Choi et el.

# Supplemental Information

# NEK8 Links the ATR-regulated Replication Stress Response and S-phase CDK Activity to Renal Ciliopathies

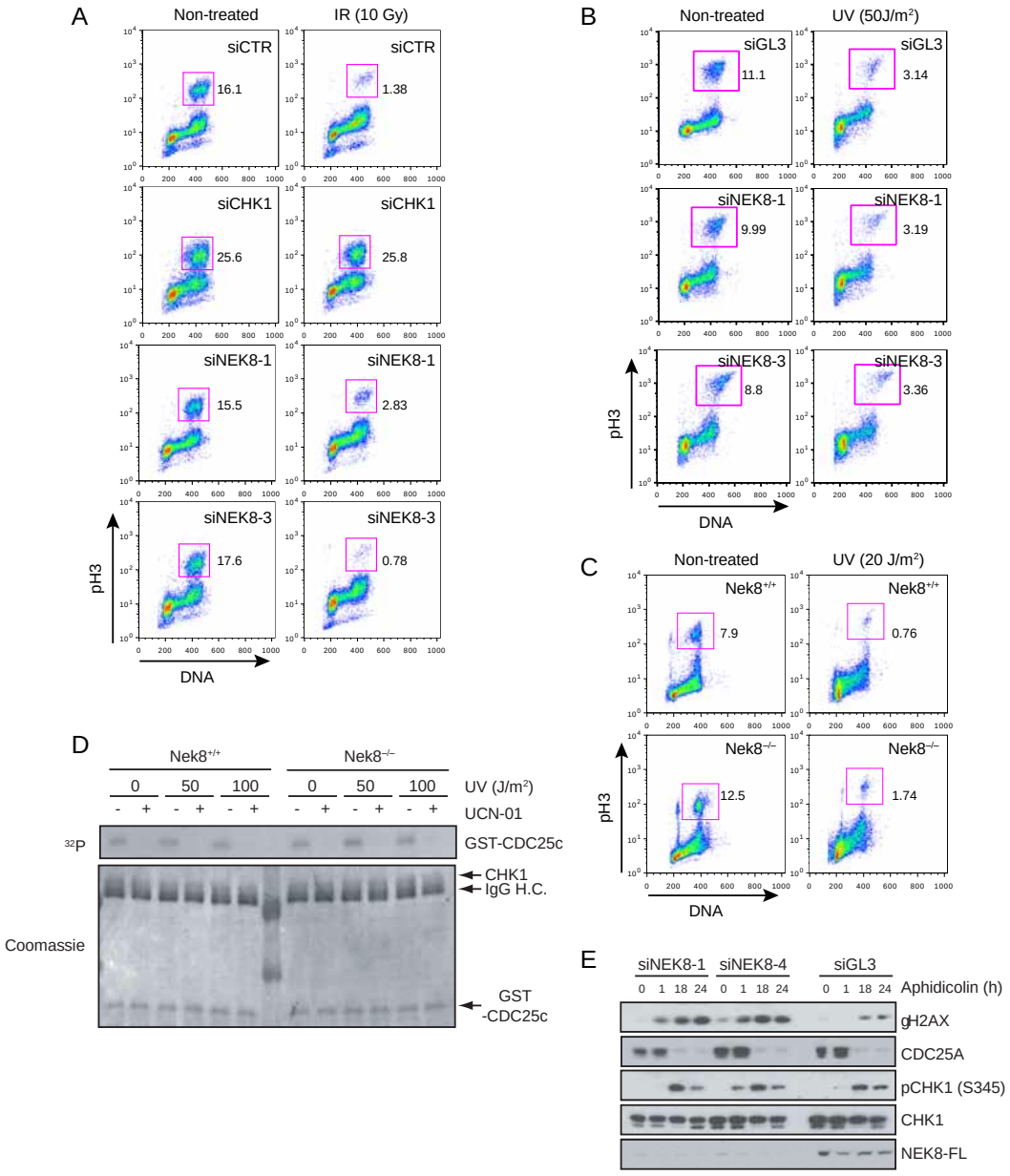
Hyo Jei Claudia Choi[1], Jia-Ren Lin[1], Jean-Baptiste Vannier[2], Gisela G. Slaats[3], Andrew C. Kile[1], Renee D. Paulsen[1], Danielle K. Manning[4], David R. Beier[4], Rachel H. Giles[3], Simon J. Boulton[2], and Karlene A. Cimprich[1§]

[1]Stanford University School of Medicine, Department of Chemical and Systems Biology,
Stanford, CA 94025
[2]London Research Institute, Clare Hall Laboratories,
Blanche Lane, South Mimms, EN6 3LD, UK.
[3]Department of Nephrology and Hypertension, University Medical Center Utrecht,
Heidelberglaan 100, 3584CX Utrecht, the Netherlands
[4]Brigham and Women's Hospital, Division of Genetics, Boston MA, 02115

[§]To whom all correspondence is directed

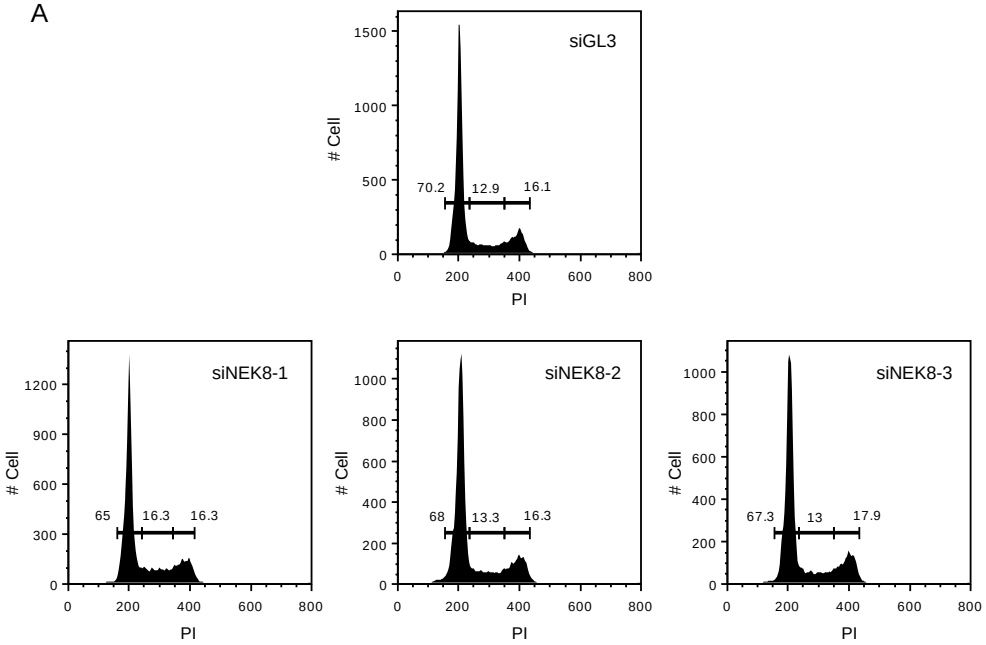
**Contents**

Choi_Supplemental Figure 1.

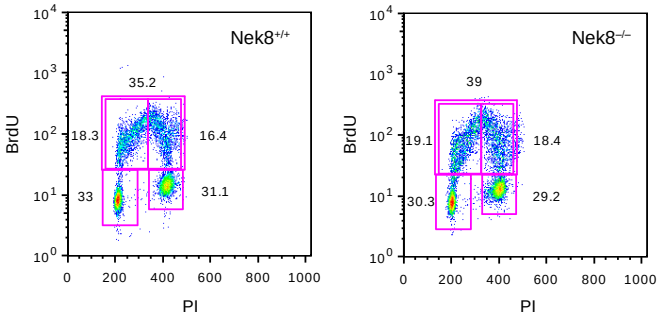


Choi_Supplemental Figure 2.

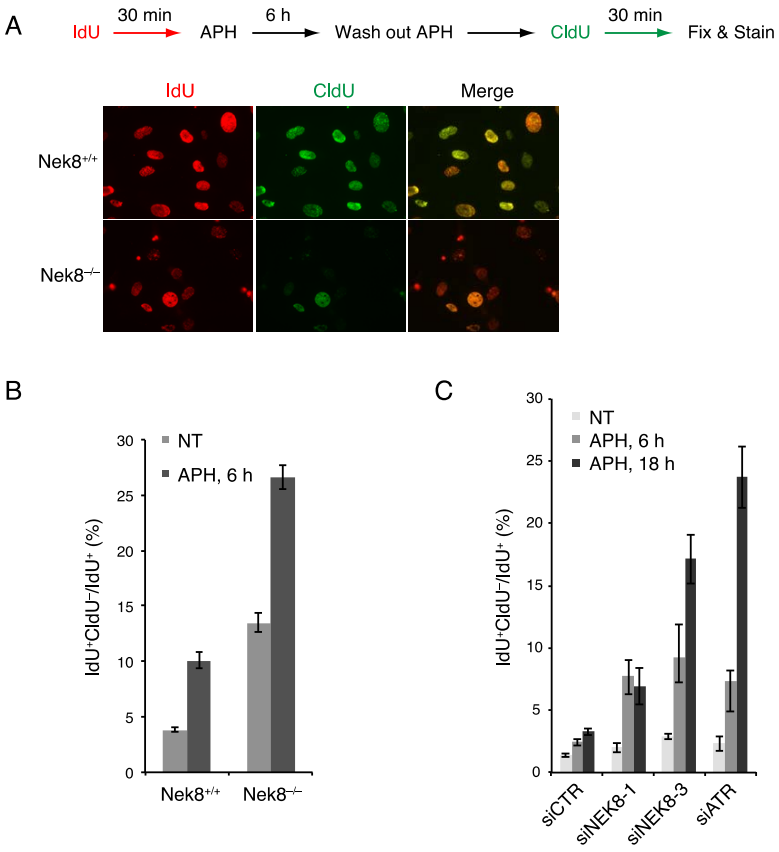
A



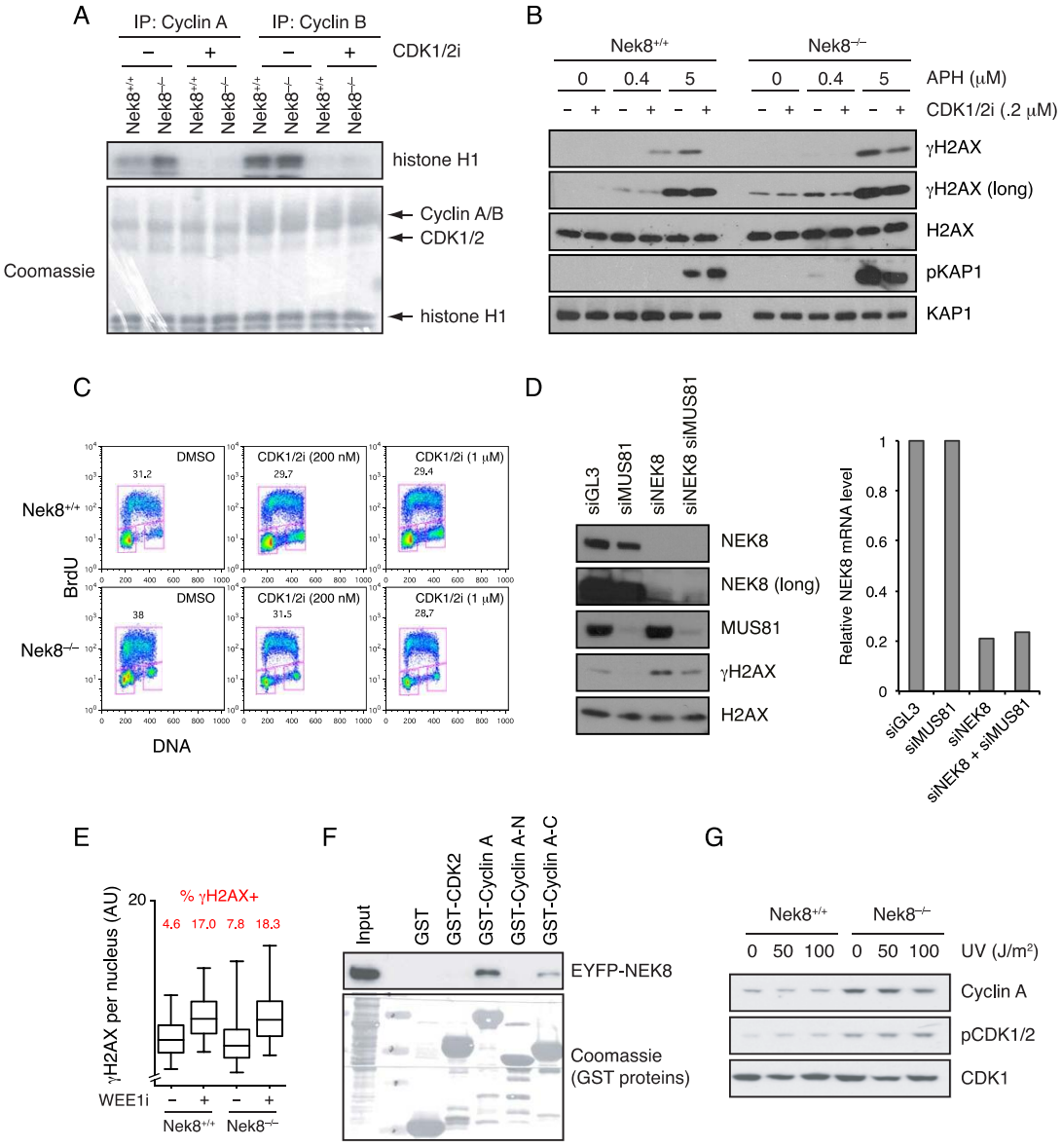

B



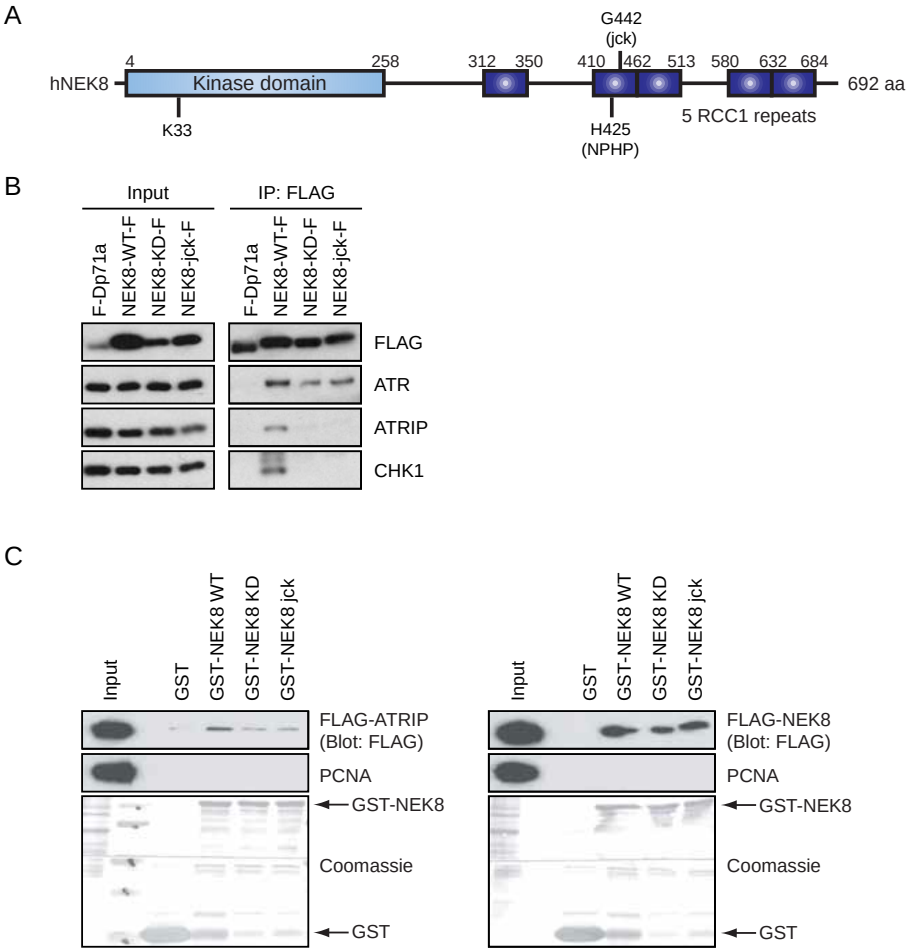
Choi_Supplemental Figure 3.



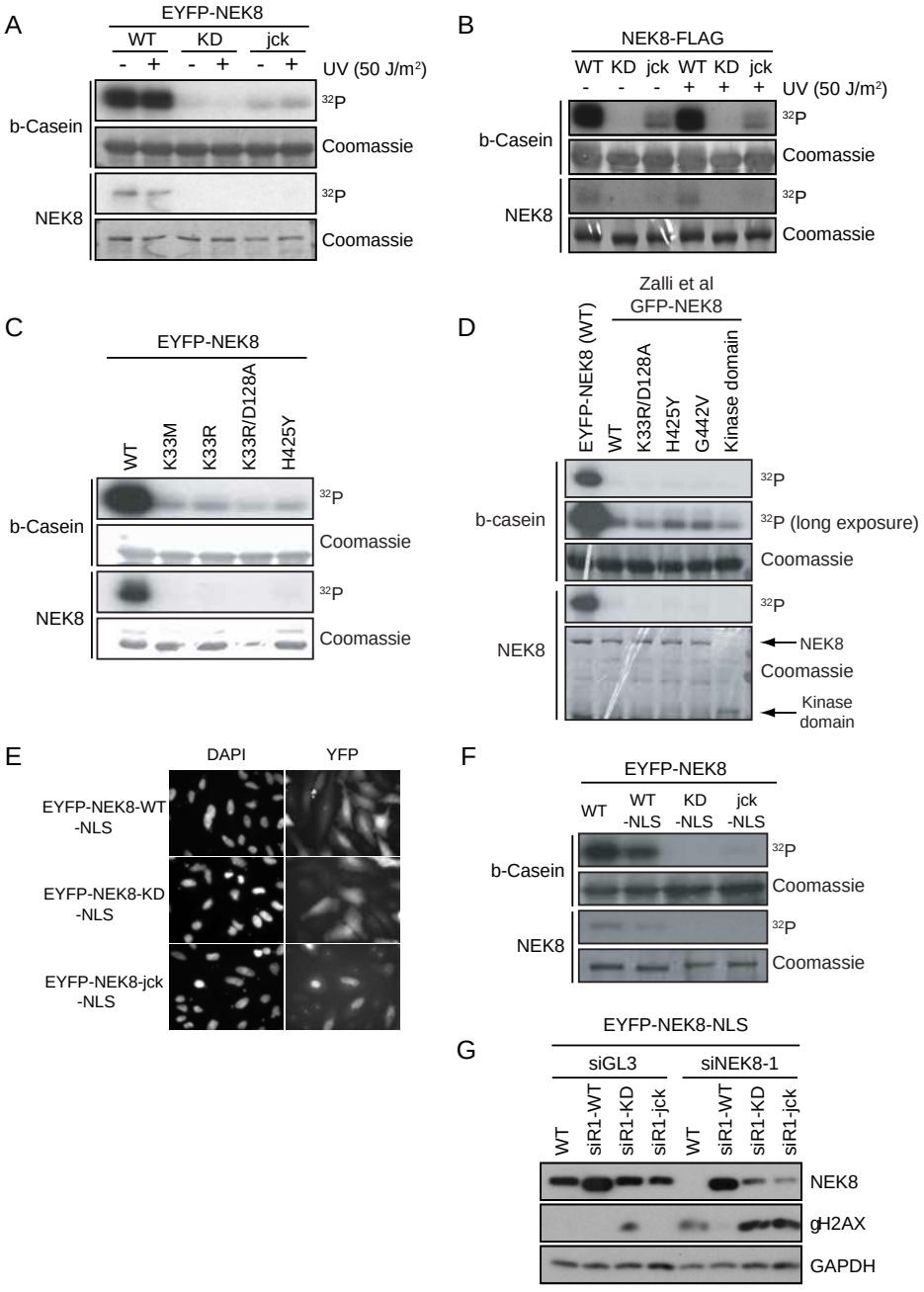
Choi_Supplemental Figure 4.



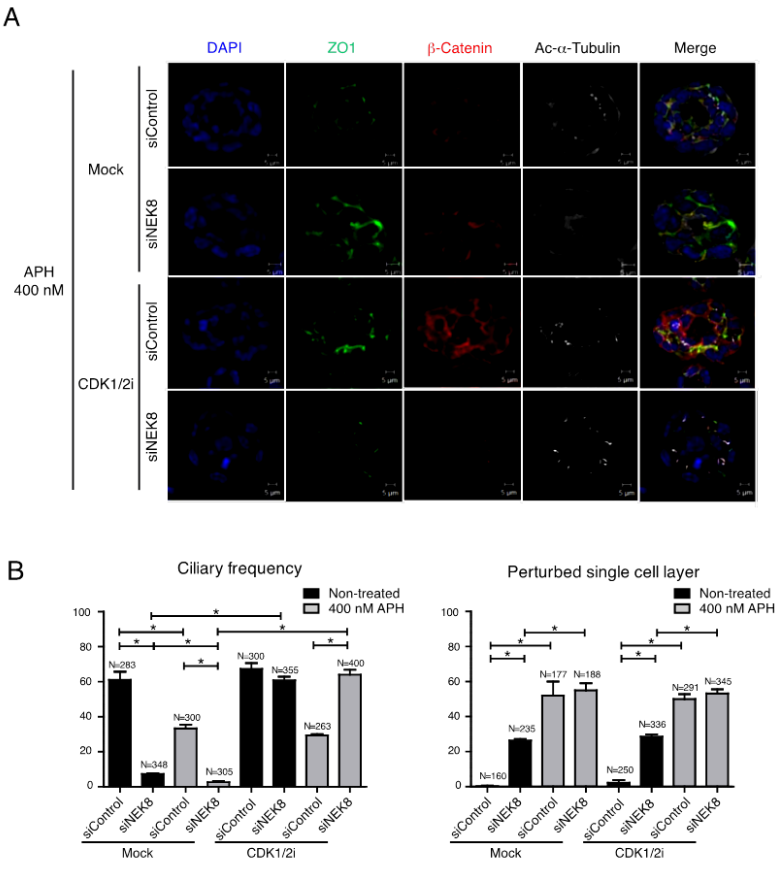
Choi_Supplemental Figure 5.



Choi_Supplemental Figure 6.



Choi_Supplemental Figure 7.



## SUPPLEMENTAL FIGURE LEGENDS

**Figure S1.** NEK8 is not required for cell cycle arrest after DNA damage. **(A-B)** HeLa cells were transfected with siRNAs targeting GL3, CHK1, or NEK8 for 48 h. Cells were untreated or irradiated with 10 Gy IR (A) or 50 J/m$^2$ UV (B), then allowed to recover with nocodazole for 8 h (A) or 6 h (B) to capture mitotic cells. **(C)** Immortalized wild-type and *Nek8*$^{-/-}$ MEFs were untreated or irradiated with 20 J/m$^2$ UV and left for 6 h in the presence of nocodazole. In A-C, mitotic cells were identified by staining for pH3 and analyzed by flow cytometry. The percentages of mitotic (pH3-positive) cells are shown. Knockdown of CHK1 was used as a positive control for loss of G2/M checkpoint in (A). **(D)** Immortalized wild-type and *Nek8*$^{-/-}$ MEFs were treated with the indicated doses of UV and allowed to recover for 1 h. Cells were lysed and endogenous CHK1 was immunoprecipitated using CHK1 antibody. Kinase reactions were performed with GST-fused CDC25c expressed and purified from bacteria in the presence of $\gamma^{32}$P-ATP for 30 min at 30 °C. Substrate and kinase loading was confirmed by Coomassie staining, and $^{32}$P-labeled products were detected by autoradiography. The CHK1 inhibitor UCN-01 was used to test for kinase specificity. **(E)** HEK293T cells stably expressing FLAG-tagged wild-type NEK8 (NEK8-FL) were transfected with siGL3 or siNEK8 for 72 h. The cells were left untreated or treated with 400 nM aphidicolin and collected at the times shown for western blotting with the indicated antibodies.

**Figure S2.** The effect of NEK8 depletion on cell cycle progression. **(A)** HeLa cells were transfected with siGL3 or siNEK8. 72 h after knockdown, cells were fixed and stained with propidium iodide (PI). **(B)** Asynchronously growing immortalized wild-type and *Nek8*$^{-/-}$ MEFs were pulsed with BrdU for 15 min, harvested, fixed, and stained with BrdU and PI. In both cases, cell cycle profiles were determined by flow cytometry.

**Figure S3.** NEK8 is essential for the recovery and restart of stalled replication forks. **(A)** Top, schematic of replication fork recovery assay to monitor the ability of cells to restart DNA replication after release from a replication block (Itakura et al., 2005). Immortalized wild-type or *Nek8*$^{-/-}$ MEFs were labeled with IdU for 30 min and treated with aphidicolin for 6 h. After the aphidicolin block was washed out, the cells were labeled with CldU for another 30 min prior to fixation. Fixed cells were stained with antibodies recognizing IdU (red) or CldU (green), and DAPI. Bottom, representative images of IdU, CldU staining, and the overlap of IdU and CldU in MEFs. **(B-C)** Loss of NEK8 leads to a profound defect in their ability to restart replication upon release from aphidicolin. The percentage of IdU$^{+}$ cells that failed to recover after replication block (IdU$^{+}$ CldU$^{-}$ /IdU$^{+}$) was scored in wild-type and *Nek8*$^{-/-}$ MEFs (B) and HeLa cells (C). HeLa cells were transfected with GL3, NEK8, or ATR siRNAs, labeled with IdU and CldU, and processed as described in A. The percentage of cells that failed to recover after replicative damage was quantified as in B. ATR was knocked down as a positive control. All error bars show SEM (n = 3).

**Figure S4.** NEK8 regulates cyclin A-CDK activity. **(A)** NEK8 does not regulate mitotic CDK activity. Both cyclin A and cyclin B-bound CDKs were immunoprecipitated from wild-type and *Nek8*$^{-/-}$ MEFs and processed for *in vitro* kinase reactions using histone H1. The CDK1/2 inhibitor (CDK1/2i) was used as a negative control. Cyclin B-associated CDK activity is unaffected in *Nek8*$^{-/-}$ MEFs. **(B)** Abrogation of γH2AX by CDK1/2 inhibition in *Nek8*$^{-/-}$ MEFs. MEFs were treated with APH (400 nM or 5 μM) and CDK1/2i (200 nM) for 18 h and processed for western blotting with the indicated antibodies.
**(C)** CDK1/2 inhibition does not affect cell cycle progression in MEFs. Asynchronous wild-type and *Nek8*$^{-/-}$ MEFs were treated with CDK1/2i (200 nM or 1 μM) for 18 h, pulsed with BrdU for 15 min, harvested, fixed, and stained with BrdU and PI. Cell cycle profiles were determined by flow cytometry. **(D)** MUS81 knockdown rescues γH2AX induced by NEK8 knockdown. HeLa cells stably expressing NEK8-FLAG were transfected with indicated siRNA for 72 h, then

γH2AX levels were analyzed by western blotting (left panel) and mRNA levels of NEK8 were measured by RT-qPCR to confirm the knockdown of endogenous NEK8 (right panel). **(E)** γH2AX levels were analyzed by immunofluorescence in MEFs treated with or without WEE1 inhibitor (WEE1i, 10 μM) for 18 h. γH2AX intensity is plotted in box and whisker format and analyzed as in Figure 4F. Numbers in red indicate the percentage of γH2AX-positive cells. AU, arbitrary units. **(F)** NEK8 interacts with the C-terminal region of cyclin A. The GST-fused CDK2, cyclin A, N-terminal cyclin A (cyclin A-N) and C-terminal cyclin A (cyclin A-C) proteins were expressed, purified from bacteria, and incubated with extracts from cells expressing EYFP-NEK8. The GST-fused and the associated proteins were isolated by glutathione-agarose beads and analyzed by western blotting. **(G)** CDK1/2 is still phosphorylated in *Nek8*$^{-/-}$ MEFs. Wild-type and *Nek8*$^{-/-}$ MEFs were treated with UV as indicated, and after 1 h they were processed for western blotting with the indicated antibodies.

**Figure S5.** Analysis of interactions between NEK8 and ATR pathway components. **(A)** Domain architecture of human NEK8. NEK8 consists of a kinase domain in the N-terminus and 5 RCC1 repeats in the C-terminus. The amino acid residues mutated in this study are shown. **(B)** Co-immunoprecipitation experiments using HEK293T cells stably expressing FLAG-tagged wild-type (NEK8-WT-FL), kinase-deficient (NEK8-KD-FL), *jck* (NEK8-jck-FL) NEK8, or DP71a (FL-DP71a) were performed and analyzed as described in Figure 5A and Figure 6E with the noted exceptions. Cells were lysed with high salt buffer containing 600 mM NaCl, then immunoprecipitations were carried out at 150 mM NaCl after dilution of the initial lysate. **(C)** Interaction of recombinant NEK8 with ATRIP. Bacterially expressed GST or GST-fused NEK8 (WT, KD, *jck*) bound to glutathione sepharose beads was incubated with lysates from HEK293T cells expressing FLAG-ATRIP (left panel) or FLAG-NEK8 (right panel) for 2 h. The association of FLAG-ATRIP or FLAG-NEK8 from cell lysates with glutathione-beads was monitored by immunoblotting with FLAG antibody. GST-proteins bound to glutathione beads were assessed by Coomassie staining.

**Figure S6.** Regulation of NEK8 kinase activity and its localization. **(A-D)** *In vitro* kinase assays were performed with the indicated NEK8 wild-type and mutant proteins as described in Figure 6A. EYFP (A) or FLAG-tagged (B) wild-type (WT), kinase deficient (KD), or *jck* NEK8 were expressed in HEK293T cells for 48 h. The cells were untreated or irradiated with UV and allowed to recovery for 1 h prior to lysis. (C) HEK293T cells were transfected with EYFP-tagged wild-type (WT), kinase deficient NEK8 (K33M, K33R, or K33R/D128A), or NPHP patient NEK8 mutant (H425Y) for an *in vitro* kinase assay. (D) The kinase activities of EYFP-tagged wild-type (WT) NEK8 used throughout this study and GFP-tagged NEK8 constructs from Zalli et al (Zalli et al., 2012) were compared as indicated. The discrepancy in our data with Zalli et al might be due to the change in the first amino acid of their NEK8 construct as well as the difference in the linker between NEK8 and the epitope tags. **(E)** Localization of NLS-fused wild-type and mutant NEK8. U2OS cells stably expressing EYFP-tagged wild-type (YFP-NEK8-WT-NLS), kinase deficient (YFP-NEK8-KD-NLS), or *jck* (YFP-NEK8-*jck*-NLS) NEK8 were subjected to immunofluorescence. **(F)** NLS-fused NEK8 variants were expressed in HEK293T cells and immunoprecipitated using GFP antibody. Kinase activity was measured as described in Figure 6A. **(G)** U2OS cells stably expressing NLS-fused wild-type (WT), siNEK8-1-resistant wild-type (siR1-WT), kinase-deficient (siR1-KD), and *jck* (siR1-*jck*) EYFP-NEK8 were transfected with siGL3 or siNEK8-1 and processed for western blotting with the indicated antibodies.

**Figure S7.** NEK8 knockdown or replication stress leads to loss of cilia and a perturbed single cell layer. **(A)** Immunostaining of spheroids for cilia (acetylated tubulin, white), tight junctions (ZO1, green) and adherens junctions (β-catenin, red) with DAPI counterstaining (blue) shows loss of cilia and perturbed single cell layer in cells treated with siNEK8, APH (400 nM) and/or CDK1/2i 1 μM for 18 h. **(B)** Quantification of ciliary frequency (* $p < 0.006$) and perturbed single layer events (* $p < 0.003$) in spheroids show significant differences between siControl

and siNEK8-treated spheroids with an additive effect of APH (400 nM, 18 h) and a rescue of ciliary frequency upon CDK1/2i (1 μM, 18 h). Number of events scored is indicated in bar graphs (N). Error bars represent SEM (n=4).

## EXTENDED EXPERIMENTAL PROCEDURES

### Cell Culture and Plasmids

To generate stable cell lines, HeLa, U2OS, and HEK293T cells were transfected with the indicated plasmids using Fugene6 (Roche) and selected with either Geneticin (for EYFP-NEK8; Invitrogen) or puromycin (for FLAG-Dp71a, NEK8-FLAG; Sigma). Cells with stably integrated FLAG-tagged plasmids were maintained in standard medium containing 0.5 μg/mL puromycin. U2OS cells carrying EYFP-tagged constructs were expanded from a single colony and maintained with 1 mg/mL Geneticin. Mouse embryonic fibroblasts (MEFs) were derived from E14.5 embryo as previously described (Manning et al., 2013). Full-length human NEK8 (1-692) was amplified from NEK8 cDNA purchased from Open Biosystems (clone ID 8327708) and subcloned into mammalian expression vectors, pEYFP-C1, pEYFP-Nuc (Clontech), pcDNA3.1 (Invitrogen), pBMN (Addgene) or the bacterial expression vector, pGEX4T-1 (GE Healthcare). To introduce point mutations (K33M, K33R, D128A, G442V, H425Y) and siRNA-resistant silent mutations in NEK8, site-directed mutagenesis was performed using the Quikchange site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions. The cyclin A2, CDK1, CDK2 containing plasmids were kindly provided by Dr. James E. Ferrell Jr., and then PCR amplified and subcloned into pcDNA3-FLAG vector. The FLAG-tagged WEE1 constructs was a gift from Dr. Raimundo Freire (Dominguez-Kelly et al., 2011). All constructs were verified by DNA sequencing.

### Antibodies and Reagents

Antibodies to γH2AX and phospho S345-CHK1 (Cell signaling); phospho S824-KAP1 (Bethyl Laboratory); phospho S10-H3 (Upstate); CHK1, CDC25A, cyclin A, cyclin B, CDK1, CDK2, WEE1 and PCNA (Santa Cruz Biotechnologies); NEK8, M2 FLAG and γ-tubulin (Sigma); BrdU and IdU (BD Bioscience); CldU (BU1/75), H3, H2AX, and GAPDH (Abcam); and RAD18 (Novus) are all commercially available. Antibodies to ATRIP and mouse NEK8 have been previously published

(Bomgarden et al., 2006; Liu et al., 2002). The non-targeting control siRNA (siControl; D-001210-01, D001810-10) and the synthetic siRNAs targeting NEK8 (D-004866-01/siNEK8-1, D-004866-02/si-NEK8-3, D-004866-03/si-NEK8-4, D-004866-04/si-NEK8-2, L-044403-00/ON-TARGETplus SMARTpools), ATR (M-003202-05-0005), ATRIP (M-180657-00-0005), CHK1 (D-003255-04-0005) MUS81 (D-016143-17-0005, J-016143-09, J-016143-10), and luciferase (GL3; D-001400-01-20) were purchased from ThermoFisher. siRNA-resistant NEK8 constructs were generated against D-004866-01. siGL3 and siControl were used as negative controls throughout the study. All cell transfections were performed using 20-25 nM siRNA with Dharmafect 1 (ThermoFisher) or RNAimax (Invitrogen) following the manufacturer's protocols. The ATR inhibitor (Sulfone 45) was synthesized by the Medicinal Chemistry shared resource at Ohio State University (Charrier et al., 2011). The CHK1 inhibitor (CHIR-124, S-2683) was purchased from Selleckchem (Tse et al., 2007). Inhibitors of CDK1/2 (217714) and WEE1 (681641) were purchased from Millipore (Dominguez-Kelly et al., 2011; Gavet and Pines, 2010).

**Flow Cytometry and Cell Cycle Analysis**

To monitor S-phase progression, cells were pulse-labeled with 10 mM BrdU for 30 min, 48 h after siRNA knockdown or cDNA expression, and washed three times with PBS. Aphidicolin (400 nM) and/or nocodazole (50 ng/mL) were added at the indicated time points. After fixing samples with ice-cold 70 % ethanol, cells were permeabilized with 0.25 % Triton X-100/PBS for 15 min on ice, blocked in 2 % BSA/PBS for 15 min, and incubated in primary BrdU antibody (BD Bioscience) for 2 h. Cells were then washed three times in PBS, incubated in AlexaFluoro-488 secondary antibody for 1 h, and washed three times with PBS. Propidium iodide (PI; 0.1 μg/mL; Sigma) and RNase A (10 μg/mL; QIAGEN) was added to determine DNA content and cells were analyzed on a FACS Caliber (BD Bioscience) and FlowJo software. Ratios of early S-phase cells (left box) to total S-phase cells (left plus right box) observed in the presence vs absence of

aphidicolin for a given siNEK8 were normalized to the same number for siGL3 sample.

**RT-qPCR**

HeLa cells were transfected with siGL3 or siNEK8 for 72 h and harvested to isolate total RNA using TRIzol reagent (Invitrogen). A total of 1.5 μg of RNA was used for reverse transcription with random hexamer primers and SuperScript III Reverse Transcriptase Kit (Invitrogen). An equal amount of cDNA was mixed with iTaq SYBR Green Supermix (Bio-Rad). The PCR reactions were run on 7900HT Fast Real-Time PCR System (Applied Biosystems) using the conditions that follow: initial step of 3 min at 95 °C, then 40 cycles of 15 sec at 95 °C and 60 sec at 60 °C. Changes in mRNA expression were measured by setting up the comparative threshold cycle with β-actin.

**Neutral Comet Assay**

Comet assays were performed with the Single Cell Gel Electrophoresis Assay-kit (Trevigen) according to the manufacturer's protocols. DNA was stained with SYBR-gold (Invitrogen) and comet tail moments were calculated by counting 50 to 100 cells for each sample and analyzed with Comet Assay Software Program (CASP Lab). Box-and-whiskers graphs were plotted with Prism v6.0 (GraphPad Software). Top and bottom of the boxes correspond to 25 - 75 %, respectively. The line near the middle of the box marks the median ($50^{th}$ percentile) and whiskers correspond to 10 - 90 %. Statistical analysis was performed with Prism v6.0 using the non-parametric Mann-Whitney rank sum t-test.

**Clonogenic Cell Survival Assay**

Immortalized MEFs were seeded onto 6 cm plates at a density of $10^3$ cells. Cells were treated with aphidicolin at the concentrations indicated and/or CDK1/2 inhibitor (200 nM) for 18 – 24 h, and then released into fresh media. After 9-12 days, cells were fixed in 1 % formaldehyde in PBS, stained with 0.5 % crystal

violet (Sigma) for 10 min, and rinsed with tap water. Resulting colonies were manually counted. Each point was performed as three experimental replicates.

**DNA Combing**

DNA combing was performed and processed as previously described (Bianco et al., 2012; Michalet et al., 1997). Briefly, early passages of primary MEFs were pulsed-labeled with IdU (Sigma) for 30 min, quickly washed with PBS and pulse-labeled with CldU (Sigma) for 30 min. Genomic DNA fibers were extracted in agarose plugs and stretched on silanized coverslips with the molecular combing system (Genomic Vision). DNA fibers were visualized using specific primary antibodies specific for IdU and CldU (BD Bioscience and AbCys, respectively) and stained with AlexaFluoro-488 and AlexaFluoro-594-conjugated secondary antibodies (Invitrogen). The DNA fibers were captured with Zeiss Axio Imager M1 microscope equipped with an ORCA-ER camera (Hamamatsu) controlled by Volocity 4.3.2 software (Improvision). Representative images of DNA fibers were assembled from different fields of view. Box-and-whiskers graphs were plotted with Prism v5.0. Top and bottom of the boxes correspond to 25 - 75 %, respectively. The line near the middle of the box marks the median and whiskers correspond to 10 - 90 %. Statistical analysis was performed with Prism v5.0 using the non-parametric Mann-Whitney rank sum t-test.

**Replication Fork Recovery and Restart Assay**

HeLa cells were transfected with the indicated siRNAs for 48 h and labeled with IdU for 30 min. After washing cells three times with PBS, cells were untreated or treated with 5 μM aphidicolin for 6 or 18 h. Aphidicolin was thoroughly washed out with PBS and DMEM and cells were labeled with CldU for 30 min. CldU and IdU-labeled cells were fixed with ice-cold 70 % ethanol, denatured with 2 N HCl for 30 min at room temperature (RT), and neutralized with 100 mM sodium borate pH 8.5. The samples were blocked and subjected to immunofluorescence using primary BrdU antibodies, specific for IdU and CldU (BD Bioscience and Abcam, respectively) followed by incubation with AlexaFluoro-594 and

AlexaFluoro-488-conjugated secondary antibodies (Invitrogen). For pH3 recovery assay, siRNA-transfected HeLa cells were labeled with EdU (Invitrogen) for 30 min 48 h post-transfection and treated with 5 μM aphidicolin for 6 h. Aphidicolin block was washed out with PBS and DMEM, and cells were treated with nocodazole (50 ng/mL; Sigma) for 12 h and were fixed with 4 % paraformaldehyde (PFA) in PBS for 15 min at RT. Samples were processed for immunofluorescence using Click-it EdU AlexaFluor-488 Image Kit (Invitrogen) according to the manufacturer's protocols. A cell was considered positive if its IdU/CldU intensity was greater than a threshold set three times the mean intensity observed in control cells.

### *In Vitro* Kinase Assay

HEK293T cells or MEF cells were lysed in high-salt buffer (300 mM NaCl, 50 mM Hepes, pH 7.5, 0.8 % Triton X-100, 8 % Glycerol, 1 mM EDTA, 1 mM DTT, protease inhibitors and phosphatase inhibitors). Cell lysates were clarified by centrifugation and were immunoprecipitated with M2 agarose beads (Sigma) or CHK1 (Santa Cruz)/cyclinA (Santa Cruz)/cyclin B (Santa Cruz)/GFP (Abcam) antibody-bound protein G sepharose beads (Sigma) for 2 h at 4 °C. Precipitates were washed three times with high-salt buffer and once with kinase buffer (20 mM Hepes pH 7.5, 5 mM $MnCl_2$, 5 mM $MgCl_2$, 5 mM beta-glycerophosphate, 1 mM NaF, 1 mM DTT, 1 mM $Na_3VO_4$). Kinase reactions were carried out by incubating immunoprecipitated NEK8 with 50 μl of kinase buffer with 10 μM ATP, 5 μM $\gamma^{32}$P-ATP and 5 μg of substrates (β-casein, histone H1 or GST-CDC25C fragment) for 30 min at 30 °C, and stopped by adding 20 μl of 4 X SDS sample buffer. The proteins were then analyzed by SDS-PAGE and autoradiography.

### Co-immunoprecipitation and GST Pull-down Assays

Cells were lysed in buffer A (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 % NP-40, 1 mM DTT, 1 mM PMSF, protease and phosphatase inhibitors) or buffer B (600 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 % NP-40, 1 mM DTT, 1 mM PMSF, protease inhibitors and phosphatase inhibitors)

and clarified by high speed centrifugation. The supernatants were collected and incubated with M2 beads (Sigma), anti-ATRIP/protein G, or rabbit IgG/protein G (Sigma) for 2 h at 4 °C. The protein content of the supernatants was determined using the BCA protein determination kit (Pierce). An equal amount of total protein was added to the beads for each sample. The beads were washed three times with buffer A and precipitates were eluted from the beads either with 3X FLAG peptide (50 μg/mL; Sigma) or SDS sample buffer. For GST-pull down assays, GST alone or GST-fused protein-conjugated glutathione-sepharose beads (Sigma) were incubated with the indicated FLAG or EYFP proteins in buffer A for 2 h at 4 °C and washed three times with buffer A. The immunoprecipitates were resuspended in SDS sample buffer and analyzed by western blotting using anti-FLAG antibodies. The amount of GST proteins was examined by Coomassie blue staining after western blotting.

**G2/M Checkpoint Assay**

HeLa cells transfected with the indicated siRNAs for 48 h or immortalized MEFs were irradiated with 10 Gy of IR or 50 J/$m^2$ UV and allowed to recover for 1 h. Then, 50 ng/ml of nocodazole was added. IR and UV-irradiated cells were harvested 8 h and 6 h later, respectively, fixed with ice-cold 70 % ethanol and processed for flow cytometry. The mitotic index and the DNA content were determined with pH3 (S10) antibody and PI staining, respectively.

**Chromatin Fractionation**

Chromatin fractionations were processed as previously described (Smits et al., 2006). Briefly, approximately $10^7$ cells were washed with ice-cold PBS, resuspended in 200 ml of buffer C (50 mM Hepes [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M sucrose, 10 % glycerol, 1 mM DTT, protease and phosphatase inhibitors) containing 0.1 % Triton X-100, and incubated on ice for 5 min. After centrifugation at 1,300 g for 4 min at 4 °C, the supernatant (cytoplasmic fraction, S1) was collected and pellets were lysed in 200 ml of buffer D (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, protease and phosphatase inhibitors) for 30 min on ice.

The supernatant (soluble nuclear fraction, S2) was separated by centrifugation at 1,700 g for 4 min at 4 °C and the pellets (chromatin fraction, P2) were further washed with buffer D and spun down at 10,000 g for 1 min. Fractions were resuspended in SDS sample buffer and analyzed by western blotting. Fractions S1 and S2 were pooled to one soluble fraction (S1 + S2). Fraction P2 was sheared by sonication. Protein concentrations were determined with the Lowry protein assay and equal loading of fractions was verified by immunoblotting against GAPDH and Orc2 for soluble and chromatin fractions, respectively.

**Isolation of Proteins on Nascent DNA (iPOND)**

The iPOND procedure was performed as reported (Sirbu et al., 2011) with the following modifications. HEK293T cells stably expressing FLAG-tagged wild-type NEK8 (NEK8-FL) were pulsed with 10 μM EdU for 10 min, chased into 10 μM thymidine-containing media, and collected as previously described. Click reactions were performed using 10 μM Azide-PEG4-Biotin Conjugate (AZ104P4, Click Chemistry Tools). Cell lysis and wash steps were carried out with RIPA buffer (100 mM Tris, pH 7.5, 150 mM NaCl, 1 % NP-40, 0.1 % SDS, 0.5 % sodium deoxycholate). Cells were lysed on ice with RIPA buffer at a concentration of 0.75 X $10^7$ cells/mL and sonicated 6 times for 10 sec at 40 % amplitude using a Branson Digital Sonifier 450 with 2 min of rest between pulses. Input samples were collected (0.1 % total lysate) from cleared lysates and resuspended in 2X laemmli sample buffer. High Performance Streptavidin Sepharose (GE Healthcare) was used to capture biotin-tagged DNA complexes overnight at 4 °C with rotation. After washing with RIPA buffer, samples were resuspended in 2X laemmli sample buffer and analyzed by SDS-PAGE and western blotting. No click reaction (no clk) is the input control processed with no biotin-azide. PCNA, RAP70, ATRIP, and CHK1 were immunoblotted as positive controls. Tubulin was used as a negative control and the constant levels of histone H3 was used to confirm chromatin maturation.

**γH2AX Immunofluorescence of Cells and Kidney Sections**

All experiments were performed and analyzed as previously described (Paulsen et al., 2009). Briefly, the percent γH2AX positive population (% γH2AX positive) was calculated by applying an intensity cutoff approximately three times greater than the average γH2AX intensity of all siControl or siGL3-treated wells. Mouse lines for wild-type and homozygous *jck* mice have been previously described (Liu et al., 2002; Manning et al., 2013; Trapp et al., 2008). Kidneys from three-week-old mice were fixed in 4 % PFA for 30 min, transferred to 30 % sucrose overnight at 4 °C, and embedded in paraffin, and 10-μm sections were cut and mounted on slides. Sections were dewaxed, rehydrated, and fixed with 90 % methanol. The sections were blocked in 2% BSA/TBS for 1 h at RT and stained with rabbit antibody to γH2AX (1:200, Cell Signaling) overnight at 4 °C. The sections were then stained with DAPI and goat anti-rabbit AlexaFluoro-488-conjugated secondary antibody (1:1000, Invitrogen) for 1 h at RT and mounted in Prolong Gold (Invitrogen). Confocal imaging was performed using Zeiss LSM500 confocal microscope and processed with the ZEN 2009 software. Four sections per kidney were scanned and four to six random fields were analyzed per section. P-values were calculated using a two-tailed Student's t-test.

**IMCD3 Spheroid Growth Assay and Immunofluorescence**

10,000 IMCD3 cells were seeded per well of a 48 well and transfected with 20 nM of ON-TARGET*plus* siRNA SMARTpools (ThermoFisher) for non-targeting pool (D-001810-10) or mouse NEK8 (L-044403-00) using Lipofectamine RNAimax (Invitrogen, 13778-075) 6 h after plating, according to the manufacturer's protocol. Cells were trypsinized 24 h post-transfection and resuspended cells were then mixed 1:1 with growth factor-depleted matrigel (BD Bioscience) in Labtech chamber slides. After polymerization for 20 min at 37 °C, warm medium was dripped over the matrix until just covered. The IMCD3 cells formed spheroids with cleared lumens 3 days later. During the last 18 or 6 h, spheroids were cultured with medium containing 400 nM or 5 μM aphidicolin, respectively, with or without 1 μM CDK1/2i for 18 h. Medium was removed by pipetting and the gels were washed three times for 10 min with warm PBS

supplemented with calcium and magnesium. The gels were then fixed in fresh 4 % PFA for 30 min at RT. After washing three times in PBS after fixation, the cells were permeabilized for 15 min in gelatin dissolved in warm PBS (350 mg/50 mL) with 0.5 % Triton X-100. Primary antibody (mouse anti-acetylated tubulin, Sigma, 1:20,000), rat anti-ZO1 (1:400, Santa Cruz) and rabbit anti-β-catenin (1:400, BD Bioscience) was diluted in permeabilization buffer and incubated overnight at 4 °C. After washing the spheroids three times for 30 min in permeabilization buffer, goat anti-mouse-Cy5, donkey anti-rabbit-TRITC, and goat anti-rat-FITC secondary antibodies (1:400, Invitrogen) were each diluted in permeabilization buffer and incubated with the spheroids for 4 h at RT. Spheroids were washed three times in permeabilization buffer for 10 min per wash and then incubated for 1 h with DAPI, before being washed an additional three times in PBS, and finally mounted in Fluoromount-G (Cell Lab, Beckman Coulter) overnight at RT. Images and z-stacks were taken with a Zeiss LSM700 confocal microscope and approximately 50 spheroids per condition were scored. GraphPad Prism 5.0 was used to perform two-tailed Student's t-tests.

## SUPPLEMENTAL REFERENCES


Bianco, J.N., Poli, J., Saksouk, J., Bacal, J., Silva, M.J., Yoshida, K., Lin, Y.L., Tourriere, H., Lengronne, A., and Pasero, P. (2012). Analysis of DNA replication profiles in budding yeast and mammalian cells using DNA combing. Methods *57*, 149-157.

Bomgarden, R.D., Lupardus, P.J., Soni, D.V., Yee, M.C., Ford, J.M., and Cimprich, K.A. (2006). Opposing effects of the UV lesion repair protein XPA and UV bypass polymerase eta on ATR checkpoint signaling. Embo J *25*, 2605-2614.

Charrier, J.D., Durrant, S.J., Golec, J.M., Kay, D.P., Knegtel, R.M., MacCormick, S., Mortimore, M., O'Donnell, M.E., Pinder, J.L., Reaper, P.M.*, et al.* (2011). Discovery of potent and selective inhibitors of ataxia telangiectasia mutated and Rad3 related (ATR) protein kinase as potential anticancer agents. J Med Chem *54*, 2320-2330.

Dominguez-Kelly, R., Martin, Y., Koundrioukoff, S., Tanenbaum, M.E., Smits, V.A., Medema, R.H., Debatisse, M., and Freire, R. (2011). Wee1 controls genomic stability during replication by regulating the Mus81-Eme1 endonuclease. J Cell Biol *194*, 567-579.

Gavet, O., and Pines, J. (2010). Progressive activation of CyclinB1-Cdk1 coordinates entry to mitosis. Dev Cell *18*, 533-543.

Itakura, E., Sawada, I., and Matsuura, A. (2005). Dimerization of the ATRIP protein through the coiled-coil motif and its implication to the maintenance of stalled replication forks. Mol Biol Cell *16*, 5551-5562.

Liu, S., Lu, W., Obara, T., Kuida, S., Lehoczky, J., Dewar, K., Drummond, I.A., and Beier, D.R. (2002). A defect in a novel Nek-family kinase causes cystic kidney disease in the mouse and in zebrafish. Development *129*, 5839-5846.

Manning, D.K., Sergeev, M., van Heesbeen, R.G., Wong, M.D., Oh, J.H., Liu, Y., Henkelman, R.M., Drummond, I., Shah, J.V., and Beier, D.R. (2013). Loss of the ciliary kinase Nek8 causes left-right asymmetry defects. J Am Soc Nephrol *24*, 100-112.

Michalet, X., Ekong, R., Fougerousse, F., Rousseaux, S., Schurra, C., Hornigold, N., van Slegtenhorst, M., Wolfe, J., Povey, S., Beckmann, J.S.*, et al.* (1997). Dynamic molecular combing: stretching the whole human genome for high-resolution studies. Science *277*, 1518-1523.

Paulsen, R.D., Soni, D.V., Wollman, R., Hahn, A.T., Yee, M.C., Guan, A., Hesley, J.A., Miller, S.C., Cromwell, E.F., Solow-Cordero, D.E.*, et al.* (2009). A genome-wide siRNA screen reveals diverse cellular processes and pathways that mediate genome stability. Mol Cell *35*, 228-239.

Sirbu, B.M., Couch, F.B., Feigerle, J.T., Bhaskara, S., Hiebert, S.W., and Cortez, D. (2011). Analysis of protein dynamics at active, stalled, and collapsed replication forks. Genes Dev *25*, 1320-1327.

Smits, V.A., Reaper, P.M., and Jackson, S.P. (2006). Rapid PIKK-dependent release of Chk1 from chromatin promotes the DNA-damage checkpoint response. Curr Biol *16*, 150-159.

Trapp, M.L., Galtseva, A., Manning, D.K., Beier, D.R., Rosenblum, N.D., and Quarmby, L.M. (2008). Defects in ciliary localization of Nek8 is associated with cystogenesis. Pediatr Nephrol *23*, 377-387.

Tse, A.N., Rendahl, K.G., Sheikh, T., Cheema, H., Aardalen, K., Embry, M., Ma, S., Moler, E.J., Ni, Z.J., Lopes de Menezes, D.E.*, et al.* (2007). CHIR-124, a novel potent inhibitor of Chk1, potentiates the cytotoxicity of topoisomerase I poisons in vitro and in vivo. Clin Cancer Res *13*, 591-602.

Zalli, D., Bayliss, R., and Fry, A.M. (2012). The Nek8 protein kinase, mutated in the human cystic kidney disease nephronophthisis, is both activated and degraded during ciliogenesis. Hum Mol Genet *21*, 1155-1171.