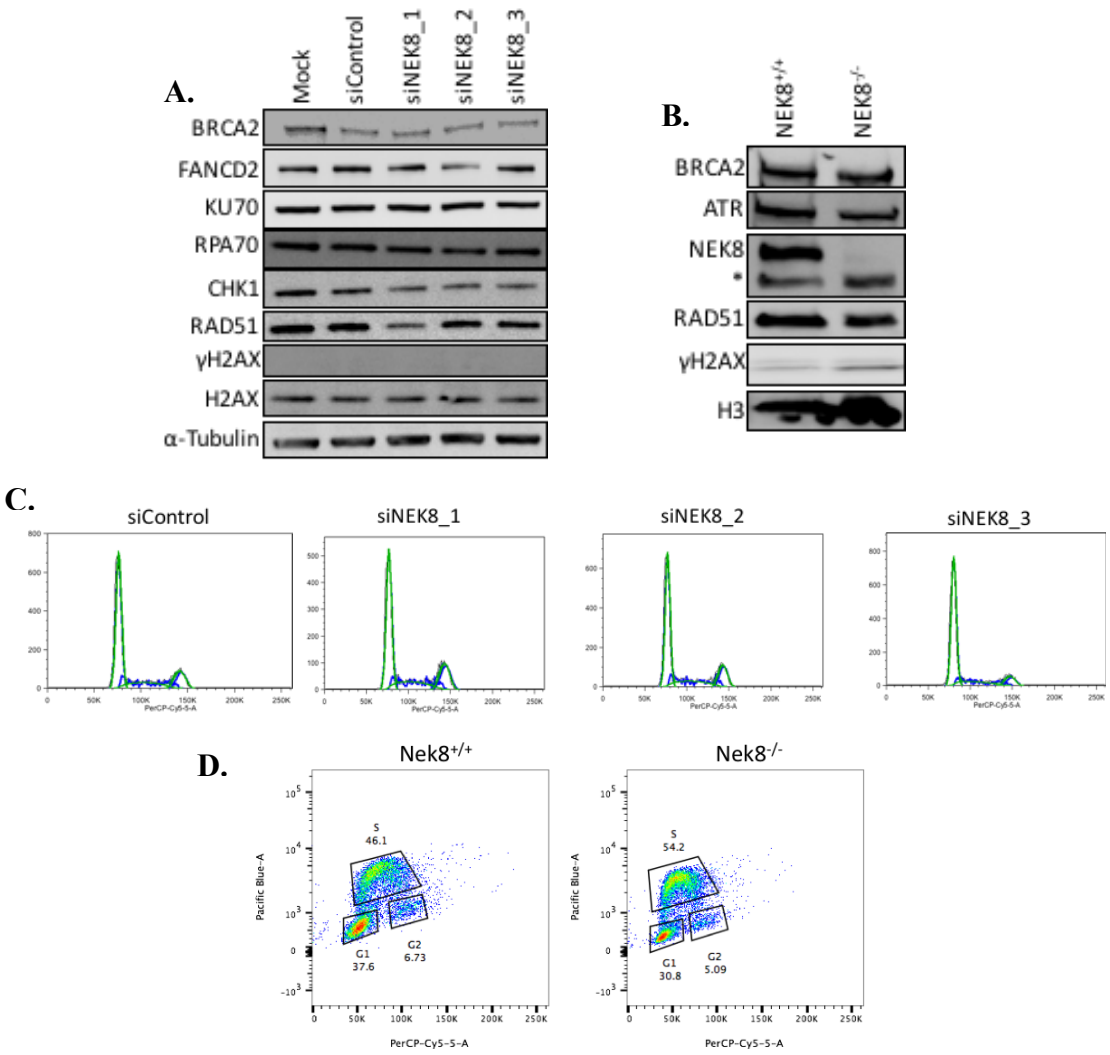


Figure 3.3.**Effect of NEK8 on protein expression of key DNA repair proteins and cell cycle distribution A.** Western blot of key DNA repair protein expression in U-2 OS cells depleted of NEK8. **B.** Western blot of key DNA repair protein expression in Nek8 MEFS. **C.** Three independent siRNAs targeting NEK8 were transfected into U-2 OS cells (20nM), 48h later, cells were collected, fixed and stained with P.I. prior to FACS. **D**. *Nek8*$^{+/+}$ or *Nek8*$^{-/-}$ MEFs were collected, fixed and subjected to Click-IT chemistry and stained with P.I. prior to FACS. All experiments, n=3.

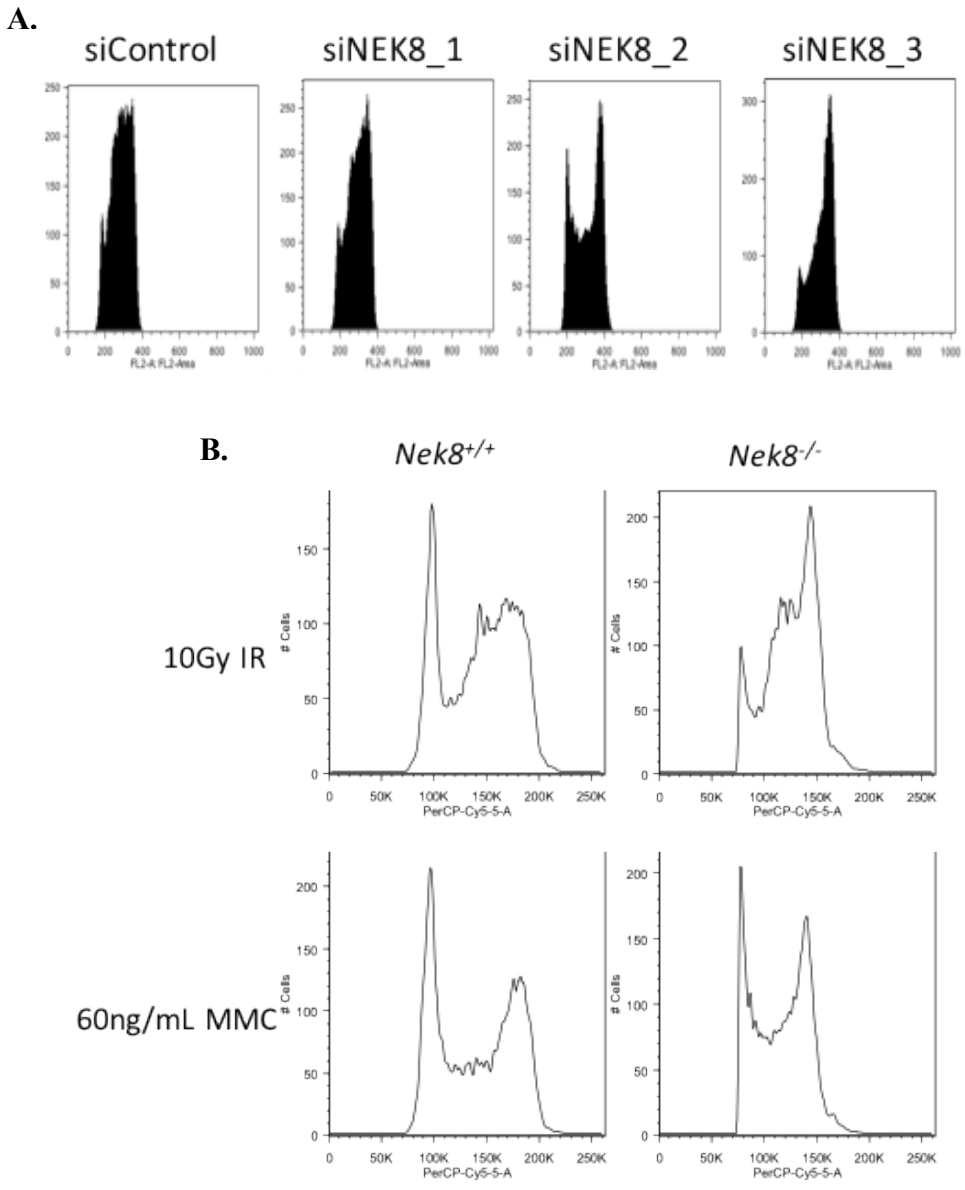


Figure 3.4. **NEK8 is not required for a functional S-phase checkpoint in U-2 OS cells or MEFs. A.** Three independent siRNAs targeting NEK8 were transfected into U-2 OS cells (20nM), 48h later, cells were treated with MMC (60ng/mL, 24h). Cells were then collected, fixed and stained with P.I. prior to FACS. **B.** MEFs ($Nek8^{+/+}$ or $Nek8^{-/-}$) were treated with IR (10Gy, 6h) or MMC (60ng/mL, 24h), then collected, fixed and stained with P.I. prior to FACS.

NEK8 re-expression does not effectively rescue RAD51 focus formation

A long standing gold standard in RNAi studies is the phenotypic rescue via re-expression of an RNAi-resistant target protein in RNAi-depleted cell lines [110, 111, 113, 114, 116]. As such, we proceeded to generate constructs resistant to siRNA #2 to re-express NEK8 in U-2 OS cells (Figure 3.5D and E). However, NEK8 constructs containing either C- or N-terminal tags did not reproducibly restore RAD51 focus formation in NEK8-depleted U-2 OS cells (Figure 3.5A, B, C).

Next, we attempted to complement Nek8 deficiency in the *Nek8*$^{-/-}$ MEFs using a transiently expressed murine Nek8 FLAG-tagged construct (mNek8-FLAG). Similar to results observed in U-2 OS cells and in murine cells expressing hNEK8, mNek8-FLAG failed to complement RAD51 focus formation following DNA damage with MMC (Figure 3.6).

While complementation is considered an important and strong supporting element in validating RNAi screening targets, it is not, in our opinion, an absolute necessity. In the case of NEK8, the effect observed on the efficiency of RAD51 focus formation following depletion of NEK8 by siRNA in the human cell line U-2 OS is strongly supported by the fact that a genetic knockout of Nek8 in murine cells strongly replicates this phenotype. As such, we remain confident that NEK8 is modulating RAD51 focus formation in response to DNA damage and replication stress.

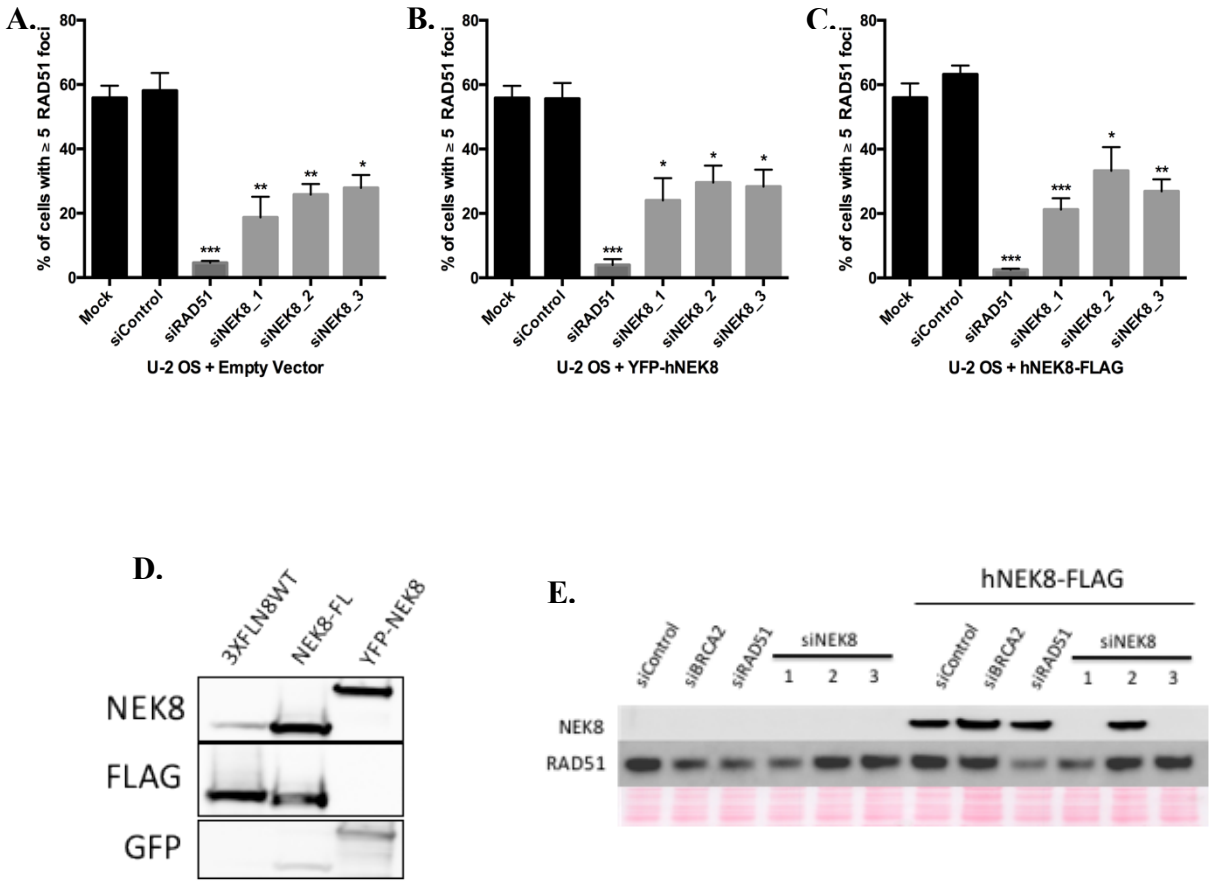
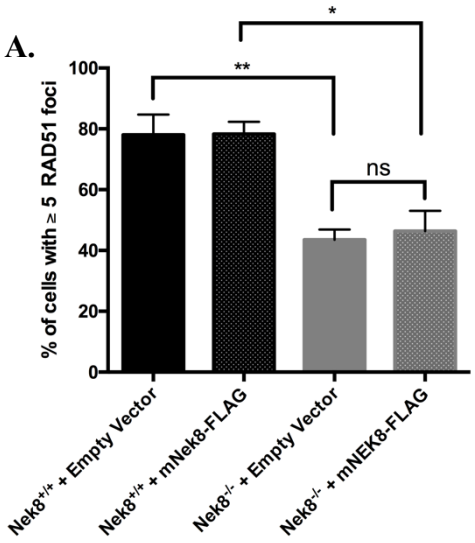


Figure 3.5. **Exogenous NEK8 does not rescue RAD51 focus formation in NEK8 depleted U-2 OS cells. A-C.** U-2 OS cells were transfected with **A.** empty vector, **B.** YFP-hNEK8 or **C.** hNEK8-FLAG plasmids constructs. 48h post transfections cells were treated with MMC (60ng/mL, 24h), fixed and immunostained for RAD51 and construct tag (YFP or FLAG). Quantitation is % of cells ≥5 foci per cell (n=3, +/- SEM). **D-E.** Western blots of transfected U-2 OS cells showing **D.** expression of NEK8 constructs and **E.** expression of NEK8 siRNA resistant construct. * = $p<0.05$, ** = $p<0.05$, *** = $p<0.001$



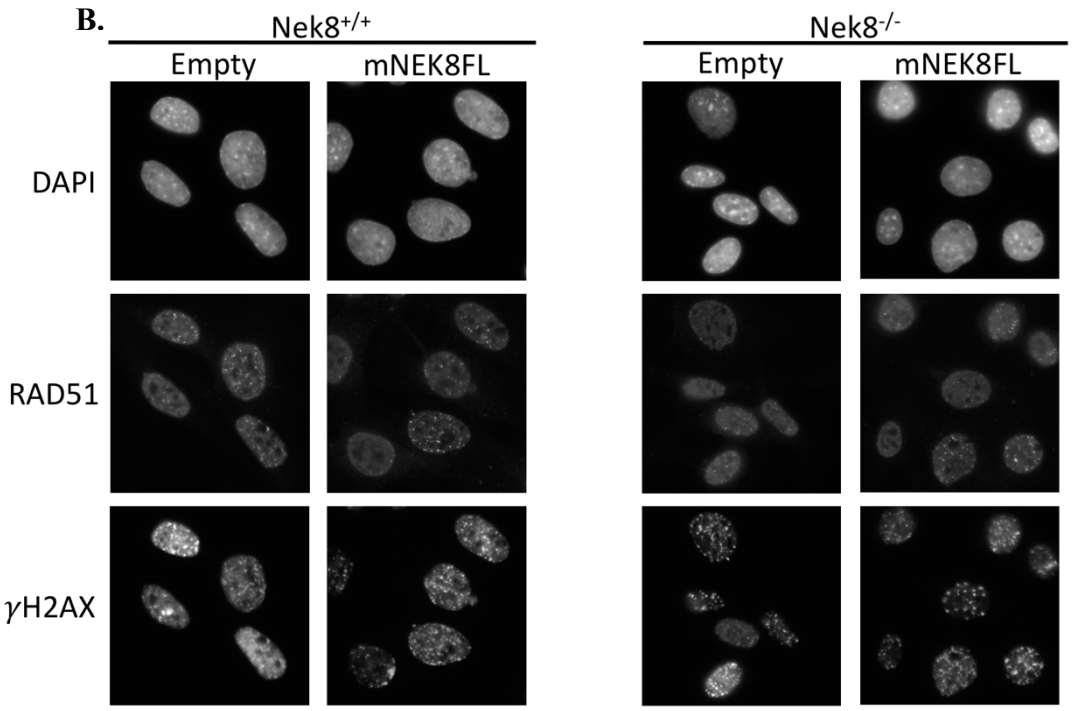


Figure 3.6. **Exogenous Nek8 does not rescue RAD51 focus formation in Nek8$^{-/-}$ MEFs.**

A. Nek8 MEFs were transfected with empty vector or mNek8-FLAG plasmid constructs. 48h post transfections cells were treated with MMC (60ng/mL, 24h), fixed and immunostained for RAD51. Quantitation is % of cells ≥5 foci per cell (n=3, +/- SEM). **B.** Representative image of MMC (60ng/mL,24h) induced RAD51 foci. * = p<.05, ** = p<.01

The effect of NEK8 depletion on focus formation of various DNA repair factors

RAD51 focus formation is contingent on many key factors in the HR pathway. Many of these factors also form immunofluorescence detectable nuclear foci [9, 12, 46]. We began by testing formation of phosphorylated-H2AX (γH2AX) foci, which is believed to be the key upstream signaling and DNA repair protein recruitment marker for DSBs [145, 146]. Interestingly, NEK8 was recently implicated in the ATR-mediated replication response [93] via a genome wide screen where an increase in γH2AX foci in undamaged cells was observed in NEK8-depleted cells, suggesting that NEK8 may play a role in general genome stability [99]. Using our three independent siRNAs targeting NEK8 in U-2 OS cells, we observed an only slight and insignificant increase in γH2AX focus formation (Figure 3.7A). As measured by Western blot, we also did not detect a significant increase in H2AX phosphorylation in undamaged U-2 OS Cells (Figure 3.7B). Following damage with MMC, γH2AX foci were all at similar levels in control and NEK8 siRNA depleted U-2 OS cells (Figure 3.7A). Next, we tested focus formation of the breast and ovarian cancer susceptibility protein, BRCA1 in NEK8 depleted cells. Interestingly, siRNA #2 and #3 targeting NEK8 decrease the levels of BRCA1 focus formation in response to IR (Figure 3.7D). BRCA1 foci still appear to form in the NEK8 depleted cells, but in a much more dispersed pattern as compared to control cells (Figure 3.7C).

To validate these findings we also tested γH2AX foci in Nek8 MEFs. Unfortunately, there is no antibody available for the reliable detection of BRCA1 foci in murine cells. Interestingly, in the *Nek8*$^{-/-}$ MEFs, γH2AX focus formation is slightly, yet not significantly increased in undamaged cells as compared to *Nek8*$^{+/+}$ MEFs (Figure 3.8A). However, by Western blot we are able to detect an increased level of phosphorylated H2AX protein in *Nek8*$^{-/-}$

MEFs as compared to *Nek8*$^{+/+}$ MEFs (Figure 3.8B). This difference in phosphorylation, however, became undiscernible following treatment with HU (Figure 3.8B).

Lastly, we tested if NEK8 itself is able to form distinct nuclear foci following DNA damage. As endogenous NEK8 protein is undetectable by the NEK8 antibody in human cell lines, we employed an experimental strategy of overexpression using our tagged NEK8 constructs. In the absence of DNA damage, NEK8 is primarily localized to the cytoplasm (Figure 3.9A). In response to DNA damage induced by MMC, NEK8 remains primarily cytoplasmic (Figure 3.9B). Of note, overexpression of a construct containing an NLS, where NEK8 is primarily localized to the nucleus, also does not form NEK8 foci in response to DNA damage (Figure 3.9C).

Taken together, these data show that NEK8 modulates RAD51 focus formation following multiple types of damage including IR, MMC, CDDP and replication stress induced by HU, in multiple human cell lines as well as in MEFs. While we observed mild decreases in total RAD51 protein levels in NEK8 depleted U-2 OS cells and *Nek8*$^{-/-}$ MEFs, we do not believe that this level of decrease is sufficient to account for the reduction in RAD51 focus formation.

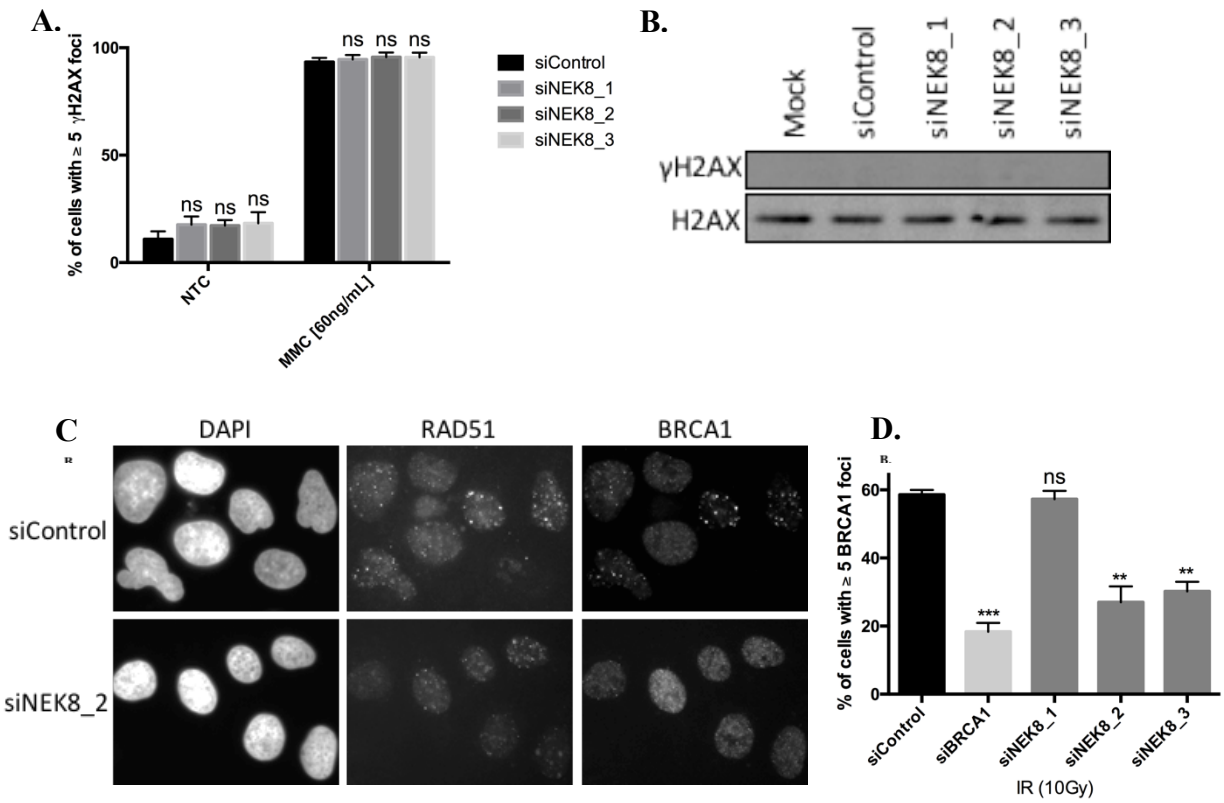


Figure 3.7. **Effect of NEK8 depletion on DNA repair protein foci important for RAD51 focus formation in U-2 OS cells. A-D.** Three independent siRNAs targeting NEK8 were transfected into U-2 OS cells (20nM), 48h later treated with **A**. MMC (60ng/mL, 24h) or **C-D.** IR (10Gy, 6h) and then fixed and immunostained for **A.**γH2AX and **C-D.** BRCA1 or **B.** NTC cells collected for Western blot. Quantification is % of cells with ≥ 5 foci per cell (n=3, +/- SEM). **C.** Representative image of IR induced RAD51 and BRCA1 focus formation in U-2 OS cells. ** = $p<0.01$, *** = $p<0.001$

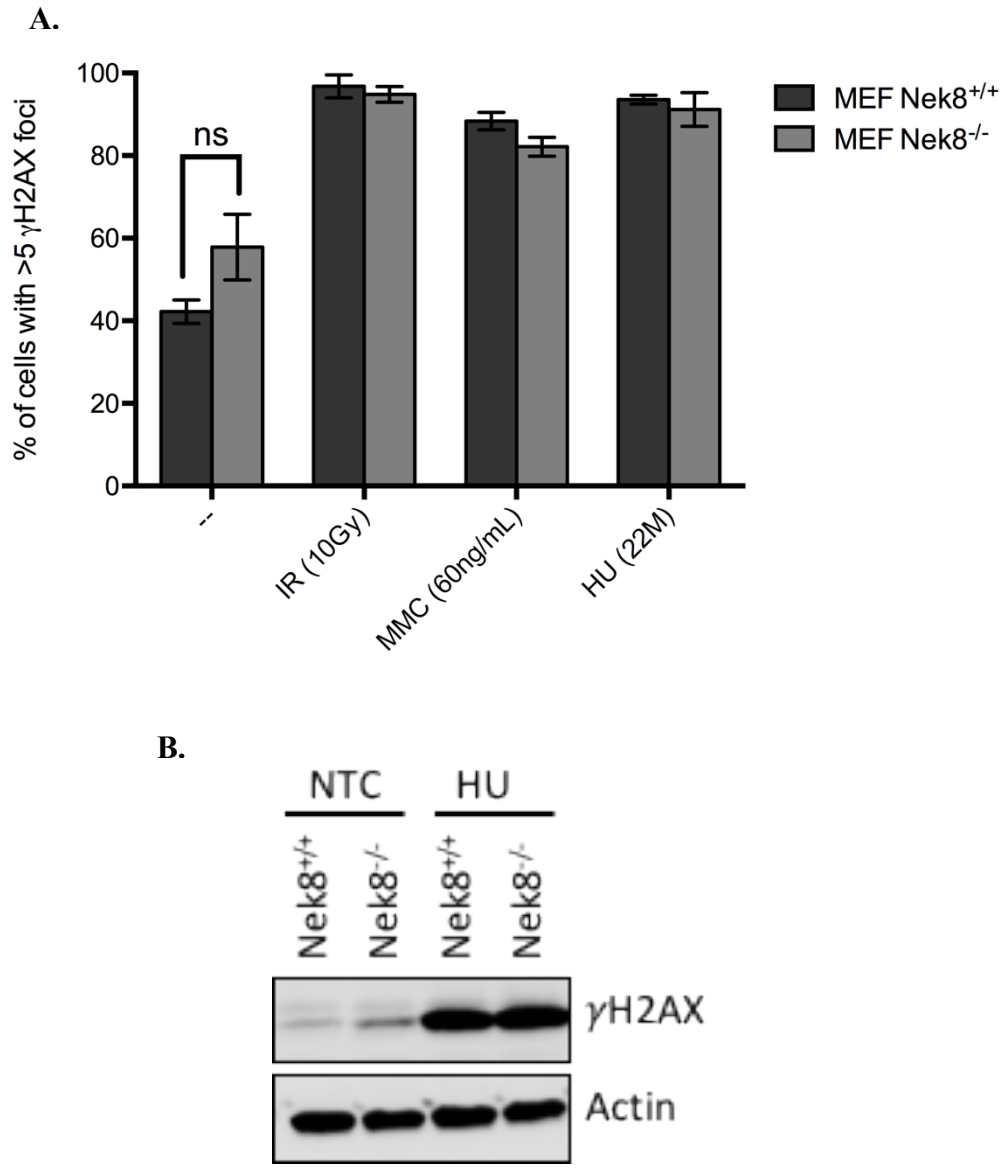


Figure 3.8. **Loss of Nek8 mildly increases H2AX phosphorylation in MEFs. A.** Nek8 MEFs were treated with IR (10Gy, 6h), MMC (60ng/mL, 24h) or HU (2mM, 6h) and then fixed and immunostained for γH2AX. Quantification is % of cells with ≥ 5 foci per cell (n=3, +/- SEM). **B.** Western blot of HU treated (2mM, 6h) Nek8 MEFs.

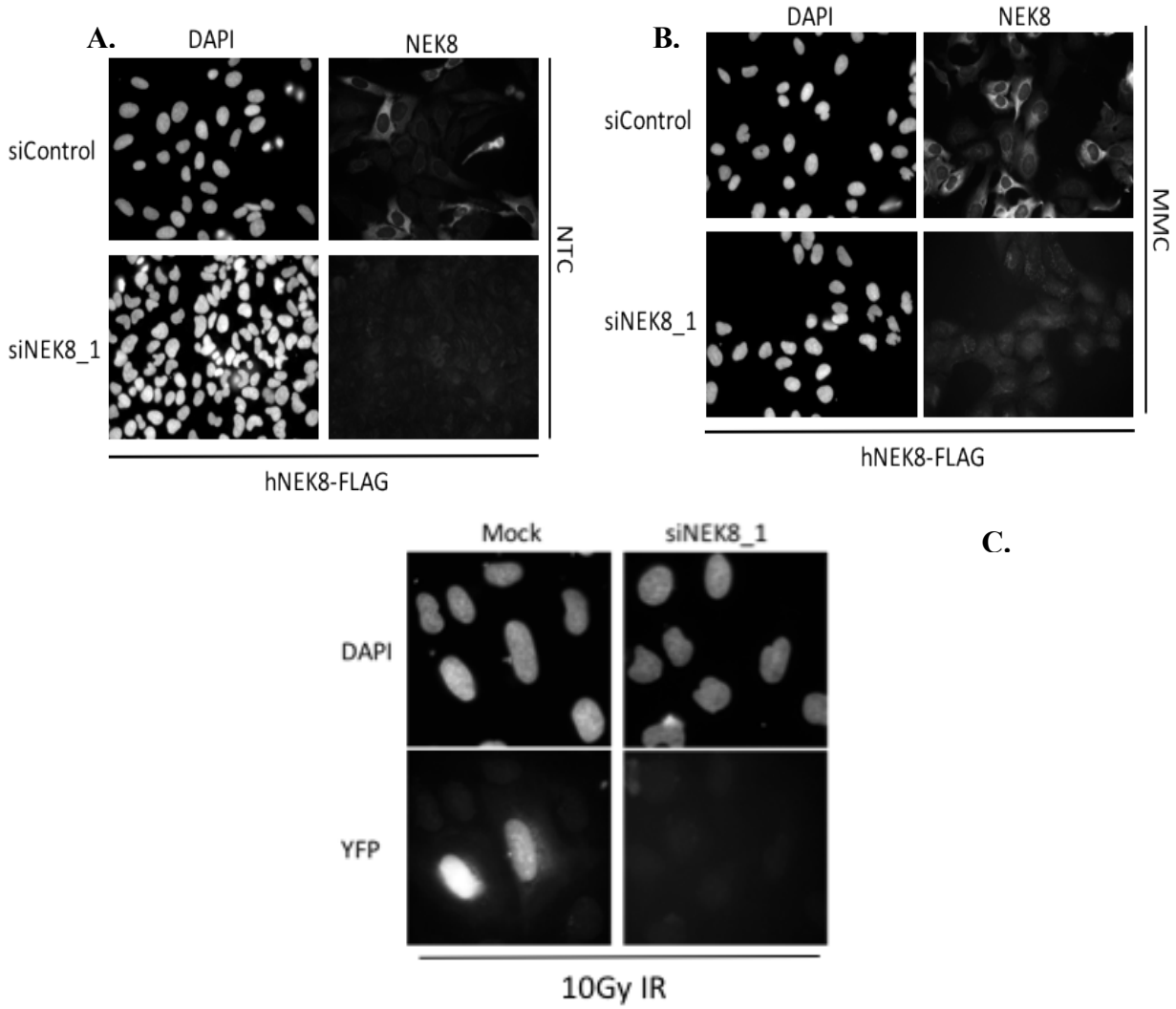


Figure 3.9. **Exogenous NEK8 does not form foci in U-2 OS cells. A-C.** U-2 OS cells were transfected with **A-B.** hNEK8-FLAG or **C.** YFP-hNEK8. 24h post transfection cells were transfected with siRNA (20nM). 24h post siRNA transfection, cells were treated with **A.** non-treated control or **B.** MMC (60ng/mL, 24h) or **C.** IR (10Gy, 6h) and then fixed and immunostained for **A-B**. FLAG or **C.** YFP.

DISCUSSION

In this study we sought to examine if the effect on RAD51 foci following the loss of NEK8 was a phenotype specific to the inter-strand crosslinking agent MMC, or if it was a universal response to many types of DNA damage as well as replication stress. We found that following depletion of NEK8 via siRNA in U-2 OS cells, RAD51 foci fail to form efficiently after treatment with MMC, IR, CDDP or HU (Figure 3.1). Interestingly, we observed that this phenotype is not specific to human cells, but is also reproducible in murine embryonic fibroblasts which have had Nek8 genetically knocked-out (*Nek8*$^{-/-}$MEFs) (Figure 3.2). This is the strongest line of evidence we currently have to validate that the effect of NEK8 depletion on RAD51 focus formation in human cells is not an off-target effect of siRNA depletion, as a genetic knock-out of Nek8 is a much cleaner system than siRNA. These data strongly suggest that NEK8 is modulating RAD51 focus formation following multiple types of DNA damage as well as following replication stress, although, unsurprising, as to-date it appears that RAD51 focus formation under these conditions is regulated via a similar pathway [9, 12, 46, 144].

We also confirmed that depletion of NEK8 did not lead to an altered cell cycle profile in the absence of DNA damage (Figure 3.3C, D). As RAD51 foci primarily form in S- and G2-phase of the cell cycle [105], it is important that depletion of NEK8 did not lead to an accumulation of U-2 OS cells in G1-phase of the cell cycle. Interestingly, *Nek8*$^{-/-}$MEFs exhibit a higher number of cells in S- and G2-phase of the cell cycle as compared to *Nek8*$^{+/+}$MEFs (Figure3.3D). This likely does not account for the decrease of RAD51 foci detectable in these cells as it would be expected for these cells to have increased levels of RAD51 foci with this cell cycle profile. Also, *Nek8*$^{-/-}$MEFs have been described as progressing through S-phase at a slower rate than *Nek8*$^{+/+}$MEFs [93], which likely explains this observed phenotype.

As complementation studies play a key role in validating siRNA targets [111, 113-116], we set out to rescue the RAD51 phenotype observed following NEK8 depletion in U-2 OS cells or loss of Nek8 in *Nek8*$^{-/-}$MEFs. Unfortunately, we were not successful in complementing this phenotype in either cell line. There are many possible reasons why this may occur, including but not limited to: we postulated that NEK8 expression is tightly regulated and the extent of overexpression in our experimental system was detrimental to normal NEK8 function. Secondly, it is possible that our NEK8 plasmid constructs may not encode fully functional kinase enzymes. This appears to be an issue that has been experienced previously with NEK8 [93]. As NEK8 kinase activity is reported to be required for its ATR-mediated replication stress response [93], this may explain our inability to complement the phenotype. Attempts to determine the functionality of our kinase constructs via *in-vitro* kinase assays were not successful (data not shown). However, as we have an isogenic pair of Nek8 MEFs, we remain confident that the RAD51 phenotype we observe following DNA damage and replication stress is not an off-target effect of siRNA.

Surprisingly, we did not detect a significant increase in the phosphorylation of H2AX protein in cells not expressing NEK8. As reported by Choi et. al., loss of NEK8 increases endogenous DNA damage as measured by γH2AX foci and γH2AX Western blot [93]. While we see a slight increase in γH2AX foci in both U-2 OS cells depleted of NEK8 and *Nek8*$^{-/-}$MEFs (Figures 3.7A and 3.8A), it is not statistically significant. We do, however, detect an increase in endogenous γH2AX by Western blot in *Nek8*$^{-/-}$MEFs (Figure 3.8B). This finding can possibly be attributed to the manner in which foci are scored as positive in our experimental system. While we did not quantify foci intensity in these experiments, quantitation of foci intensity may reveal a statistically significant difference between *Nek8*$^{-/-}$ and *Nek8*$^{+/+}$ MEFs.

Interestingly, of the other DNA repair foci we tested, we detected a decrease in BRCA1 foci after depletion of NEK8 by siRNA in U-2 OS cells. While we only observed this phenotype with two of three siRNAs, further validation is required to verify this phenotype. However, as BRCA1 is required for efficient RAD51 focus formation via complex formation with PALB2-BRCA2 [147], this finding may prove to lend insight into the mechanism by which NEK8 modulates RAD51 focus formation. Unfortunately, there appears to be no antibody available for the detection of murine BRCA1, limiting our study to human cells depleted of NEK8 by siRNA. We therefore cannot definitively conclude that NEK8 depletion also modulates BRCA1 focus formation following DNA damage.

Lastly, we observe that exogenously expressed NEK8 does not form distinct nuclear foci. NEK8 has previously been described to localize to the centrosome, cilia and nucleus [85-87]. We were hopeful that NEK8 itself may form nuclear foci, as another ciliary localized NIMA-related kinase member, NEK1, has been reported to form nuclear foci following multiple types of DNA damage [148]. Interestingly, NEK1 was also a top twenty hit in our initial siRNA kinome screen (Chapter 2) however it did not pass our validation procedures. It is possible that, for the same reasons that we are unable to complement RAD51 foci in NEK8-depleted cells using exogenous NEK8, the same NEK8 constructs may not be able to form nuclear foci. Therefore, while under our experimental conditions NEK8 does not form foci, we cannot definitively conclude that NEK8 is unable to form nuclear foci in response to DNA damage.

In summary, we show that NEK8 modulates RAD51 focus formation following multiple types of DNA damage and replication stress. We observed a decrease in BRCA1 focus formation following DNA damage in NEK8-depleted cells, suggesting a possible mechanism by which NEK8 modulates RAD51 focus formation. Lastly, we were not able to observe NEK8 focus

formation following DNA damage. Thus, NEK8 modulates RAD51 focus formation following DNA damage and replication stress, however a mechanism has yet to be described.

MATERIALS AND METHODS

Cell Lines

U-2 OS and were purchased from the American Type Culture Collections. Nek8 MEFs were a gift of the Cimprich lab (Stanford) with permission from the Beier lab (Seattle Children's). Cell lines were cultured in DMEM containing 10% FBS, 2mM L-glutamine and 1X Pen/Strep in a humidified 5% CO_2 containing atmosphere at 37^{o}C.

siRNAs and Plasmids

siRNAs targeting BRCA2 [136], BRCA1 [149] RAD51 **(**target sequence: 5'-AACTAATCAGGTGGTAGCTCA-3'), and NEK8 (#1 target sequence: 5'-TCACTCTTCTGGTTGTAGG-3', #2 target sequence: 5'- TCAGAGGAGAAGCAATATC-3', #3 target sequence: 5'- AGAGATAGGTGCAAAGGTG-3') were transfected at 20nM using Lipofectamine RNAiMAX (ThermoFisher). AllStars siRNA (Qiagen) was used as a negative control.

GFP-NEK8 plasmid was a gift from the Fry lab (University of Leicester). YFP-NEK8 was a gift from the Cimprich lab (Stanford). hNEK8 coding sequence was PCR amplified from the YFP-NEK8 plasmid to contain a C-terminal FLAG tag and cloned into pLentiX1-pUB-puro [149] using SalI and XbaI sites. mNEK8-FLAG was a gift from the Beier lab (Seattle Children's)

Immunofluorescence microscopy

Immunofluorescence microscopy was conducted as previously described [137]. Briefly, transfected cells were grown on coverslips, treated with MMC (60ng/mL, 24h), IR (10Gy, 6h), CDDP (5nM, 24h) and HU (2mM, 24h U-2 OS, 6h Nek8 MEFs) and then simultaneously fixed

and permeabilized (2% PFA and 0.5% Triton X-100 in PBS for 20 minutes) or for NEK8 staining (ice cold methanol). Cells were immunostained for RAD51, RPA, γH2AX and BRCA1. 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 three times independently

Western Blotting

Whole-cell extracts were prepared and resolved by polyacrylamide gel electrophoresis as described [137]. Proteins were transferred onto nitrocellulose membranes. Antibodies against α-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), γH2AX (JBW301, Millipore), GFP (Life Technologies), H2AX (Millipore), H3 (Abcam), Ku70 (Abcam), mNEK8 (gift from David Beier), hNEK8 (N-17, Santa Cruz), RAD51 (H-92, Santa Cruz) 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 MEFs were treated with MMC (60ng/mL, 24h), IR (10Gy, 6h) or not treated. Cells were then fixed and stained for DNA content. Nek8 MEFs were subjected to Click-IT chemistry for EdU detection (Life Technologies, C10636). Flow cytometry was performed to determine the cell cycle phase distribution (Canto or LSR-2).

CHAPTER 4. NEK8 IS REQUIRED FOR MAINTENANCE OF GENOME STABILITY FOLLOWING REPLICATION STRESS

ABSTRACT

In the previous chapters, we identified the kinase, NEK8, as a novel candidate regulator of DNA damage-induced RAD51 focus formation. In this chapter, we demonstrate that NEK8 is an important factor in the regulation of HR. We also demonstrate that NEK8 is required for the cellular resistance to a replication inhibitor, hydroxyurea (HU). Interestingly, we observed that NEK8 deficient cells are not sensitive to other DNA damaging agents which require HR for repair of their lesions. Furthermore, we observe that Nek8 deficient cells are sensitive to ATR inhibition and further sensitized to replication stress inducing agents in this ATR-deficient background. We also characterized the role of Nek8 in maintaining replication fork and genome stability in response to replication fork stall by HU. Nek8 deficient cells exhibit decreased chromatin loading of factors important for both HR and replication fork protection including RAD51 and BRCA2. This phenotype directly correlated with an increase in replication fork degradation in Nek8 deficient cells. We also observed a decreased ability for Nek8 deficient cells to restart replication following treatment with HU. Lastly, we observed an increase in genome instability as measured by metaphase analysis of chromosomes in Nek8 deficient cells. Our findings provide a more complete picture on the role of NEK8 in modulating replication fork protection and genome stability possibly via regulation of the RAD51 recombinase.

INTRODUCTION

The human kinase, NEK8, is a member of the NIMA-related kinases (NEKs) family, which contains 11 serine/threonine protein kinases. Recently, NEK8 was linked to the ATR mediated

replication stress response via regulation of the protein kinase CDK2 [93]. Cells deficient in NEK8 are characterized by an increase in endogenous 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 [93]. It has also been observed that kidneys of Nek8 mutant mice accumulate DNA damage, and loss of Nek8 or replication stress similarly disrupts renal cell architecture in these mice [93].

Replication fork reversal is one mechanism mediated by the recombinase, RAD51, which is important for the protection of stalled replication forks from collapse, breakage and degradation [72]. Key proteins in the Fanconi anemia and HR pathways, including RAD51 and BRCA2, have been described as being required for replication fork protection in an HR independent manner [76, 77, 144]. Recent evidence supports the notion that proteins required for proper RAD51 function have important roles in replication fork protection and genome stability. One such protein, PTEN, has been implicated in both RPA- [150] and RAD51-mediated replication fork protection [151]. A novel protein, BOD1L, is required to maintain stable RAD51 filaments on replication forks to prevent DNA2-dependent fork degradation [152]. Also, the RAD51 paralogs, which are important for HR-mediated restart of collapsed replication forks, have been observed to play an important physiological role in the protection of stalled replication forks in the absence of DSBs [153]. The central cell cycle kinase, ATR, is a key mediator of many of these processes and is central to RAD51-mediated replication fork protection [59, 60].

Through a high-throughput siRNA screen targeting the human kinome, we identified NEK8 as candidate kinase for the regulation of MMC-induced RAD51 focus formation (Chapter 2). We further described that NEK8 modulates RAD51 focus formation in response to many

types of DNA damage across multiple cell lines and across mammalian species (Chapter 3). In this study, we characterize the role of NEK8 in regulating replication fork protection via its modulation of RAD51.

RESULTS

NEK8 modulates homologous recombination

NEK8 modulates efficient RAD51 focus formation following DNA damage and replication fork stress (Chapters 1 and 2). As RAD51 is an essential protein for HR, we hypothesized that NEK8 also modulates HR. Our data coupled with the observation that spontaneous DNA damage accumulates in NEK8 deficient cells [93] as well as the assumption that HR is required for the repair of DNA replication associated spontaneous DNA damage [154, 155] led us to test the efficiency of HR in the absence of NEK8. Using the U-2 OS DR-GFP reporter assay [156], we found that depletion of NEK8 with three independent siRNAs lead to a roughly two-fold decrease in HR efficiency when compared to siControl transfected U-2 OS cells (Figure 4.1A). This decrease in HR approximately correlates with the decrease in DNA damage-induced RAD51 focus formation observed following depletion of NEK8 via siRNA (Chapter 2).

Using a 3X-FLAG tagged hNEK8 construct resistant to siRNA #2 targeting NEK8 (Figure 4.1C), we overexpressed NEK8 in the U-2 OS DR-GFP cell line. After induction of a double strand break, all siRNAs targeting NEK8 lead to a roughly two-fold decrease in HR efficiency (Figure 4.1B). Similar to our complementation studies in an attempt to rescue RAD51 focus formation in U-2 OS cells, we also unsuccessfully attempted to complement HR in NEK8-depleted U-2 OS DR-GFP cells. This was not completely surprising as using alternate human NEK8 constructs in U-2 OS cells and mouse Nek8 constructs in MEFs failed to rescue RAD51 focus formation (Chapter 2). These results suggest that NEK8 regulates HR in U-2 OS DR-GFP

cells, possibly through a similar mechanism by which it regulates RAD51 focus formation and ectopic overexpression of NEK8 is not sufficient to support this function.

Nek8 is required for cellular resistance to replication stress

As a key component of the HR pathway, tightly controlled regulation of RAD51 function is known to mediate the resistance to various anti-cancer drugs including inter-strand DNA crosslinking agents and PARP inhibitors [157, 158]. As we have previously identified NEK8 as being required for RAD51 focus formation and efficient HR, we next tested cellular sensitivity of *Nek8*$^{-/-}$ MEFs to multiple DNA damaging agents. Surprisingly, *Nek8*$^{-/-}$ MEFs were sensitive to the replication inhibitor hydroxyurea (HU), but not to any other DNA damaging agents which require HR for repair of their lesions (Figure 4.2). This phenotype was expected for replication inhibitors such as HU as it has been previously reported that *Nek8*$^{-/-}$ MEFs are sensitive to the replication inhibitor aphidicolin [93].

We next tested the effect of NEK8 depletion by siRNA in U-2 OS cells on cellular sensitivity to DNA damaging agents. Similar to the *Nek8*$^{-/-}$ MEFs, we observed that depletion of NEK8 only renders U-2 OS cells sensitive to the replication inhibitor, HU (Figure 4.3A and B). These data suggested to us that the role NEK8 has in modulating RAD51 focus formation and HR may be in a non-canonical function of RAD51. It has previously been suggested that RAD51 along with other essential HR proteins have a non-canonical function in the early stages of replication fork protection, where RAD51 is loaded onto a reversed replication fork [144] and other HR proteins, including BRCA2, are required for replication fork protection [76, 77]. To test if NEK8 modulates this function of RAD51 at stalled replication forks, we designed an experiment where *Nek8*$^{-/-}$ MEFs were treated with HU for six hours and then released into fresh media. The cells were allowed to recover from the HU-induced replication stress and then

measured for their relative survival. We chose this time point as it has been demonstrated that a six-hour time pulse of HU is sufficient to induce replication stall, but not sufficient to induce double strand breaks [144]. Interestingly, *Nek8*$^{-/-}$ MEFs were hypersensitive to HU under these conditions as compared to *Nek8*$^{+/+}$ MEFs (Figure 4.3C).

Similar to NEK8 depletion leading to hypersensitivity to HU, recent data shows that acute inhibition of the cell cycle and replication kinase ATR causes rapid cell death in cells experiencing replication stress [159]. As NEK8 has previously been linked to the ATR-regulated replication stress response via its regulation of CDK activity during S-phase of the cell cycle [93], we set out to further explore the role NEK8 has in the ATR-mediated replication stress response. To do this, we tested the effect of inhibition of ATR via the selective kinase inhibitor VE-821 on cellular survival in the *Nek8*$^{-/-}$ MEF background. Interestingly, *Nek8*$^{-/-}$ MEFs are sensitive to ATR inhibition as compared to *Nek8*$^{+/+}$ MEFs (Figure 4.4A). We next tested the effect of ATR inhibition on cellular survival following treatment with HU in *Nek8*$^{-/-}$ MEFs. We first determined the optimal concentration of ATR inhibition by a dose response curve using inhibition of phosphorylation on the ATR target protein CHK1 as a readout. We chose to use a concentration of 1.25μM of the ATR inhibitor, which effectively decreases phosphorylation of CHK1 roughly two-fold following HU treatment (Figure 4.4B). This is also the concentration of ATR inhibitor where we first observed a difference in cellular survival in *Nek8*$^{-/-}$ MEFs (Figure 4.4A). Interestingly, ATR inhibition leads to increased sensitivity of both *Nek8*$^{-/-}$ MEFs and *Nek8*$^{+/+}$ MEFs to continuous treatment of HU (Figure 4.4C) as well as to a short term six-hour pulse of HU (Figure 4.4D), 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 and may act independently of ATR.

This role of NEK8 may be an essential early response to replication fork stall mediated by RAD51.

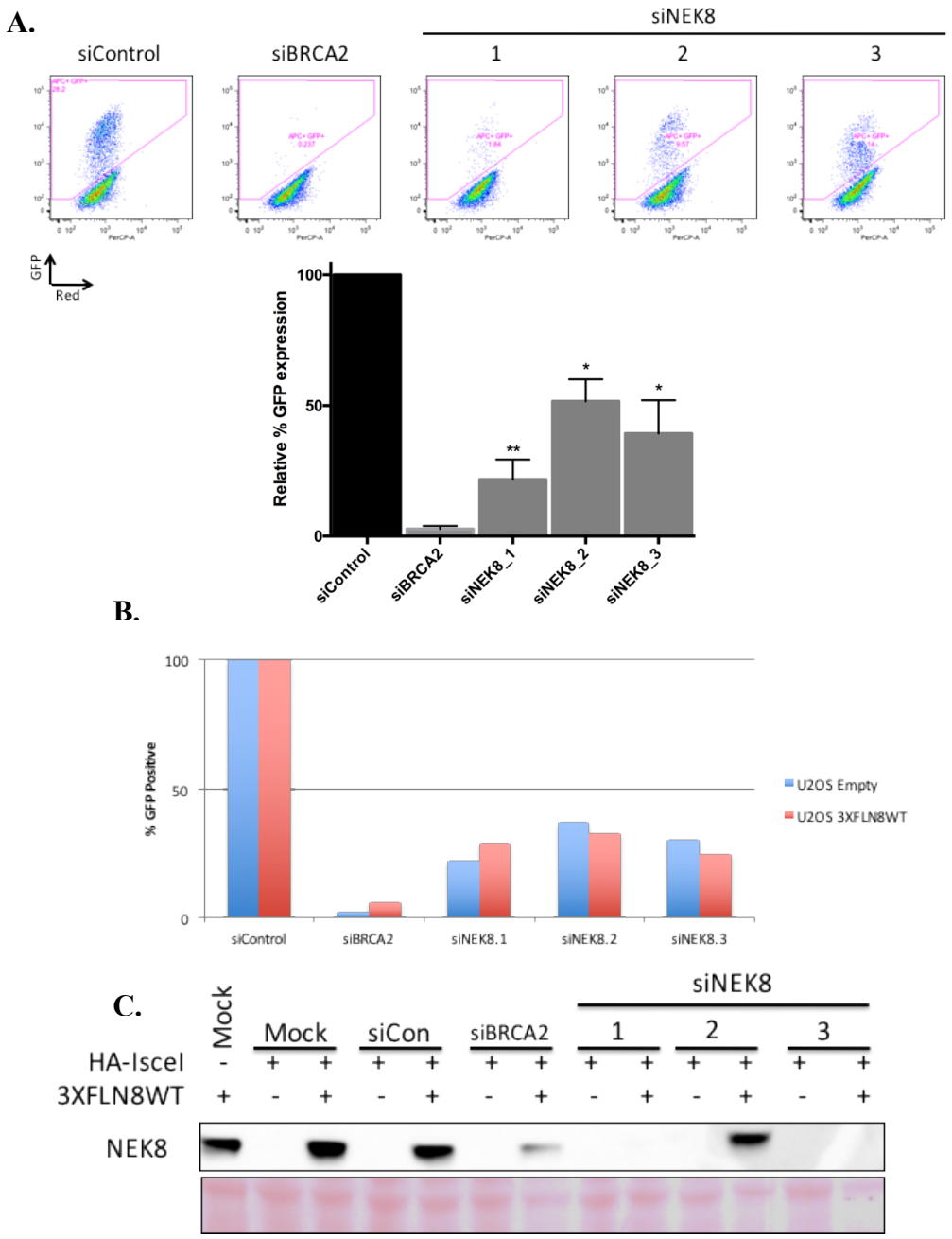


Figure 4.1. **NEK8 depletion decreases HR efficiency. A.** U-2 OS DR-GFP cells were transfected with siRNA (20nM). 24h post transfection cells were transfected with pCBASce or control plasmids. GFP expression was detected and quantified via flow cytometry and FloJo software. (n=3) **B.** U-2 OS cells were transfected with 3XFLAG hNEK8. 24h later cells were transfected with siRNA (20nM). Cells were then treated as in **A.** (n=2). **C.** Representative Western blot of NEK8 overexpression in U-2 OS DR-GFP. *p<.05, **p<.01

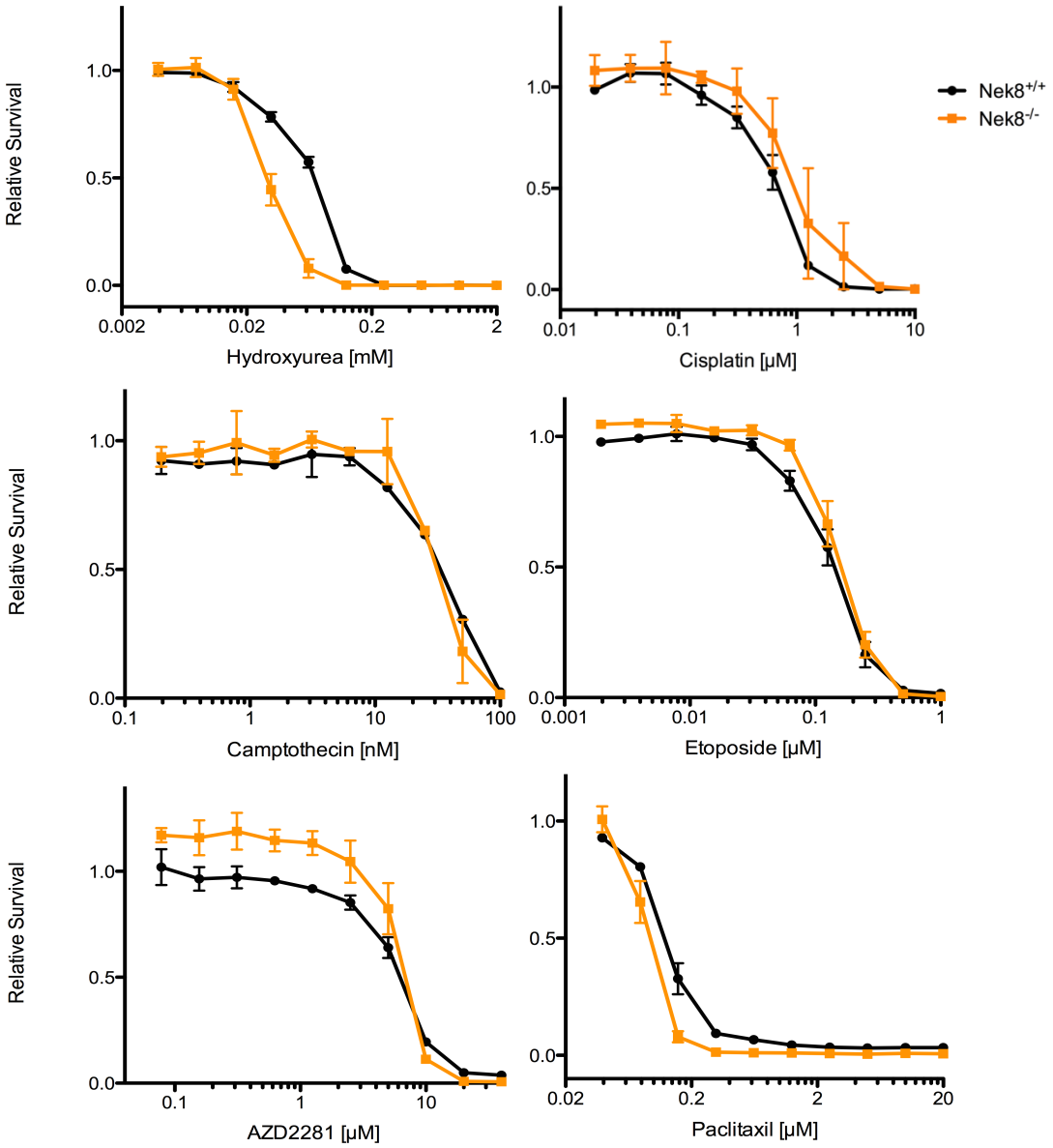


Figure 4.2. **Nek8 is required for resistance to replication stress in MEFs**. Cell survival in Nek8 MEFs in response to increasing doses of hydroxyurea, cisplatin, camptothecin, etoposide, PARP inhibitor (AZD2281) or paclitaxel as indicated. Cell survival was assayed by crystal violet staining and expressed as a fraction of the untreated control (n=4, +/-SEM).

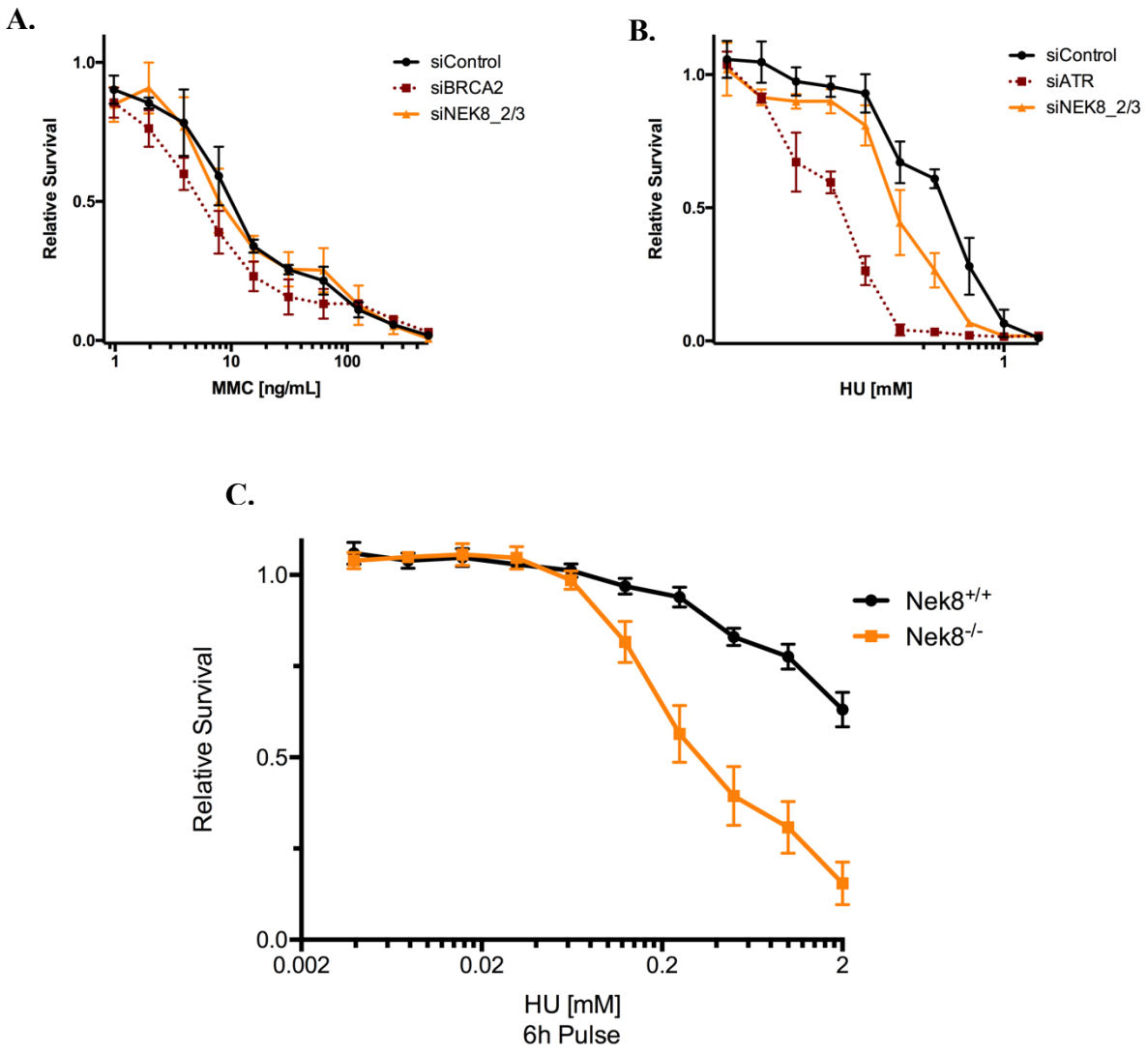


Figure 4.3. **NEK8 is required for resistance to replication stress in U-2 OS and MEFs. A-B.** Cell survival in U-2 OS cells transfected with siRNA (20nM) and treated with **A.** MMC and **B.** HU. (n=3, +/-SEM). **C.** Cell survival in *Nek8* MEFs in response to a 6h pulse of HU. Cell survival was assayed by crystal violet staining and expressed as a fraction of the untreated control (n=7, +/-SEM).

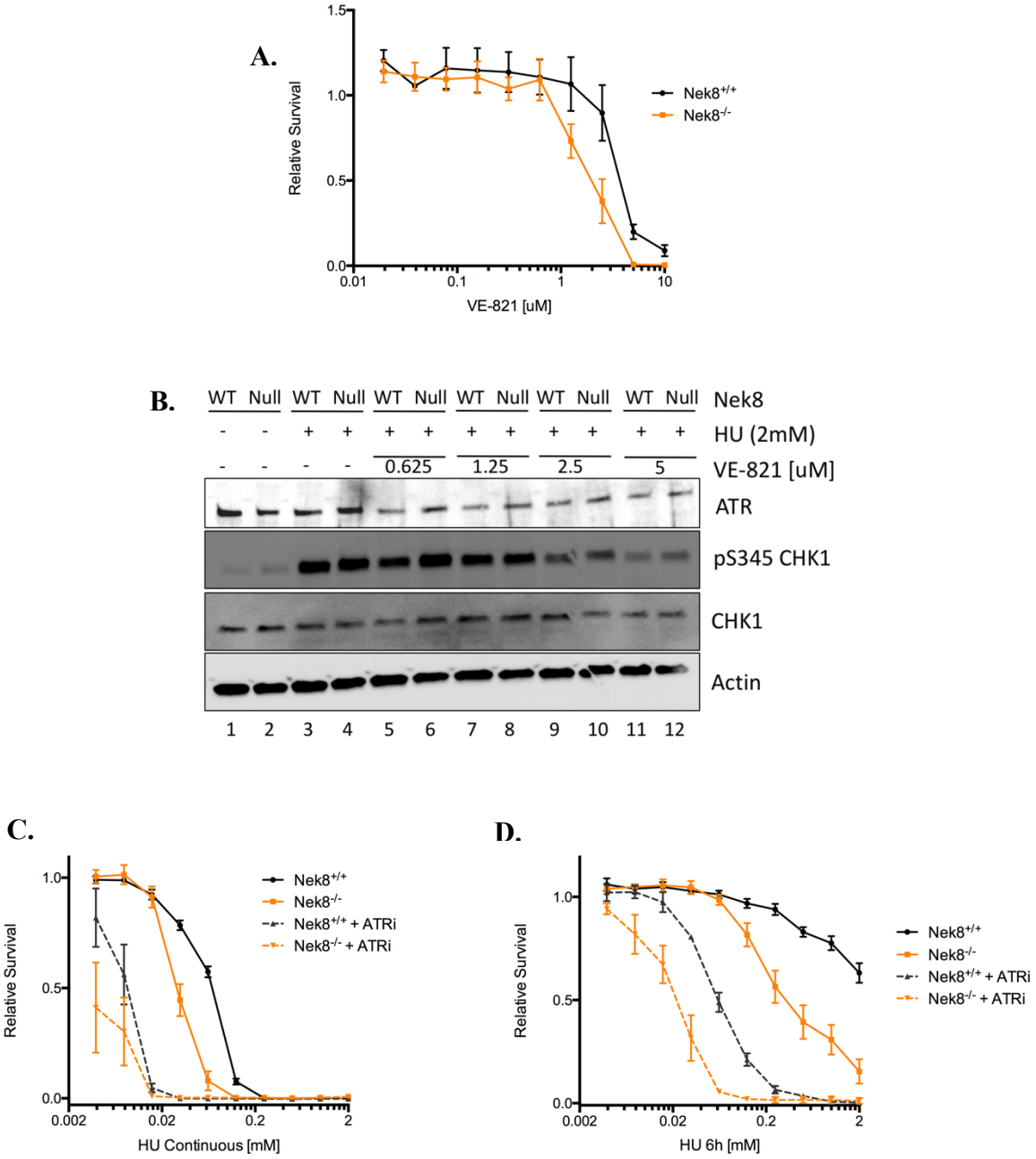


Figure 4.4. **Nek8 MEFs are sensitive to ATR inhibition. A.** Cell survival in Nek8 MEFs treated with increasing doses of ATR inhibitor as indicated (n=3, +/-SEM). **B.** Nek8 MEFS were treated with ATR inhibitor followed by treatment with HU. Cells were collected for Western blot. **C.** Cell survival in Nek8 MEFs in response to continuous treatment or a 6h pulse of HU with or without ATR inhibition. Cell survival was assayed by crystal violet staining and expressed as a fraction of the untreated control (n=3, +/-SEM).

Nek8 is required for replication fork protection and genome stability

Since the *Nek8*$^{-/-}$ MEFs were only sensitive to HU, we next tested the hypothesis that NEK8 is regulating the function of RAD51 in replication fork protection. As previously mentioned, recent data suggests that many proteins in the Fanconi anemia and HR pathways are required for replication fork protection following replication fork stall [76, 77]. To test this hypothesis, we first treated U-2 OS cells with HU followed by chromatin fractionation to determine the efficiency of chromatin loading of important replication fork protection machinery in the presence and absence of NEK8. As expected, following treatment with HU, RAD51 chromatin loading was decreased in NEK8-depleted cells compared to control cells (Figure 4.5A, Lanes 4 and 12). Interestingly, the loading of both BRCA1 and BRCA2 was also abrogated in both cell lines (Figure 4.5A, Lanes 4 and 12), suggesting that the decrease in RAD51 focus formation and chromatin loading was due to an effect of NEK8 on the efficient localization of BRCA1 and BRCA2 to chromatin. This phenotype may reflect total expression levels of the proteins, as we observed a mild decrease in overall BRCA2 protein expression following depletion of NEK8 in U2OS cells (Chapter 3). We also observed an increase in chromatin bound ATR in the absence of replication stress in NEK8 depleted cells, while there is no difference in chromatin loading following replication stress (Figure 4.5A, Lanes 2 and 10, 4 and 12). Lastly, we also observed an increase in the amount of phosphorylated CHK1 bound to chromatin both in untreated and HU treated cells (Figure 4.5A, Lanes 2 and 10, 4 and 12). Importantly, the effect of efficient chromatin loading of RAD51, BRCA2, while mild, is also observed in *Nek8*$^{-/-}$ MEFs (Figure 4.5C, Lanes 4 and 8). Interestingly, we also observed an increase in ATR bound to chromatin both in untreated and HU treated *Nek8*$^{-/-}$ MEFs (Figure 4.5C, Lanes 2, 4, 6 and 8). We also observed an increase in the phosphorylated form of chromatin bound RPA32 following HU

treatment in these same MEFs (Figure 4.5C, Lanes 4 and 8). The effect on chromatin loading of these proteins was not limited to replication inhibition with similar effects on loading of RAD51 and BRCA2 were observed in U-2 OS cells following DNA damage induced with MMC (Figure 4.5B). We also observed that Nek8 is primarily in the soluble fraction in *Nek8*$^{+/+}$ MEFs (Figure 4.5C, Lanes 1 and 3).

Cells having defective Fanconi anemia and HR pathways also exhibit increased levels of replication fork degradation following treatment with replication fork inhibitors. These cells also show increased levels of genomic instability in response to replication stress [76, 77]. Similarly, a recent report suggests that cells defective in NEK8 have defective replication fork stability, and an increased sensitivity to the replication inhibitor aphidicolin [93], but 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 mediating the proper function of important HR proteins, including RAD51, BRCA1 and BRCA2.

To test this hypothesis, we first used DNA fiber analysis to investigate replication fork dynamics in *Nek8*$^{-/-}$ 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) (Figure 4.6A). Surprisingly, in our hands, *Nek8*$^{-/-}$ MEFs did not exhibit a significant difference in replication tract length compared to *Nek8*$^{+/+}$ MEFs as measured by IdU incorporation (Figure 4.6B). This is in contrast to a previous report showing *Nek8*$^{-/-}$ MEFs having slowed rates of fork progression [93]. Our results may differ based on the type of assay used to determine replication tract progression.

We next measured the length of CldU tracts following treatment with HU. *Nek8*$^{-/-}$ MEFs consistently exhibit shorter CldU tract lengths following HU as compared to HU-treated *Nek8*$^{+/+}$

MEFs (Figure 4.6C). To determine the directionality of resection, we calculated the ratio of CldU to IdU incorporation. *Nek8*$^{-/-}$ MEFs had a ratio of 0.794 as compared to *Nek8*$^{+/+}$ MEFs with a ratio of 0.957 following HU treatment (Figure 4.6D). This suggest that resection of DNA at stalled replication forks in the absence of NEK8 occurs in the 3'-5' direction on the leading strand and in the 5'-3' direction on the lagging strand.

Next, we tested the ability of *Nek8*$^{-/-}$ MEFs to restart replication following replication stall. Cells were treated with HU for six hours and then released into fresh media. We measured the ability of *Nek8*$^{-/-}$ MEFs to resume replication via incorporation of EdU. In untreated cells both wild-type and *Nek8*$^{-/-}$ MEFs exhibited a normal cell cycle profile. Following a six-hour pulse with HU, both wild-type and *Nek8*$^{-/-}$ MEFs had completely stalled replication as measured by EdU incorporation (Figure 4.7A and B). Interestingly, *Nek8*$^{+/+}$ MEFs resumed replication around two hours post release from HU and were cycling normally by 18 hours post release. In contrast, *Nek8*$^{-/-}$ MEFs also began resuming replication but at a much reduced rate at two hours post release from HU. The *Nek8*$^{-/-}$ MEFs also failed to fully resume normal replication as determined by their cell cycle profile 18 hours after release from HU (Figure 4.7B). This data, coupled with the replication fork protection phenotype shows that the loss of replication fork protection in the absence of NEK8 leads to degradation of the stalled fork which consequently leads to a failure to restart replication following stall by HU.

Lastly, recently published data suggest that defects in replication fork protection mediated by proteins of the Fanconi anemia and homologous recombination pathways increases genomic instability in response to HU [76, 77]. To test if loss of NEK8 lead to similar results, we tested if treatment with HU increased levels of genomic instability in *Nek8*$^{-/-}$ MEFs via metaphase spread analysis. Basal levels of chromosomal aberrations were not significantly

different between *Nek8*$^{+/+}$ and *Nek8*$^{-/-}$ MEFs in the absence of replication inhibition by HU (Figure 4.7C, 0.0667±0.0463 and 0.1333±0.0631, p=0.398). However, after treatment with HU, *Nek8*$^{-/-}$ MEFs showed a drastic increase in the number of chromosomal aberrations per cell compared to *Nek8*$^{+/+}$ MEFs (Figure 4.7C, 0.2±0.0884 and 1.719±0.5576, p=0.0115). Taken together, these data suggest that NEK8 is an essential factor for the maintenance of genomic stability through replication fork protection and replication restart following replication stall with HU.

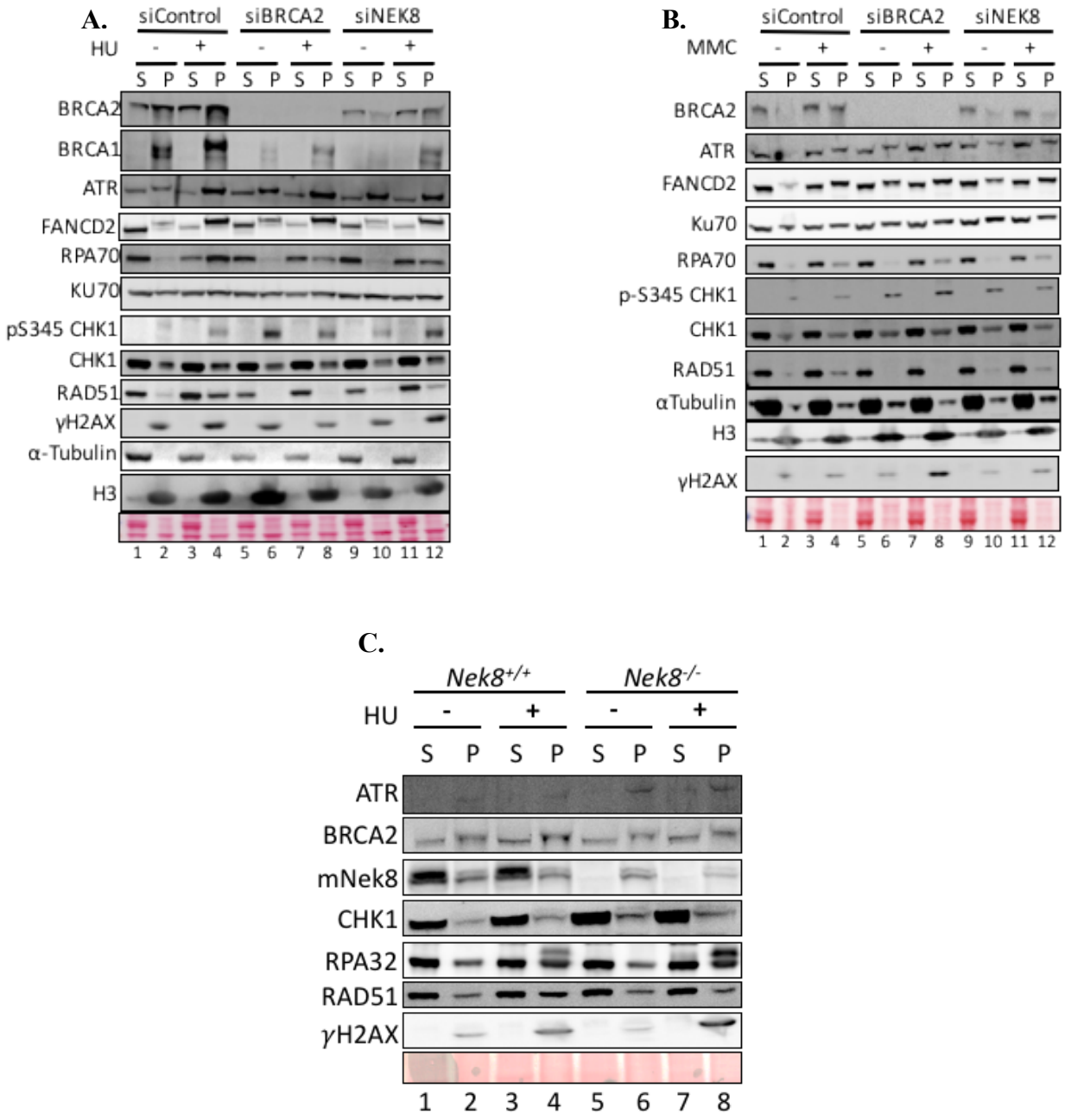


Figure 4.5. **Loss of NEK8 leads to decreased chromatin loading of important replication fork protection proteins. A-B.** U-2 OS cells were transfected with siRNA (20nM). 48h post siRNA transfection, cells were treated with or without **A.** HU (2mM 24h) **B.** MMC (60ng/mL, 24h) and then subjected to chromatin fractionation (S, soluble fraction; P, insoluble fraction) and Western blotting. **C.** *Nek8*$^{+/+}$ and *Nek8*$^{-/-}$ MEFs were treated with or without HU (2mM, 6h) and then subjected to chromatin fractionation (S, soluble fraction; P, insoluble fraction) and Western blotting.

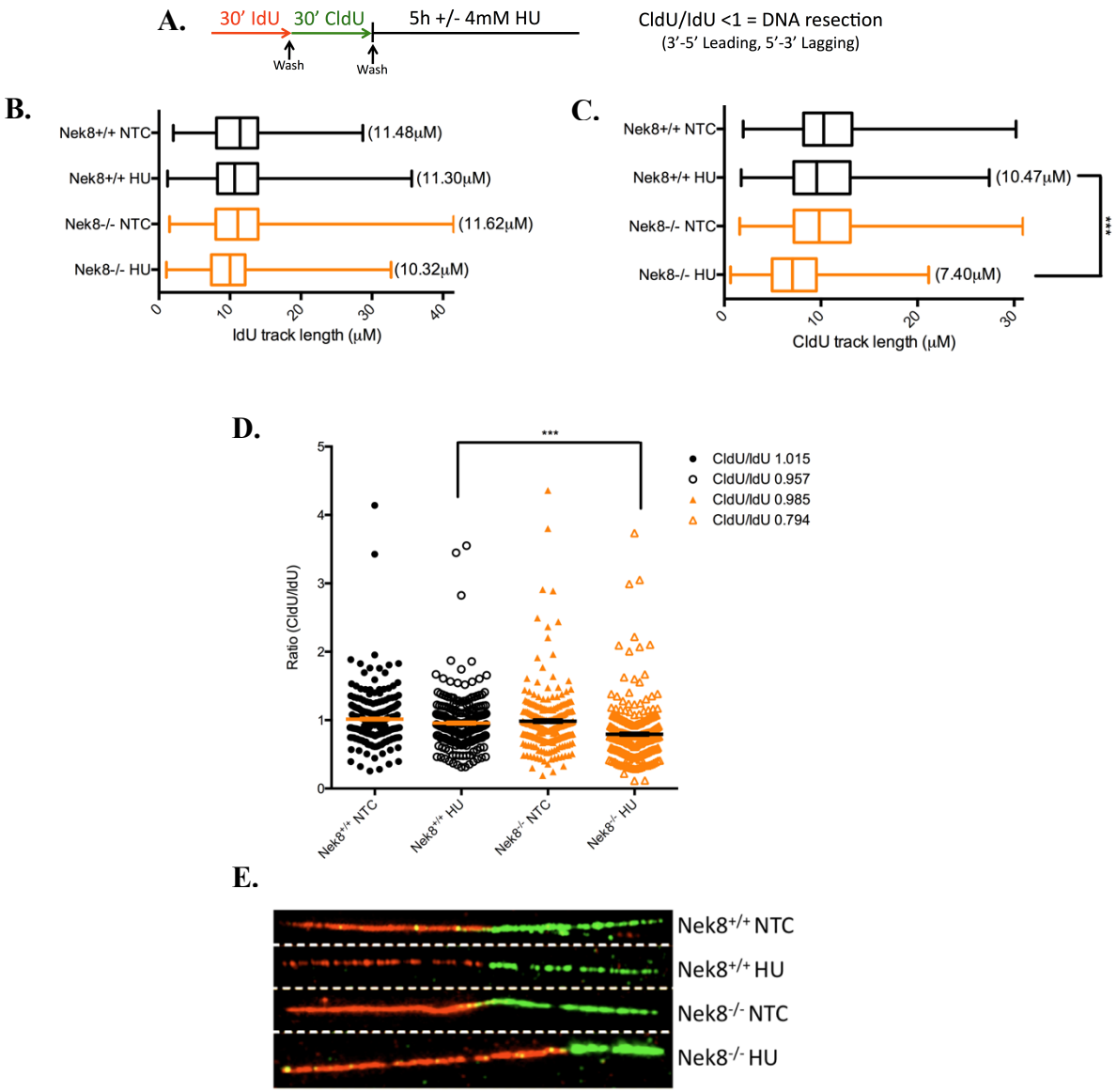


Figure 4.6. ***Nek8* prevents replication fork degradation in MEFs. A.** Schematic of experimental conditions for DNA replication tract assay. Red tracts, IdU; Green tracts, CldU **B.** IdU tract length in HU treated or untreated *Nek8*$^{+/+}$ and *Nek8*$^{-/-}$ MEFs. Mean tract length is denoted in parenthesis and designated in box and whisker plots. **C.** CldU tract length in HU treated or untreated *Nek8*$^{+/+}$ and *Nek8*$^{-/-}$ MEFs. Mean tract length is denoted in parenthesis and designated in box and whisker plots. **D.** Ratio of CldU/IdU tract lengths in *Nek8*$^{+/+}$ and *Nek8*$^{-/-}$ MEFs. Bar signifies mean. Ratio noted in legend. **E.** Representative DNA fiber images from *Nek8*$^{+/+}$ and *Nek8*$^{-/-}$ MEFs either treated or untreated with HU. (n=300 fibers/sample) *** = p<.001

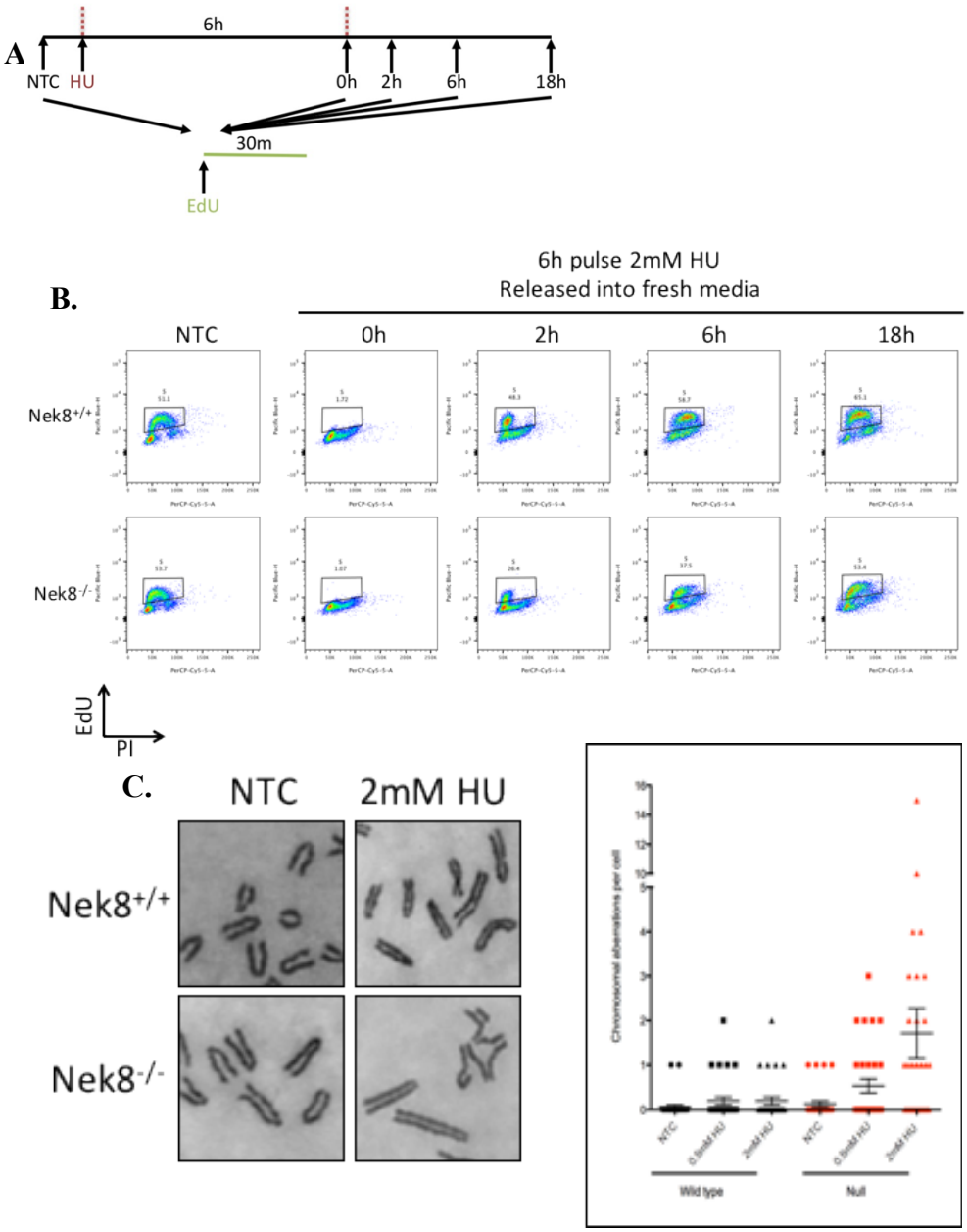


Figure 4.7. ***Nek8* is important for replication restart and genome stability. A.** Schematic diagram of experimental conditions for replication restart assay. **B.** *Nek8*$^{+/+}$ and *Nek8*$^{-/-}$ MEFs were treated with or without HU as indicated and released into fresh media. Cells were collected and subjected to EdU Click-iT chemistry and stained for DNA content. Cell cycle distribution was determined by flow cytometry. **C.** *Nek8*$^{+/+}$ and *Nek8*$^{-/-}$ MEFs were treated with or without HU for 6h and then released into fresh media for 16h. The cells were then treated with colcemid and processed for metaphase analysis. Quantification is chromosome aberrations per metaphase. Mean chromosome aberrations per metaphase are denoted on graph. (n=30/sample, +/- SEM)

DISCUSSION

In this study we sought to elucidate a possible mechanism by which NEK8 regulates RAD51 focus formation in response to DNA damage and replication fork stall as well as phenotypic outcomes due to this regulation. Not surprisingly, we observed that NEK8 depletion by siRNA decreases HR as measured in the U-2 OS DR-GFP system (Figure 4.1A). This phenotype was expected as RAD51 is the key modulator of HR [9, 12, 46] and as such, a decrease in RAD51 focus formation will decrease rates of HR. We also attempted to rescue the HR defect in NEK8-depleted cells, but similar to our attempts to complement RAD51 focus formation (Chapter 3), we were unsuccessful. The validity of this phenotype therefore cannot be validated without further experimentation. One such possibility is to test the rates of HR using the DR-GFP system in the *Nek8*$^{-/-}$ MEF background. This however is an area we have yet to explore.

We found that cells deficient in NEK8 were sensitive to replication stress-inducing agents, consistent with previous reports [93]. However, these cells were not sensitive to any other type of damage requiring HR for efficient repair of their lesions (Figure 4.2). This phenotype is surprising in that no previous reports suggest a deficiency in RAD51 focus formation associated with resistance to DNA damaging agents requiring HR for their repair. This finding suggests that NEK8 maintains a complex regulatory role in the repair of DNA lesions requiring HR. One such possibility is that NEK8 has dual functions in both HR and the opposing DNA repair pathway, non-homologous end joining (NHEJ). This notion is supported by data suggesting that blocking NHEJ substantially rescues the inter-strand crosslink repair defect of FANCD2 deficient human cells [160]. However, rates of NHEJ in a NEK8 deficient background have yet to be explored. Secondly, while we observe a two-fold decrease in both RAD51 focus formation and HR (Chapter 3 and Figure 4.1A) in cells lacking NEK8, it is possible that these

cells maintain a sufficient amount of HR to repair lesions created by a multitude of DNA damaging agents, thus maintaining cellular resistance to these agents. Lastly, the RAD51 paralogs, which are key components in RAD51 focus formation and HR, are required for protection of nascent DNA at stalled replication forks [153], but do not contribute equally to the cellular resistance of replication stress agents [161]. Thus, we can speculate that NEK8 may function in regulating RAD51-mediated replication fork protection via the RAD51 paralogs. Preliminary genetic interaction data not presented in this dissertation supports the notion that NEK8 functions through one sub-complex of the RAD51 paralogs. However, this has yet to be fully explored.

Interestingly, we observed hypersensitivity to short term replication stress in *Nek8*$^{-/-}$ MEFs (Figure 4.3C). 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 non-canonical roles in the early stages of replication fork protection [76, 77, 144]. The key cell cycle kinase, ATR, is also an important factor in the early stages of replication fork protection [59, 60, 162]. The NEK8 kinase has been observed to interact with ATR, ATR-interacting protein (ATRIP), and CHK1 [93]. However, the role of NEK8 in this complex is not fully understood. We observed that *Nek8*$^{-/-}$ MEFs are sensitive to inhibition of ATR (Figure 4.4A) and are further sensitized to HU following ATR inhibition (Figure 4.4C and D). This suggests that NEK8 has a separate and distinct function from ATR in the early stages of replication fork protection.

Through chromatin fractionation we observed that key components of the HR and replication fork protection pathways, including RAD51, BRCA1 and BRCA2, are not loaded

efficiently onto DNA following replication stress and DNA damage induced by MMC in U-2 OS cells depleted of NEK8 (Figure 4.5A and B). This key piece of evidence supports the notion that NEK8 is important for maintaining important replication fork protection proteins on DNA. The decreased chromatin loading of both BRCA1 and BRCA2 proteins in the NEK8-depleted U-2 OS background provide insight into the possible mechanism by which NEK8 is regulating RAD51 focus formation and resistance to replication stress inducing agents as BRCA1 and BRCA2 play key roles in both phenotypes [31, 76, 77, 147]. We also observed similar phenotypes for RAD51 and BRCA2 in the *Nek8*$^{-/-}$ MEFs (Figure 4.5C). Unfortunately, we have not encountered an antibody suitable for the detection of murine BRCA1 and were unable to determine if efficient chromatin loading occurred in the *Nek8*$^{-/-}$ MEFs in response to treatment with HU.

Following chromatin fractionation in the *Nek8*$^{-/-}$ MEFs, we observed a higher level of chromatin bound ATR and CHK1 kinases in both untreated and HU treated conditions. The ATR phenotype is consistent with published reports that ATR accumulates on chromatin in the presence of replication stress [163]. The CHK1 kinase associates with chromatin in a replication dependent manner via interaction with 14-3-3 proteins [164], but once phosphorylated on Serine345 in a DNA damage response manner, quickly dissociates from chromatin, a process dependent on PIKK-checkpoint signaling, to initiate the downstream DNA damage checkpoint signaling [165]. Interestingly, in our NEK8-depleted U-2 OS cell background we also observed an increase in chromatin bound ATR in untreated conditions (Figure 4.5A) in line with previous reports of increased endogenous replication stress in NEK8-depleted cells [93]. Notably, we also observed an increase in chromatin bound pS345 CHK1 in NEK8-depleted U-2 OS cells in both untreated and HU-treated backgrounds (Figure 4.5A). This suggests that NEK8 may also

regulate the dissociation of phosphorylated CHK1 from chromatin. This hypothesis is intriguing as NEK8 is reported to compete for binding with another 14-3-3 interacting protein TAZ [166]. It is tempting to speculate that NEK8 may be competing with 14-3-3 in binding of CHK1 to modulate its association with chromatin. NEK8 is, in-fact, an interacting partner of CHK1 [93]. However, the role of this interaction has yet to be investigated.

We observed an increase in degradation of stalled replication forks in the *Nek8*$^{-/-}$ MEF background (Figure 4.6). 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 [76, 77]. We have yet to determine the nuclease which is responsible for this degradation, but it is possible that, similar to previously published data, the nuclease MRE11 could be the responsible protein. Similar to cells deficient in replication fork protection [76], *Nek8*$^{-/-}$ MEFs also exhibit an increase in genomic instability following treatment with HU (Figure 4.7C). This phenotype is likely explained by a decrease in HR (Figure 4.1) coupled with the inability to efficiently restart replication following treatment (Figure 4.7) ultimately manifesting as genome instability (Figure 4.7) and cellular sensitivity to HU (Figure 4.2).

In summary, we describe multiple phenotypes associated with the loss of NEK8 in mammalian cells. Our data provide a clearer picture into the function of NEK8 in key aspects of the replications stress response. However, these data also raise questions about the role of NEK8 in fine tuning the response to DNA damage, an area which remains cloudy. Thus, a mechanistic description of NEK8 regulation of RAD51 focus formation remains to be identified.

MATERIALS AND METHODS

Cell lines

U-2 OS were purchased from the American Type Culture Collections. U-2 OS DR-GFP cells were a gift of the Jasin Lab (Memorial Sloan Kettering) [167]. Nek8 MEFs were a gift of the Cimprich lab (Stanford) with permission from the Beier lab (Seattle Children's). All cell lines were cultured in DMEM containing 10% FBS, 2mM L-glutamine and 1X Pen/Strep in a humidified 5% CO2 containing atmosphere at 37oC.

siRNAs and Plasmids

siRNAs targeting BRCA2 [136], RAD51 (target sequence: 5'-AACTAATCAGGTGGTAGCTCA-3'), and NEK8 (#1 target sequence: 5'-TCACTCTTCTGGTTGTAGG-3', #2 target sequence: 5'- TCAGAGGAGAAGCAATATC-3', #3 target sequence: 5'- AGAGATAGGTGCAAAGGTG-3') were transfected at 20nM using Lipofectamine RNAiMAX (ThermoFisher). AllStars siRNA (Qiagen) was used as a negative control.

hNEK8 coding sequence was PCR amplified from the YFP-NEK8 (gift from Cimprich lab) plasmid to contain a N-terminal 3XFLAG tag and cloned into pLentiX1-pUB-puro [149] using SalI and XbaI sites. I-SceI expression vector, pCBASce, was a gift from the Jasin lab (Memorial Sloan Kettering).

Homologous recombination assay

U2OS DR-GFP cells were transfected with siRNAs or miRNA mimics and then transfected 24 hours later with pCBASce (I-SceI expression vector) or empty 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.

Western blotting

Whole-cell extracts were prepared and resolved by SDS-polyacrylamide gel electrophoresis as described [137]. Proteins were transferred onto nitrocellulose membranes. Antibodies against α-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), pS345 CHK1 (CST),FANCD2 (Abcam), γH2AX (JBW301, Millipore), H3 (Abcam), Ku70 (Abcam), mNEK8 (gift from David Beier), hNEK8 (N-17, Santa Cruz), RAD51 (H-92, Santa Cruz) 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 fractionation

Cell fractionations were prepared as described [149]. Briefly, cells were resuspended in buffer CSK (10mM PIPES, pH = 6.8, 100mM NaCl, 1mM EGTA, 1mM EDTA, 300mM Sucrose, 1.5mM MgCl2, 0.1% Triton-X-100 and protease inhibitors) and incubated in ice for 5min. Samples were centrifuged at 1500g for 5min. 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% β-mercaptoethanol) and boiled for 5min. Western blotting was performed as described

Survival assay

Cell survival was measured by a crystal violet absorbance-based assay. Cells were seeded onto 12-well plates at a density of 6-9x106 cells/well. The next day, cells were treated with increasing concentrations of drug and incubated for five to eight more days. After that, cellular monolayers were fixed, stained with crystal violet and re-solubilized as previously described [137].

DNA fiber assay

DNA fiber assay was conducted as previously described with some changes [76]. Briefly, cells were labeled with IdU (50 μM), washed, and labeled with CldU (50 μM). Cells were then exposed to hydroxyurea (4 mM), or untreated media. DNA fibers were essentially spread as described [168] before standard detection of IdU and CldU tracts (primaries: α-IdU, α-BrdU from BD Biosciences; α-CldU, α-BrdU from Novus Biologicals and secondaries: 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

7×105 cells were seeded 24h prior to treatment with HU (4 mM) and treated with colcemid (0.1μg/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).

Cell cycle analysis

Nek8 MEFs were treated with HU (2mM, 6h), or untreated media. Following treatment with HU, cells were washed and chased into fresh media for indicated times. Cells were then fixed, permeabilized and subjected to Click-iT Plus EdU chemistry (Life Technologies) and also stained for DNA content. Flow cytometry was performed to determine the cell cycle phase distribution (Canto or LSR-2).

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 ± SEM. A *P* value < 0.05 was considered significant

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

Post-translational modifications (PTMs) play an important and vital role in the regulation of most cellular processes. The complex regulation of HR is mediated, in part, through a sequence of PTM events, but the complexity of such events is currently not fully understood. As such, we set out to identify kinases that have novel roles in the regulation of HR in response to DSBs created by the inter-strand crosslinking agent MMC. Using the human cell line U-2 OS, we conducted a high-throughput screen of the human kinome using RAD51 focus formation as a readout of HR efficiency. We found multiple kinases which are putative regulators of RAD51 focus formation and HR including, but not limited to, the kinases PAK4 and NEK8 (Chapter 2). While we focused on NEK8, it is possible that other putative hits from our screen are interesting targets to pursue as novel regulators of RAD51 focus formation and HR. We went on to show that the kinase, NEK8, functions to regulate RAD51 focus formation in response to multiple types of DNA damage as well as across species (Chapter 3). Lastly, we showed that NEK8 has important functions in the maintenance of HR, replication fork protection and overall genome stability (Chapter 4). Taken together these data provide evidence for NEK8 having novel roles in regulating important aspects of DNA repair and replication.

One kinase of interest for future studies is PAK4. We identified PAK4 in our initial screen (Table 2.1) as a kinase whose depletion lead to decreased RAD51 focus formation. Interestingly, PAK4 was also the only validated kinases to decrease RPA focus formation (Table 2.3). PAK4 is one member of the p21-activated kinase family [125], where it has been reported to be required for the oncogenic transformation of breast cancer cells [126], likely through its role in regulating proper cell cycle transitions [127-129]. As a p21-activated kinase, PAK4 may play an interesting role in the regulation of RAD51 focus formation, as depletion of p21 via

RNAi leads to decreased rates of RAD51 foci [130]. Therefore, it would be of great interest to pursue the role of PAK4 in regulating RAD51 focus formation and HR via characterization similar to that of NEK8 presented in this dissertation.

Based on the difficulty we had in complementing RAD51 focus formation in NEK8 depleted U-2 OS cells, this is an area requiring further exploration. Other groups have reported difficulty in complementation, which is likely due to defective kinase activity or mis-localization of the ectopically expressed NEK8 constructs [93]. This is likely a reason for our similar observations in U-2 OS cells. We attempted complementation with multiple NEK8 constructs, some of which have been reported to be non-functional [93] as well as with a construct containing an SV40 nuclear localization signal [93], none of which were able to effectively rescue RAD51 focus formation. We hypothesize that the tightly regulated expression levels of NEK8 are in place to regulate NEK8 activity, protein interactions or cellular localization, and as such, overexpression of the protein may initiate mechanisms to inactivate normal functions of the overexpressed protein. Such examples exist in nature, where overexpression of proteins presumably interferes at some level with the function of the protein or its complex resulting in the protein essentially acting as an antimorph, even with the absence of a mutation [169]. Therefore, it would be worthwhile to attempt NEK8 complementation under tightly regulated inducible conditions or using constructs which have been observed to complement other known NEK8 phenotypes.

We examined the function of NEK8 in resistance to DNA damaging agents and, surprisingly, found that cells deficient in NEK8 are only sensitive to agents which cause replication inhibition (Figure 4.2 and 4.3). This phenotype suggests a complex role for NEK8 in regulating the response to DNA damage. As mentioned previously, NEK8 may function as a fine

tuning kinase in regulating HR. It is also possible that NEK8 has dual functions in both HR and NHEJ. This notion is supported by data suggesting that blocking NHEJ substantially rescues the inter-strand crosslink repair defect of FANCD2 deficient human cells [160], where FANCD2 is important both for inter-strand crosslink repair as well as HR. As such, it would be worthwhile to measure the rates of NHEJ and other DNA repair mechanisms in a NEK8 deficient background. It is also possible that while we observe a two-fold decrease in both RAD51 focus formation and HR (Chapter 3 and Figure 4.1A) in cells lacking NEK8, that these cells maintain a sufficient amount of HR to repair lesions created by a multitude of DNA damaging agents, thus maintaining cellular resistance to these agents. Our data also suggests that the function of NEK8 early in replication fork protection may be more important in maintaining overall genome stability than its function in mediating HR. This can also explain the sensitivity to replication stalling agents as opposed to other DNA damaging agents. The discovery that NEK8 functions in resistance to replication inhibiting agents raises the possibility that NEK8 may have clinical utility as a therapeutic target for tumor chemosensitization through the development of a novel kinase inhibitor specific to NEK8.

We observe that NEK8 deficient cells can also be sensitized to inhibition of the checkpoint kinase ATR (Figure 4.4A) and can be further sensitized to HU in this same background (Figure 4.4C and D). Interestingly, we also observe an increase in chromatin bound ATR and CHK1 in the absence of NEK8 in both HU treated and untreated conditions. As NEK8 has previously been shown to interact with both ATR and CHK1 [93], this data suggests that NEK8 has an important function in replication fork protection that is independent of ATR function in this same phenotype. Further investigation into the function of NEK8 in this

phenotype is required. To address this, we plan to determine the functional domains of NEK8 that are required for replication fork protection.

Interestingly, we observed that some proteins required for replication fork protection and HR are not properly loaded onto chromatin following replication stall with HU (Figure 4.5). This data suggests that NEK8 functions to maintain normal loading of these key proteins on DNA following replication stall or DNA damage. However, this data does not directly show that these key proteins are not present at a stalled replication fork to aid in the protection of replication fork collapse. As such, we propose to use a recently developed method for the identification of proteins on nascent DNA (iPOND) [170] to determine if key proteins, such as RAD51, are present or absent at stalled replication forks in NEK8 deficient cells. Based on our data showing a decrease of chromatin bound RAD51 (Figure 4.5), increased replication fork degradation (Figure 4.6), NEK8 deficient cells failure to restart replication (Figure 4.7B), and an increase in genomic instability (Figure 4.7C) all in cells deficient in NEK8 following treatment with HU, we speculate that iPOND will reveal a decrease in replication fork associated RAD51, which would account for these observed phenotypes.

Lastly, 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 [85, 93]. 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.

In summary, we identified the kinase NEK8 as a protein having novel functions in RAD51 focus formation, HR, replication fork protection and genome stability (Figure 5.1). We also present a hypothetically testable model of NEK8 mediated replication fork protection (Figure 5.2). The identification of NEK8 provides further insight into the intricate regulatory mechanisms behind an already complex cellular machine responsible for the response to DNA damage. Furthermore, the identification of NEK8 provides further evidence to the importance of the interplay between centrosomal proteins and the DNA repair machinery, highlighting the importance for future research in this area.

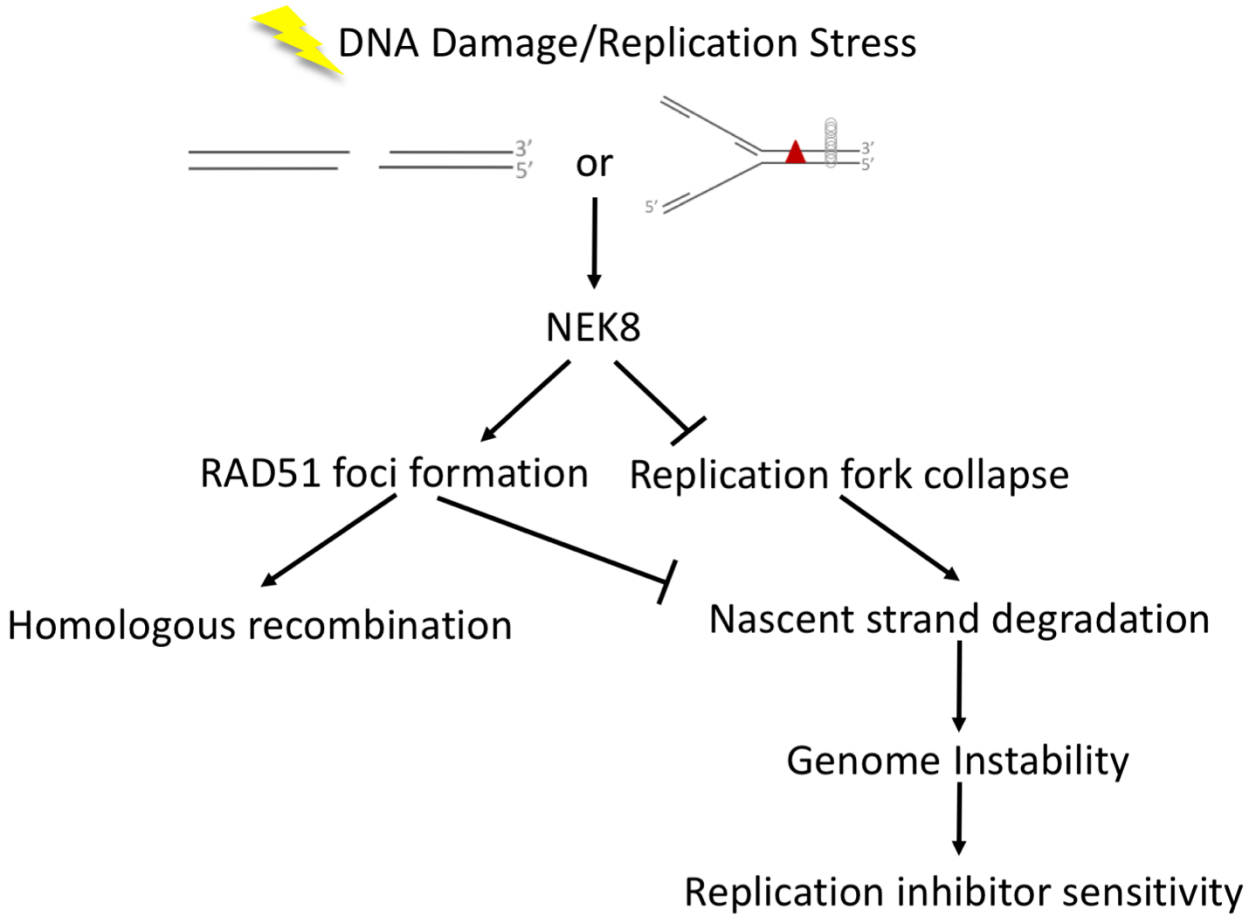


Figure 5.1. **Schematic model of NEK8 mediated replication fork protection and genetic stability.** Schematic diagram of NEK8 mediated response to DNA DSBs and replication fork stall shows following DNA damage and replication fork stall, NEK8 is required for RAD51 focus formation and HR. NEK8 inhibits replication fork collapse and nascent DNA degradation in a RAD51 dependent manner. This RAD51-mediated replication fork stability is required for maintenance of genome stability and resistance to replication inhibiting agents.

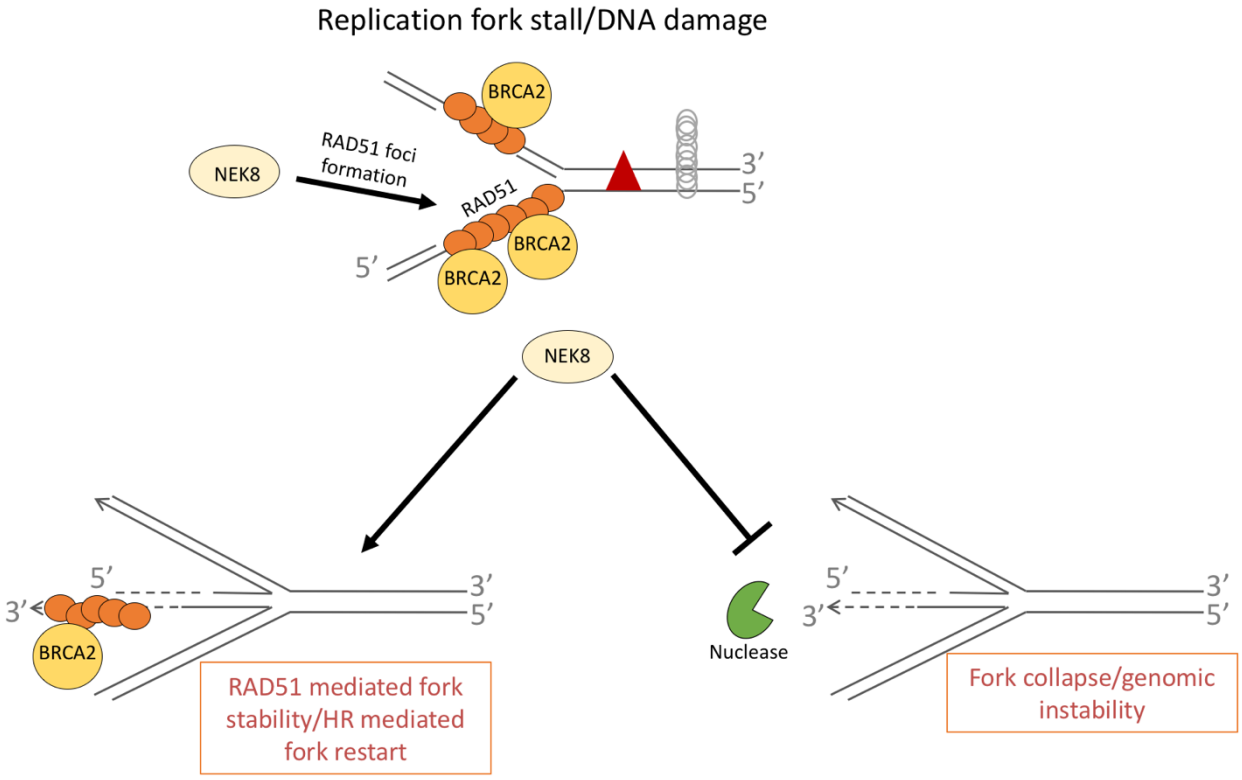


Figure 5.2. **Hypothetical model of NEK8 mediated replication fork stability.** In response to DNA damage and replication fork stall, NEK8 is required for BRCA2-mediated RAD51 focus formation. NEK8 mediates protection of a reversed replication fork 'chicken foot' structure by RAD51 and BRCA2. This structure allows for HR mediated restart of the replication fork. In the absence of NEK8, nascent DNA on stalled and reversed replication forks is not protected by RAD51 and BRCA2, leading to nuclease-mediated resection of the stalled fork.

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VITA

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