Cell Cycle 10:4, 655-663; February 15, 2011; © 2011 Landes Bioscience

Nek1 kinase functions in DNA damage response and checkpoint control through a pathway independent of ATM and ATR

Yumay Chen,[1,*] Chi-Fen Chen,[2] Daniel J. Riley[3,4] and Phang-Lang Chen[2,*]

[1]Department of Medicine; Division of Endocrinology; [2]Department of Biological Chemistry; University of California at Irvine; Irvine, CA USA; [3]Department of Medicine; Division of Nephrology; [4]Transplant Center; The University of Texas Health Science Center; San Antonio, TX USA

Key words: checkpoint control, DNA damage response, Nek1, ATM, ATR

Never-in-mitosis A related protein kinase 1 (Nek1) is involved early in a DNA damage sensing/repair pathway. We have previously shown that cells without functional Nek1 fail to activate the more distal kinases Chk1 and Chk2 and fail to arrest properly at G_1/S or M-phase checkpoints in response to DNA damage. As a consequence, foci of damaged DNA in Nek1 null cells persist long after the instigating insult, and Nek1 null cells develop unstable chromosomes at a rate much higher than identically cultured wild-type cells. Here we show that Nek1 functions independently of canonical DNA damage responses requiring the PI3 kinase-like proteins ATM and ATR. Chemical inhibitors of ATM/ATR or mutation of the genes that encode them fail to alter the kinase activity of Nek1 or its localization to nuclear foci of DNA damage. Moreover ATM and ATR activities, including the localization of the proteins to DNA damage sites and phosphorylation of early DNA damage response substrates, are intact in *Nek1*$^{-/-}$ murine cells and in human cells with Nek1 expression silenced by siRNA. Our results demonstrate that Nek1 is important for proper checkpoint control and characterize for the first time a DNA damage response that does not directly involve one of the known upstream mediator kinases, ATM or ATR.

Introduction

DNA damage initiates a complex signaling network that rapidly senses the lesions and sends response proteins into macromolecular nuclear foci at sites of damage. This network activates a series of checkpoints that temporarily halt progression of the cell cycle and prevent the cell from duplicating its DNA or from undergoing mitosis until after DNA can be assessed and repaired. For prompt and accurate DNA repair, signals must be conveyed rapidly and precisely. ATM, a member of the phosphatidylinositol-3-kinase-like kinase (PIKK) family, is activated at the site of a double strand DNA breaks (DSBs). Via phosphorylation of key substrates, ATM triggers a cascade of signals that activates and amplifies multiple downstream pathways,[1] including those that modulate DNA repair and cell cycle checkpoints.

The ATM and Rad3-related kinase (ATR) may be even more fundamental in DNA damage sensing and repair than ATM, since homozygous mutations of *ATR* have not been found in humans and since biallelic *Atr* inactivation in mice is lethal.[2] ATR has similar and intersecting downstream targets as ATM.[3,4] Whereas ATM is functions primarily in response to DSBs, ATR is primarily activated by DNA replication intermediates. ATR is thought to be the more important upstream PIKK for signaling and repairing UV radiation- and nucleoside analog-induced DNA damage, both of which cause stalled replication forks.[3,4] To date, ATM and/or ATR have been shown to be crucial, proximal signaling molecules in all forms of DNA damage sensing and repair.

Previously, we have shown that Nek1 (a.k.a. Nrk1), the mammalian ortholog of NIMA (never in mitosis A) in *Aspergillus nidulans*, is involved in DNA damage responses.[5,6] Nek1 was partially cloned after screening an expression library with anti-tyrosine antibodies and identified as the first mammalian NIMA-related kinase.[7] Nek1 has dual serine-threonine and tyrosine kinase activity. *Nek1* mRNA is abundantly expressed in mouse gonads and in specific neurons, and authors have surmised that Nek1 may play a direct and unique role in meiosis or in regulating the cell division cycle.[7,8] Nek1 is also important for proper development in mammals. Germline *Nek1* mutations in two strains of mice, the so-called kidneys-anemia-testis (kat and kat2J) strains, result in pleiotropic and ultimately fatal defects including growth retardation, facial dysmorphism, chorioid plexus and neurologic abnormalities, male sterility, anemia and progressive polycystic kidney disease (PKD).[9,10]

We first discovered the role of Nek1 in DNA damage sensing when we observed Nek1-deficient cells to be much more sensitive to the effects of ionizing radiation (IR)-induced DNA damage than otherwise identical wild-type cells.[6] The expression and kinase activity of Nek1 are quickly upregulated in cells treated with IR. Very early, at the same time that kinase activity is

*Correspondence to: Yumay Chen and Phang-Lang Chen; Email: yumayc@uci.edu and plchen@uci.edu
Submitted: 12/20/10; Revised: 01/10/11; Accepted: 01/11/11
DOI: 10.4161/cc.10.4.14814

upregulated, a portion of Nek1 consistently redistributes in cells from cytoplasm to discrete nuclear foci at sites of DNA damage. There it colocalizes with key proteins involved very early in the response to IR-induced DNA double strand breaks (DSBs), including γ-H2AX and MDC1/NFBD1. The response to DNA damage is not limited to IR since Nek1 also localizes to DNA damage sites induced by alkylating agents, UV, crossing linking agents and oxidative injury. Nek1-deficient cells fail to activate the checkpoint kinases Chk1 and Chk2 and are defective in G_1/S and M-phase checkpoints in response to DNA damage. As a result, Nek1-deficient cells fail to repair damaged DNA after relatively low dose DNA damage, and that they ultimately develop chromatid breaks.[5] To date, therefore, we know that Nek1 is important for DNA damage responses and repair, and that deficiency of Nek1 leads to defects in some of the known mediators on DNA damage response signaling pathways. What we do not know yet is whether Nek1 fits upstream, downstream or parallel to the key mediator kinases ATM and ATR.

To determine where Nek1 fits into known DNA damage and repair pathways, we characterized the consequences of ATM and ATR inactivation on Nek1 functions, and vice versa. We report here that Nek1 activities are independent of ATM or ATR. We show that Nek1 expression, kinase activity and localization to DNA damage nuclei foci are intact in ATM or ATR deficient cells. Key ATM and ATR activities are also the same in Nek1-deficient cells as they are in wild-type cells. Nek1 is therefore a unique protein kinase in DNA damage signaling, one that does not directly depend on the activity of either ATM or ATR.

Results

Nek1 responses are intact when ATM or ATR is inactivated. To determine whether Nek1 is upstream, downstream or independent of ATM and ATR, we examined Nek1 responses in cells with ATM or ATR inactivated either chemically or genetically. Since ATM and ATR are PI3K-like molecules, their activities can be inhibited effectively by micromolar concentrations of Wortmannin or LY294002.[11,12] In normal cells, IR increased Nek1 activity~2.2-fold in phosphorylating an in vitro substrate, β-casein (**Fig. 1A and B**). Although DMSO vehicle increased basal Nek1 kinase activity, neither Wortmannin nor LY294002 inhibited the increase in Nek1 kinase activity after IR. Furthermore, the chemical inhibitors of ATM and ATR failed to inhibit the relocalization of Nek1 from cytoplasm to nuclear foci of DNA damage after IR, even at high doses toxic in general to the cells (**Fig. 1C**).

Next we examined Nek1 activities in HK2 cells, normal human fibroblasts, GM9607A fibroblasts from a patient with ataxia-telangiectasia,[13] or similar fibroblasts with ATR inactivated by doxycycline-regulated overexpression of a dominant negative mutant form (ATR-KD cells).[14] Since ATM activation is dependent on ATR,[15] we used ATM phosphorylation at S1981 to confirm the dominant negative effect of ATR-KD upon addition of doxycycline. GM9607A ATM cells have no wild-type ATM and no ATM-S1981P signal was detected in them (**Fig. 2A**). Before addition of doxycycline, IR-induced ATM-S1981P signal was readily detected in the ATR-KD cells (**Fig. 2A** and lane 6), suggesting the normal response to DNA double strand breaks. Consistent with the dominant activity of kinase-dead ATR, no ATM-S1981P was detected in response to IR in ATR-KD cells induced with doxycycline (**Fig. 2A** and lane 8). Next, we assessed the expression level of Nek1 in normal skin fibroblasts as well as in ATM- and ATR-deficient cells. The expression level of Nek1 was comparable in normal cells as well as in ATM- and ATR-deficient cells (**Fig. 2A**); interestingly, an elevation in Nek1 protein level was detected in ATM-deficient cells (**Fig. 2A**, lanes 3 and 4). We have previously shown that Nek1 protein kinase activity is upregulated after DNA damaging, ionizing radiation in HK2 cells.[6] Nek1 kinase activity likewise is upregulated after IR in wild-type, ATM and ATR-KD fibroblasts. In GM9607A fibroblasts and in ATR-KD cells (especially those with the dominant mutant construct turned on by doxycycline), baseline Nek1 kinase activity in untreated cells was already high, even without IR, but increased even further after IR (**Fig. 2B and C, and Sup. Fig. 1**). Localization of Nek1 to nuclear foci of DNA damage also remained intact in ATM- or ATR-deficient cells (**Fig. 2D**). Note that both ATM-deficient and ATR-KD cells with the dominant negative mutant turned on (+doxycycline) have baseline DNA damage foci even before exposure to IR, but that additional nuclear foci become evident after IR (**Sup. Fig. 2**). Taken together, the data indicate that Nek1's role in the DNA damage response is dependent on neither ATM nor ATR.

To strengthen our data, and to make sure that potential genomic alterations associated with a cell line from an individual with ataxia telangiectasia were not confounding our results, we also examined Nek1 in additional cells with ATM or ATR inactivated. First we investigated untransformed renal tubular epithelial cells (RTEs) from *Atm*$^{-/-}$ mice and from sex-matched, wild-type littermates. In all cells types, Nek1 expression, kinase activity, and localization to DNA damage nuclei foci were intact (**Fig. 3A–C**). *Atr*$^{-/-}$ mice do not survive early embryonic stages.[2] Currently available ATR mutant somatic cells are therefore derived either from patients with a form of Seckel syndrome, or have been genetically engineered from a cancer line. To help avoid the potential for irrelevant gene inactivation or activation associated with Seckel cells, we chose to use ATR-flox cells to examine the dependence of Nek1 activation on ATR. ATR-flox cells, derived from human colon tumor line HCT116, harbor one inactivated *ATR* allele and another allele in which exon 2 of *ATR* is flanked by two *loxp* cassettes, such that *ATR* can be deleted by cre-recombinase to generate *ATR* null cells.[3] When ATR was deleted in ATR-flox cells, Nek1 expression, kinase activity and localization to nuclear DNA damage sites, were still intact in cells treated with UV, whether the second ATR allele was expressed (Ad-GFP-treated cells) or inactivated (cells expressing GFP-Cre recombinase) (**Fig. 3D and E**). Treatment with recombinant adenoviruses and/or inactivation of ATR increased Nek1 kinase activity even before UV irradiation, but UV still resulted in additional increases in Nek1 kinase activity (**Fig. 3E**). Taken together, our results in two separate types of ATM and ATR-deficient cell lines are consistent. They again indicate that Nek1 activity is not directly dependent on ATM or ATR.

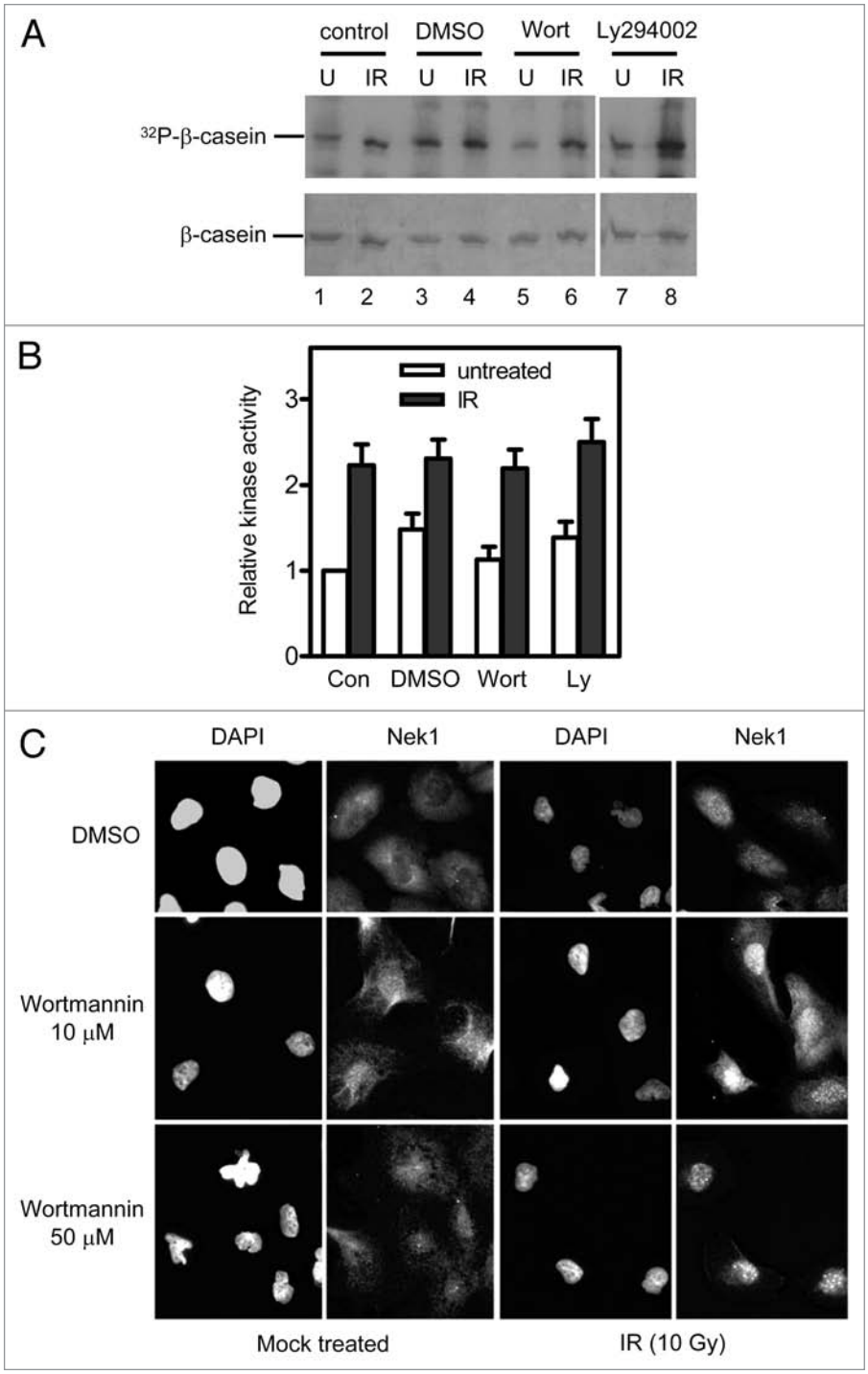

Figure 1. ATM/ATR (PI3 kinase) inhibitors do not affect Nek1 activities. (A) Nek1 kinase activity is upregulated 1 hour after DNA damage by IR (10 Gy). Activity was measured using γ-^{32}P-ATP, β-casein as an in vitro substrate, and Nek1 immunoprecipitated from HK2 cells. Kinase activity increased in response to IR in cells without any pretreatment (lanes 1–2) or after pre-treatment with DMSO vehicle alone (lanes 3–4). Chemical inhibitors of ATM, ATR, and other PI3 kinases (10 μM Wortmannin, lanes 5–6, or 10 μM Ly294002, lanes 7–8) did not prevent the upregulation of kinase activity in response to IR. (B) Histograms represent relative kinase activities compared to untreated basal state (densitometry on blots from 6–10 separate experiments, means ± SEM). Con, control cells without any pretreatment. Two-way ANOVA $p < 0.01$ when comparing IR and untreated cells for each condition. (C) DNA damage response of Nek1 is not regulated by PI3 kinase. Wortmannin at a dose sufficient to inhibit ATM and ATR activity did not prevent Nek1 from re-localizing to nuclear foci at sites of damaged DNA 1 h after IR (10 Gy). The higher dose of Wortmannin was toxic to the HK2 cells, but still did not prevent localization of Nek1 to nuclear foci after IR.

Early ATM-dependent signals are activated normally in Nek1-deficient cells. After demonstrating that Nek1 functions are intact despite inactivation of ATM or ATR, we next looked in Nek1 null cells at the functions and signals known to be dependent on ATM and/or ATR. ATM normally autophosphorylates on Ser 1987 (S1981 in human ATM), minutes after cells are exposed to DNA damaging radiation.[16,17] ATR is phosphorylated on Ser 428 after UV irradiation.[18] Using western blotting, we were able to demonstrate that genomic stress-induced activation of ATM by phosphorylation on S1987 (**Fig. 4A**), and of ATR by phosphorylation on S428 (**Fig. 4B**), are intact in wild-type and Nek1 null cells. In contrast to these very early events in the ATM- and ATR-dependent DNA damage signaling response, however, more downstream events, specifically the crucial phosphorylations of the cell cycle checkpoint proteins Chk1 and Chk2,[2,19-24] are impaired in Nek1 null cells (**Fig. 4A**, lower panels and ref. 5).

ATM and ATR rapidly phosphorylate and activate downstream mediators of a signaling cascade at specific nuclear sites or foci of damaged DNA.[25,26] Using antibodies to a generic L-phospo-Ser/Thr-Q peptide, which represents the consensus site for ATM- and ATR-dependent targets,[27-29] one can monitor ATM and ATR activity. Another substrate for ATM and ATR is the key mediator histone H2AX, which is activated to γ-H2AX by phosphorylation on Ser 139.[30-32] Using the antibodies recognizing ATM-phospho-S1987, phospho-S/T-Q substrate, and γ-H2AX, we examined the presence of normally phosphorylated ATM substrates by indirect immunostaining in IR-induced immunofluorescent foci (IRIF, **Fig. 4C**). Note that relatively high percentages of Nek1 null cells have baseline IRIF containing all three ATM substrates compared to otherwise identical wild-type cells, even in the absence of specific DNA damage by IR. These results indicate that Nek1 null cells have a more activated DNA damage response at baseline than wild-type cells.

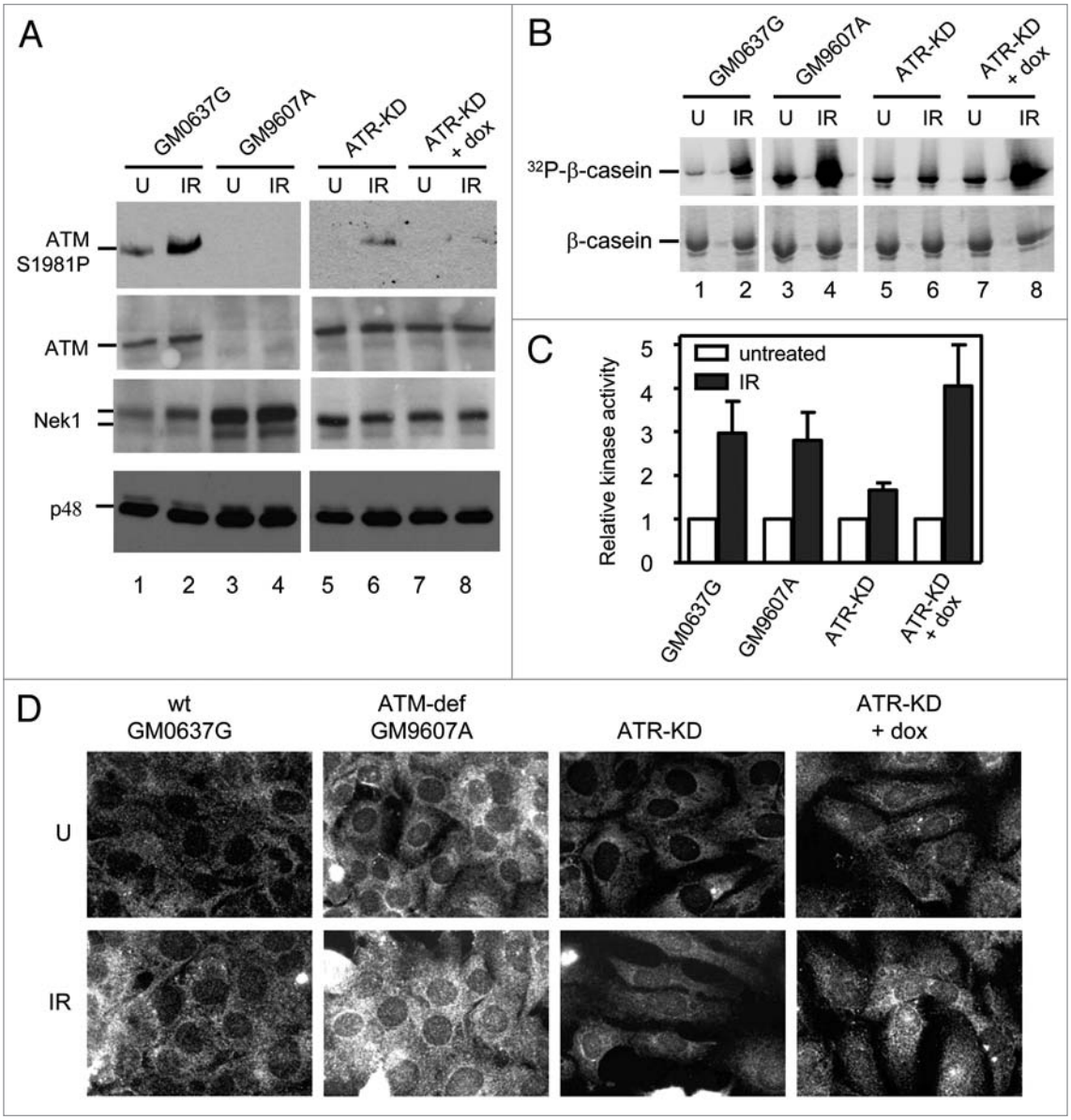

Figure 2. Nek1 expression, kinase activity and IR-induced localization of Nek1 to nuclear foci are intact in ATM and ATR mutant cells. (A) Nek1 protein is expressed in normal fibroblasts (GM0637G), fibroblasts with ATM (GM9607A) and fibroblasts without (ATR-KD) or with (ATR-KD + dox) doxycycline-regulated expression of an ATR-dominant negative mutant. In normal cells, Nek1 expression is upregulated 1 h after IR (10 Gy). Western blotting of the lower portion of the same blot with anti-p48 (retinoblastoma binding protein 4) antibodies was included to control for loading. (B) Nek1 kinase activity was measured as previously described, in the same cells as in (A). A representative blot is shown. (C) Densitometric quantification of band intensities from autoradiograph in (B) and from 5–10 other autoradiographs. In each case, ^{32}P-β-casein band intensity was normalized for loading by densitometric analysis of total β-casein in the same Coomassie blue stained gel used to measure ^{32}P-β-casein. Histograms represent relative kinase activities after IR compared to untreated basal state (means ± SEM). 2-way ANOVA $p < 0.05$ for each cell line. (D) Nek1 localizes to nuclear sites of DNA damage (IRIF) I h after IR (10 Gy) in wild-type fibroblasts as well as in ATM-deficient and doxycycline-inducible ATR-KD cells.

Nonetheless, IR results in additional and unimpaired localization of ATM-phospho-S1987, phospho-S/T-Q substrate and γ-H2AX to nuclear IRIF in almost all Nek1 null cells within ten minutes, just as it does in identically treated wild-type cells. Thus early, ATM-dependent phosphorylation events are intact in Nek1 null cells. ATR has H2AX and SQ substrates in common with ATM, and similar observations with immunoflourescent foci and nuclear sites of DNA damage were observed after UV-irradiation of wild-type and Nek1 null cells (data not shown).

Downregulation of Nek1 expression by RNA interference in normal cells results in phosphorylation of proximal ATM or ATR substrates. To make sure that our observations in murine cells were not consequences only of a specific, hypomorphic *Nek1* mutation in the kat2J mouse strain, we also examined ATM substrates in human HK2 renal tubular epithelial cells with Nek1 expression silenced by RNA interference (short inhibitory RNA). Silencing of Nek1 expression by an adenovirus-mediated shRNA was specific and efficient (**Fig. 5A**). Although infection of cells

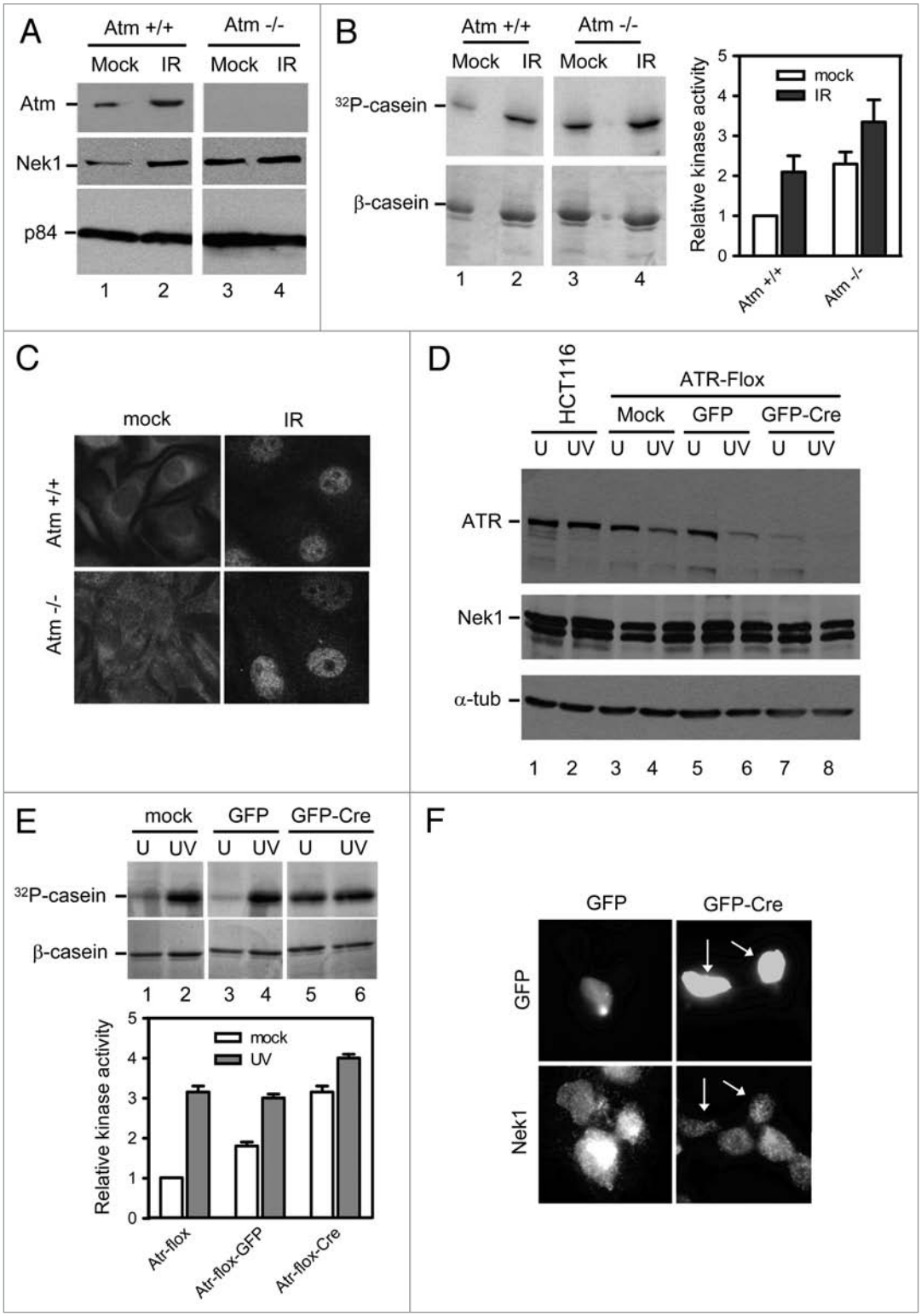

Figure 3. Nek1 expression, kinase activity and IR-induced localization of Nek1 to nuclear foci are intact in Atm null mouse renal tubular epithelial cells or ATR null human cells. (A–C) Nek1 activity in ATM null cells. *Atm*$^{+/+}$ and *Atm*$^{-/-}$ renal tubular epithelial cells were established from 6-week-old mice as described previously in references 5 and 6, and used for Nek1 activity analysis. (A) Nek1 expression in *Atm*$^{-/-}$ cells. 1 h after IR (10 Gy), cells were lysed and protein lysates were separated by SDS gel electrophoresis, transferred to membranes, and western blotted with anti-Atm (upper part), anti-Nek1 (middle part) and anti-p84 (lower part) antibodies, the latter to control for loading. (B), Nek1 kinase activity is upregulated after DNA damage by IR (10 Gy) in Atm null cells. Activity was measured using γ-^{32}P-ATP, β-casein as an in vitro substrate and Nek1 immune complexes from *Atm*$^{+/+}$ (lanes 1 and 2) or *Atm*$^{-/-}$ cells (lanes 3 and 4), as described previously in reference 6. (C) Nek1 localizes to IRIF in Atm null cells. Cells were fixed 1 hour after irradiation with IR (10 Gy). The fixed cells were immunostained with anti-Nek1 antibodies.

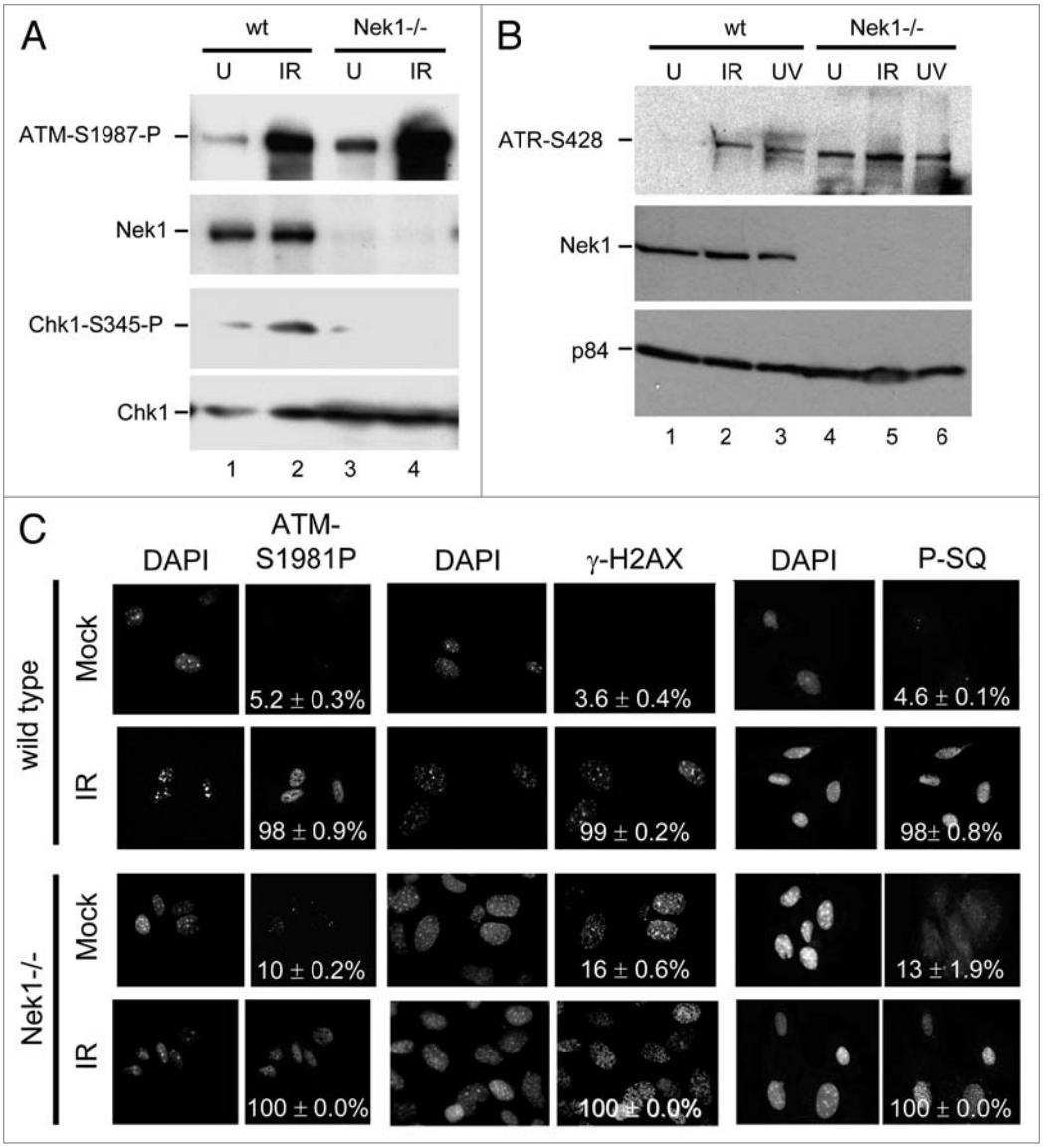

Figure 4. Phosphorylation of ATM-dependent substrates is intact in Nek1 null cells. (A and B) Activation of ATM and ATR are intact in *Nek1*$^{-/-}$ cells. Cells were treated with IR (10 Gy) or UV (10 J/m^2) irradiation. Total protein lysates were subjected to SDS-PAGE, and then immunblotted with anti-Atm (A) or Atr (B) antibodies. (C) Antibodies recognizing activated ATM-phospho-S1987, phospho-S/T-Q substrate and γ-H2AX were used for indirect immunofluorescence staining of wild-type and Nek1$^{-/-}$ renal tubular cells cultured from littermate kat2J mice. One hour after IR, activated Atm, phospho-S/T-Q Atm substrate and γ-H2AX all were present in nuclear foci at sites of DNA damage in wild-type cells and in Nek1$^{-/-}$ cells. Percentages (means ± SEM) were determined by scoring >400 individual cell nuclei for the presence of >5 IRIF, then dividing the number of positive cells by the total number of nuclei stained with DAPI.[44]

with recombinant adenoviral vectors non-specifically activated the DNA damage response to a minor degree, even in cells that were not further treated with IR,[33] IR caused further phosphorylation and activation of several early ATM substrates, including ATM itself on Ser1981, γ-H2AX and the consensus ATM/ATR sequence (L-S/T-Q) (**Fig. 5B**). These results after silencing Nek1 expression in human cells were entirely consistent to those we observed in murine Nek1$^{-/-}$ cells: upstream ATM- and ATR-dependent substrates were activated normally. Therefore, our observations about the role of Nek1 in ATM- and ATR-independent DNA damage responses are not unique only to the specific, Nek1/kat2J mutation. Rather, they are the direct consequences of Nek1 inactivation.

Discussion

We have previously shown that Nek1 is involved early in the DNA damage response.[6] It is required for efficient DNA damage checkpoint activation and for proper DNA damage repair. Without functional Nek1, cells fail to activate distal signals, including phosphorylation of the crucial ATM/ATR target kinases Chk1 and Chk2.[5] In this current report, we demonstrate, using different cell lines and several complementary methods, that inactivation of either ATM or ATR does not affect Nek1's expression, kinase activation or localization to nuclear DNA damage sites. With reciprocal experiments, we also show that early ATM and

ATR activities in phosphorylating several nuclear substrates and in recruiting them to sites of damaged DNA are also intact in Nek1-deficient cells, as well as in cells with Nek1 expression silenced by inhibitory RNA. We therefore conclude that Nek1 fits into a DNA damage signaling pathway that is not directly dependent on the activities of ATM or ATR. To our knowledge, the newly discovered function of Nek1 in DNA damage is thus unique, since all known DNA damage response/repair pathways have heretofore been thought to involve either ATM or ATR.

Aberrant or aborted DNA damage responses without significant DNA damage, a so-called pseudo-DNA damage responses have been reported. After treatment with agents that make cells senescent, but that don't actually cause antecedent DNA breaks before the responses are activated, γ-H2AX localizes to nuclear foci, but phospho-ATM-S1987 mislocalized after several days.[34] More distal DNA damage responses, however, like 53BP1 localization to nuclear foci, do not occur in such circumstances and the pseudo-DNA damage response is thought to be a delayed, secondary response of senescent cells to the reactive oxygen species and inflammatory cytokines activated in senescent cells without prior DNA damage. Similar, aborted, psudeo-DNA damage responses (i.e., activation of some DNA damage proteins) have been seen in response to other cellular stressors like heat or osmotic shock.[35-37] The aberrant, ATM- and ATR-independent DNA damage responses we documented above Nek1-deficient cells, however, are different from reported pseudo-DNA damage responses primarily because Nek1 deficiency does cause significant DNA damage, leading to chromosome breaks[5] and eventually to an unstable chromosome phenotype.[38] The DNA damage response in Nek1$^{-/-}$ cells also occurs very quickly, within minutes, and is not dependent on the cells becoming senescent. We cannot exclude the possibility that Nek1 is playing roles in both true DNA damage responses and in later, secondary, pseudo-DNA damage responses, but our data still show that the ATM- and ATR-independent effects of Nek1 appear to be unique and relevant to DNA damage in cancer and other neoplasic diseases.

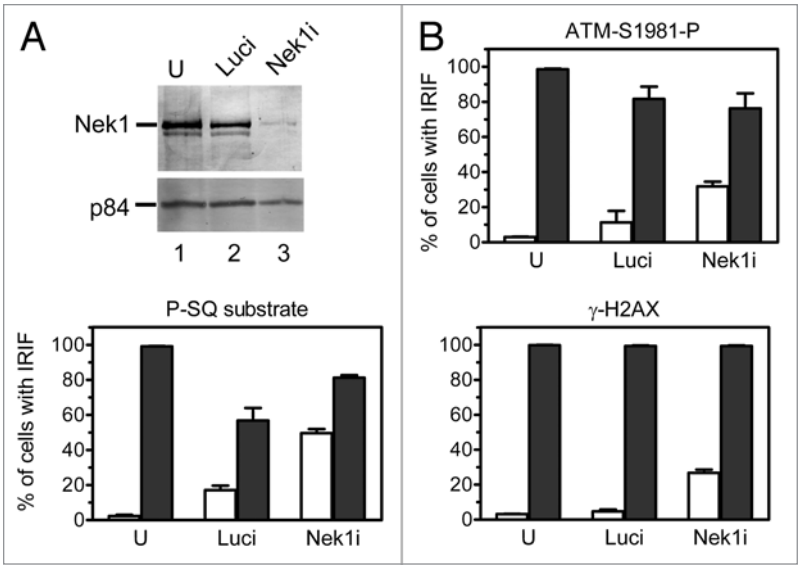

Figure 5. Early ATM substrates are activated in human cells with Nek1 expression silenced by shRNA. (A) Silencing of Nek1 expression by RNA interference in human RTEs. HK2 cells were either left untreated (U), infected with an adenovirus vector carrying shRNA specific for silencing the firefly luciferase gene (Luci) or transfected with the same backbone vector carrying shRNA specific for human Nek1 (Nek1i). 72 hours after infection, cells were lysed. Protein lysates were separated by SDS-PAGE, transferred to membranes, and western blotted with anti-Nek1 and anti-p84 antibodies, the latter to control for loading. (B) ATM's autophosphorylation on Ser 1981 (equivalent to Ser 1987 in mouse cells), phosphoryation of H2AX, and phosphorylation of generic S/T-Q substrate were all intact 24 h after pretreatment with Luci and Nek1. In each case, compare with untreated cells (U) and IR-treated cells (IR). More than 400 cells were scored in duplicate experiments for each condition. Means ± SEM are shown in the histograms.

After damage from such environmental insults and oxidative damage, the everyday equivalents of focused IR and UV irradiation used as examples in the work presented here, cells activate DNA damage responses through a series of feed-forward transducers to amplify signals and to repair the injury.[1] Nek1 seems to function in DNA damage responses in some ways like ATM and ATR. Nek1 may be a synergistic activator of key substrates. It appears to be an independent transducer of DNA damage signals to downstream responders. It may phosphorylate the downstream substrates, some of the same substrates targeted by ATM and ATR, in a hit-and-run manner, perhaps differentially depending on the stimulus and the phase of the cell cycle. Our unpublished results and those from other labs[39] indicate that Nek1 interacts with several proteins involved in DNA damage response signaling, some upstream of Chk1 and Chk2, and at least one involved in the effector arm of DNA damage repair. Detailed analysis the potential downstream targets will help to extend our understanding of the DNA damage signaling pathway and repair mechanism.

Interestingly, both IR and UV activate Nek1 and cause it to redistribute from cytoplasm to nuclear foci of damaged DNA, so Nek1 seems to be involved in responses to double-strand breaks as well as to damage of replication intermediates. Nek1 must not be as crucial as ATR in sensing and responding to damaged DNA in replication intermediates, however, since *Nek1*$^{-/-}$ cells and animals are viable through gestational stages. Replication forks must be generated during early embyrogenesis. Without ATR, rapidly dividing and differentiating embryonic cells can not survive. Without Nek1, in contrast, most cells can divide and propagate, but the fidelity of DNA repair is imperfect, such that after replication many cells die, while a smaller subset develop mutations that can lead to growth advantaged, clonal expansion. In this regard, Nek1 functions in DNA damaging signaling more akin to ATM, as a regulator of efficient and effective DNA damage repair, rather than as an absolute requirement for it. The phenotypes of ATM-deficient humans and mice are excessive cells death in certain cell types like neurons, and susceptibility to tissue-specific cancers like lymphomas.[1,40] We have speculated and continue to suggest that Nek1 has a significant role in responding to injury, which

requires efficient DNA repair prior to mitosis when injured or dead cells need to be regenerated.[5,41] Our hypothesis is based on the phenotype of Nek1-deficient kat2J mice (growth retardation, polycystic kidney disease, lymphomas in older heterozygotes) and the accelerated progression of some of these diseases after post-natal oxidative injury. Such a role for Nek1 may be part of what makes it distinct from the roles if ATM and ATR in DNA damage signaling and repair.

Although early ATM- and ATR-dependent signals are intact in Nek1-deficient cells, we have recently shown that more downstream DNA damage signals, including phosphorylation of the crucial ATM- and ATR-target kinases Chk1 and Chk2, are not properly activated in the absence of functional Nek1. The inability to activate these checkpoint kinases fully results in failure of Nek1-deficient cells to arrest at mitotic and G_1/S-phase checkpoints, which in turn leads to poorly repaired DNA and ultimately to chromosome breaks and aueuploidy.[5,38] Precisely how lack of Nek1 prevents ATM and ATR from activating the downstream kinases Chk1 and Chk2 is not clear at present. As noted above, Nek1 should be a regulatory kinase, like ATM, rather than essential protein in the cell division cycle. Unlike both ATM and ATR, Nek1 is a cytosolic protein kinase in normal, unstressed cells, and only a portion of it relocates to nuclei after DNA damage. Nek1 may regulate Chk1 and Chk2 activity directly (perhaps through a kinase-substrate interaction, since both Chk1 and Chk2 have consensus Nek1 phosphorylation sites) or indirectly.[5] It might be required for proper transport of newly synthesized Chk1 and Chk2 into nuclei, where they need to function. Experiments to explore this possibility and its details are currently being performed. The unique and apparently independent role of Nek1 in damage responses to DNA double strand breaks and to other DNA damage intermediates suggest that Nek1, and its interactions with substrates in the DNA damage signaling cascade, might make worthwhile targets for modification in diseases like polycystic disease and cancers, which are characterized by excessive cell death and unstable genomes.

Materials and Methods

Cell culture. Human HK2 human proximal renal tubular epithelial cells were obtained from American Type Tissue Collection (Rockville, MD) and cultured in 50/50 Ham's F-12/Delbecco's modified Eagle medium containing 10% fetal bovine serum and antibiotics. Primary fibroblasts and renal tubular epithelial cells were obtained from Nek1/kat2J mice or *Atm*$^{-/-}$ mice and their wild-type littermates as previously described in reference 6, and cultured in the same Ham's F-12/DMEM. SV40-large T antigen transformed, normal, human skin fibroblasts (GM0637G) and primary skin fibroblasts with ATM mutation (GM9607A) were obtained from the Coriell Institute (Camden, NJ). Human skin fibroblasts with doxycycline-regulated expression of an ATR-dominant negative mutant (ATR-KD) were obtained from E.Y-H.P. Lee (University of California, Irvine). All of the human fibroblast lines were cultured in DMEM. Human colorectal carcinoma epithelial cells, HCT 116 and their derivative, ATR-flox cells, were obtained from American Type Tissue Collection (Rockville, MD). Both of these latter cells lines were cultured in McCoy's 5A medium.

Ionizing and ultraviolet radiation. Cells were γ-irradiated using Cesium 40 at the rate of 116 cGy/min. For UV irradiation, cells were first washed with PBS twice and then placed inside a UV crosslinker (Stratagene, LaJolla, CA). The dose of UV irradiation was monitored with a UV meter.

Chemicals. The PIKK inhibitors, Wortmannin and Ly294002, were purchased from Calbiochem/EMD Chemicals (Gibbstown, NJ).

Antibodies. Rabbit polyclonal anti-Nek1 antibodies have already been described and well characterized.[5] Anti-p84 mAb 5E10,[42] anti-p48, anti-ATR mAb 2B5 and anti-ATM mAb 2C1 were purchased from GeneTex Inc., (Irvine, CA). Anti-ATM-phospho-S1981 (S1987 in mouse) antibodies were obtained from Rockland Immunochemicals (Gilbertsville, PA) (for rabbit IgG) and from Cell Signaling Technology (Danvers, MA) (for mouse IgG). Anti-ATR-phospo-S428, anti-γ-H2AX and anti-phospho-L-S/T-Q were obtained from Cell Signaling Technology. DM1A monoclonal anti-α-tubulin antibodies were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

Transfection. Transfection was accomplished using lipofectin (Invitrogen Corporation, Carlsbad, CA) and 3 μg of plasmid DNA to 1 x 10^4 cells, according to the manufacture's instructions. 12 h after transfection, the cultures were refed with fresh medium. 36 hours after transfection, the cells were treated with UV (10 J/m^2), fixed with 4% paraformaldehye 1 hour post UV irradiation and immunostainined with Nek1 antibodies.

Kinase assays. 1 x 10^6 cells were lysed and immunoprecipitated with anti-Nek1 antibodies, as described previously in reference 6. After extensive washing with lysis buffer, the immune complexes were washed twice with TBS (10 mM NaCl, 10 mM Tris-HC1, pH 7.5), twice with ddH_2O, and finally twice with kinase buffer (100 mM Tris-HCl, pH 7.5, 5 mM $MnCl_2$, 2 mM DTT, 1 μM ATP). Immune complexes were stored in 100 μl of kinase buffer at -80°C. The kinase reaction was carried out in a total volume of 30 μl, with 20 μl of immune complexes and 5 μl of β-casein in the presence of 3 μCi of γ-^{32}P-ATP. After incubation for 30 min at 37°C, EDTA were added to a final concentration of 2 mM to stop the kinase reactions and then equal volumes of SDS sample buffer were added.

Scoring for immunofluorescent nuclear foci at sites of DNA damage. A cell was scored positive for ionizing radiation immunofluorescent foci (IRIF) or UV-induced foci (UVIF) if at least five discrete, fluorescing dots were evident per nucleus. Percentages of nuclei positive for IRIF or UVIF were calculated by dividing number of nuclei with ≥5 IRIF by the total number of nuclei stained with DAPI in the same microscopic fields. The procedure has previously been validated and described in detail in reference 43 and 44.

Adenoviruses. Recombinant, GFP-tagged adenoviruses and RNA interference adenoviruses were generated using an AdEasy Vector System (Stratagene, LaJolla, CA) as previously described in reference 5 and 41. GFP-Cre recombinase adenoviruses were purchased from Vector Biolabs (Philadelphia, PA). All the

adenovirus infections were accomplished using multiplicities of infection of 30 particles per cell (MOI = 30).

Acknowledgements

This work was initiated at University of Texas Health Science at San Antonio, Texas and completed at University of California, Irvine. We thank Rosaria Polci for preliminary studies or reagents, Sergio Garcia for technical support and a Veterans Administration Renal Research Excellence Award to the South Texas Veterans Health Care System, Audie L. Murphy Division, for help with core services, equipment and institutional infrastructure. This work was supported by grants from the PKD foundation, American Society of Nephrology, the National Kidney Foundation to Y.C., and National Institute of Health to Y.C. (RO1-DK067339, P50-DK61597) and D.J.R. (RO1-DK61626).

Note

Supplemental materials can be found at:
www.landesbioscience.com/journals/cc/article/14814

References

1. Shiloh Y. The ATM-mediated DNA-damage response: Taking shape. Trends Biochem Sci 2006; 31:402-10.
2. Brown EJ, Baltimore D. ATR disruption leads to chromosomal fragmentation and early embryonic lethality. Genes Dev 2000; 14:397-402.
3. Cortez D, Guntuku S, Qin J, Elledge SJ. ATR and ATRIP: Partners in checkpoint signaling. Science 2001; 294:1713-6.
4. Hurley PJ, Bunz F. ATM and ATR: Components of an integrated circuit. Cell Cycle 2007; 6:414-7.
5. Chen Y, Chen PL, Chen CF, Jiang X, Riley DJ. Never-in-mitosis related kinase 1 functions in DNA damage response and checkpoint control. Cell Cycle 2008; 7:3194-201.
6. Polci R, Peng A, Chen PL, Riley DJ, Chen Y. NIMA-related protein kinase 1 is involved early in the ionizing radiation-induced DNA damage response. Cancer Res 2004; 64:8800-3.
7. Letwin K, Mizzen L, Motro B, Ben-David Y, Bernstein A, Pawson T. A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells. EMBO J 1992; 11:3521-31.
8. Arama E, Yanai A, Kilfin G, Bernstein A, Motro B. Murine NIMA-related kinases are expressed in patterns suggesting distinct functions in gametogenesis and a role in the nervous system. Oncogene 1998; 16:1813-23.
9. Upadhya P, Birkenmeier EH, Birkenmeier CS, Barker JE. Mutations in a NIMA-related kinase gene, Nek1, cause pleiotropic effects including a progressive polycystic kidney disease in mice. Proc Natl Acad Sci USA 2000; 97:217-21.
10. Vogler C, Homan S, Pung A, Thorpe C, Barker J, Birkenmeier EH, et al. Clinical and pathologic findings in two new allelic murine models of polycystic kidney disease. J Am Soc Nephrol 1999; 10:2534-9.
11. Helt CE, Cliby WA, Keng PC, Bambara RA, O'Reilly MA. Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related protein exhibit selective target specificities in response to different forms of DNA damage. J Biol Chem 2005; 280:1186-92.
12. Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE, Abraham RT. Inhibition of phosphoinositide-3-kinase related kinases by the radiosensitizing agent wortmannin. Cancer Res 1998; 58:4375-82.
13. Li S, Ting NS, Zheng L, Chen PL, Ziv Y, Shiloh Y, et al. Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. Nature 2000; 406:210-5.
14. Cliby WA, Roberts CJ, Cimprich KA, Stringer CM, Lamb JR, Schreiber SL, et al. Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. EMBO J 1998; 17:159-69.
15. Stiff T, Walker SA, Cerosaletti K, Goodarzi AA, Petermann E, Concannon P, et al. ATR-dependent phosphorylation and activation of ATM in response to UV treatment or replication fork stalling. EMBO J 2006; 25:5775-82.
16. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003; 421:499-506.
17. Pellegrini M, Celeste A, Difilippantonio S, Guo R, Wang W, Feigenbaum L, et al. Autophosphorylation at serine 1987 is dispensable for murine Atm activation in vivo. Nature 2006; 443:222-5.
18. Stokes MP, Rush J, Macneill J, Ren JM, Sprott K, Nardone J, et al. Profiling of UV-induced ATM/ATR signaling pathways. Proc Natl Acad Sci USA 2007; 104:19855-60.
19. Chaturvedi P, Eng WK, Zhu Y, Mattern MR, Mishra R, Hurle MR, et al. Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. Oncogene 1999; 18:4047-54.
20. Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, et al. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev 2000; 14:1448-59.
21. Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science 1998; 282:1893-7.
22. Matsuoka S, Rotman G, Ogawa A, Shiloh Y, Tamai K, Elledge SJ. Ataxia telangiectasia-mutated phosphorylates Chk2 in vivo and in vitro. Proc Natl Acad Sci USA 2000; 97:10389-94.
23. Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, et al. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science 1997; 277:1497-501.
24. Zhao H, Piwnica-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol 2001; 21:4129-39.
25. Kastan MB, Lim DS. The many substrates and functions of ATM. Nat Rev Mol Cell Biol 2000; 1:179-86.
26. Kumagai A, Dunphy WG. How cells activate ATR. Cell Cycle 2006; 5:1265-8.
27. DiTullio RA Jr, Mochan TA, Venere M, Bartkova J, Sehested M, Bartek J, et al. 53BP1 functions in an ATM-dependent checkpoint pathway that is constitutively activated in human cancer. Nat Cell Biol 2002; 4:998-1002.
28. Lou Z, Chini CC, Minter-Dykhouse K, Chen J. Mediator of DNA damage checkpoint protein 1 regulates BRCA1 localization and phosphorylation in DNA damage checkpoint control. J Biol Chem 2003; 278:13599-602.
29. Schwartz MF, Duong JK, Sun Z, Morrow JS, Pradhan D, Stern DF. Rad9 phosphorylation sites couple Rad53 to the *Saccharomyces cerevisiae* DNA damage checkpoint. Mol Cell 2002; 9:1055-65.
30. Burma S, Chen BP, Murphy M, Kurimasa A, Chen DJ. ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J Biol Chem 2001; 276:42462-7.
31. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem 1998; 273:5858-68.
32. Rogakou EP, Sekeri-Pataryas KE. Histone variants of H2A and H3 families are regulated during in vitro aging in the same manner as during differentiation. Exp Gerontol 1999; 34:741-54.
33. Sinclair A, Yarranton S, Schelcher C. DNA-damage response pathways triggered by viral replication. Expert Rev Mol Med 2006; 8:1-11.
34. Pospelova TV, Demidenko ZN, Bukreeva EI, Pospelov VA, Gudkov AV, Blagosklonny MV. Pseudo-DNA damage response in senescent cells. Cell Cycle 2009; 8:4112-8.
35. Dmitrieva NI, Bulavin DV, Burg MB. High NaCl causes Mre11 to leave the nucleus, disrupting DNA damage signaling and repair. Am J Physiol Renal Physiol 2003; 285:266-74.
36. Dmitrieva NI, Cai Q, Burg MB. Cells adapted to high NaCl have many DNA breaks and impaired DNA repair both in cell culture and in vivo. Proc Natl Acad Sci USA 2004; 101:2317-22.
37. Hunt CR, Pandita RK, Laszlo A, Higashikubo R, Agarwal M, Kitamura T, et al. Hyperthermia activates a subset of ataxia-telangiectasia mutated effectors independent of DNA strand breaks and heat shock protein 70 status. Cancer Res 2007; 67:3010-7.
38. Chen Y, Chen C, Chiang H, Pena M, Polci R, Wei R, et al. Mutation of NIMA-related kinase 1 (NEK1) leads to chromosome instability. Molecular Cancer 2011; 10:5.
39. Surpili MJ, Delben TM, Kobarg J. Identification of proteins that interact with the central coiled-coil region of the human protein kinase NEK1. Biochemistry 2003; 42:15369-76.
40. Barlow C, Hirotsune S, Paylor R, Liyanage M, Eckhaus M, Collins F, et al. Atm-deficient mice: a paradigm of ataxia telangiectasia. Cell 1996; 86:159-71.
41. Chen Y, Craigen WJ, Riley DJ. Nek1 regulates cell death and mitochondrial membrane permeability through phosphorylation of VDAC1. Cell Cycle 2009; 8:257-67.
42. Durfee T, Mancini MA, Jones D, Elledge SJ, Lee WH. The amino-terminal region of the retinoblastoma gene product binds a novel nuclear matrix protein that co-localizes to centers for RNA processing. J Cell Biol 1994; 127:609-22.
43. Peng A, Chen PL. NFBD1/Mdc1 mediates ATR-dependent DNA damage response. Cancer Res 2005; 65:1158-63.
44. Peng A, Chen PL. NFBD1, like 53BP1, is an early and redundant transducer mediating Chk2 phosphorylation in response to DNA damage. J Biol Chem 2003; 278:8873-6.