Loss of the ciliary kinase *Nek8* (*NPHP9*) causes randomization of left-right asymmetry, cardiac defects, and impaired kidney development

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Complete Methods

Generation of the *Nek8:lacZ* allele, ES cell culture, PCR genotyping, and mutants

To generate the *Nek8:lacZ* targeting construct, EL350 *E. coli* (6) were transformed with a C57BL/6 BAC containing the *Nek8* genomic locus and we subsequently performed a two-step gap-repair process (BAC RP23-163A8, CHORI: http://bacpac.chori.org). First, a 9.5kb fragment encompassing exons 2 through 15 of *Nek8* was recombined with the pPNT plasmid backbone (13). Exon three was then targeted with a cassette that encoded an internal ribosomal entry sequence-*lacZ* gene (IRES-*lacZ*) followed by a neomycin resistance gene flanked by Flip recombinase recognition sequences. *Nek8:lacZ* was electroporated into ES cells derived from B6/129 F1 mice (3) and G418 (neomycin, Sigma) resistant clones were isolated, expanded, and subjected to Southern blotting and PCR to confirm the targeting event (5). Three of 148 clones screened were positive for *Nek8:lacZ*, but two of the lines had abnormal karyotypes. Therefore, one positive clone was injected in the Brigham and Women's Hospital Transgenic Mouse facility. The resulting chimeric males were crossed to B6 females and progeny were analyzed by PCR to confirm germline transmission of the *Nek8:lacZ* allele. A 3-primer strategy was utilized as follows: a forward primer 5'-GACACCATTAGGCGCCTTCC-3' that anneals to both WT and mutant loci, a WT-specific reverse primer 5'-CTTCTCAAAGCAGGCCTTGG-3', and a mutant-specific reverse primer 5'-GGGGATCCATATTATCATCG-3'.

Male and female *Nek8:lacZ* carriers were intercrossed and litters were first analyzed at weaning for the presence of homozygous pups, but none were identified. Therefore, timed

matings were set and embryos were harvested at various gestational timepoints; examination of females for copulatory plugs was performed in the morning and if detected, noon was considered to be E0.5. Embryo tails were used for genotyping and *Nek8:lacZ* homozygotes survive gestation (E18.5), but die upon or shortly after birth and we refer to the *Nek8:lacZ* allele as *Nek8-* as the reporter is spliced out of the resulting transcript. To determine if the splicing defect is due to the neomycin cassette in the targeting construct, we crossed β-actin-FLPe deleter mice (11) with *Nek8:lacZ* carriers and generated *Nek8:lacZ*Δneo homozygotes; the neomycin-deficient transcript also lacks exon 3. Maintenance and genotyping for the *jck* mouse line has been described previously (7), and intercrosses were performed between *jck*/+ females and *Nek8*+/- males to obtain compound heterozygotes. All animals were housed in accordance with Harvard Medical School ARCM regulations.

Zebrafish morpholino injection and phenotype analysis

For *Nek8* morpholino knockdowns, WT strain TU-AB zebrafish embryos were injected at the one- to four-cell stage with 4.6 nl of a 0.125-mM, 0.25mM, or 0.5mM solution of a *Nek8* ATG translation blocking oligo: 5'-CTTCTCATACTTCTCCATGTTTTCG-3'. Heart looping was determined at 29 hours post-fertilization (hpf). Uninjected embryos or embryos injected with a scrambled control morpholino 5'-CCTCTTACCTCAGTTACAATTTATA-3' exhibited normal development. A chi square analysis was performed and the occurrence of cardiac looping defects in morpholino-injected embryos was significant, with a p value < 0.001.

Cell lines, tissue culture and Western Blot analysis

Mouse embryo fibroblasts (MEFs) were derived from E14.5 embryos using standard procedures (5). Briefly, littermates were harvested, tails were removed for genotyping, and each embryo was processed to establish independent MEF lines. The cell lines were subsequently immortalized via retroviral infection of the large T antigen (4) and selection for neomycin resistance was carried out for 14 days. MEF lines were expanded and characterized by Western blot to confirm genotyping results with respect to wild-type and null status.

Nek8 knockdown was performed in IMCD cells (1) by lentiviral infection of shRNA-expressing constructs that were generated by annealing complementary primers, the sequences of which were obtained from the Broad Institute shRNA Consortium, and cloning into the pLKO-puromycin vector. The construct expressing a small hairpin against nucleotide 1570 (sh1570) conferred the most robust knockdown when analyzed in a single infection and 48-hour drug selection, so we subsequently isolated clonal lines and screened by Western blot for the loss of NEK8 expression. 5/12 lines lacked detectable levels of NEK8 and line N8KD10 maintained the loss of NEK8 after the cells were expanded. MEFs and IMCD cell lines were maintained in DMEM/10% FBS with 100 units/mL penicillin/0.1 mg/mL streptomycin, and incubated in a humidified, 5.0% CO_2 atmosphere at 37°C.

For shear stress experiments, IMCD cell lines were cultured in DMEM/10% FBS supplemented with GlutaMAX, 100 units/mL penicillin, 0.1 mg/mL streptomycin, and 0.1 mM nonessential amino acids. Wild-type, N8KD10 and PC-depleted IMCD cell lines stably lines expressing GCaMP3 were generated using a blasticidin-resistant retroviral vector as previously described (2). GCaMP3-positive cells were transferred from a culture flask after detachment

using Trypsin-EGTA into 35 mm petri dishes with a bottom coverslip insert (No. 1.5; MatTek). The cells were grown in MatTek chambers to 40-50 % confluency prior to serum starvation (0.5 % FBS). Cells were serum starved for 96 hours prior to fluid shear stress experiments in order to reach their highest frequency of ciliation.

For Western blot analyses, cells and tissues were lysed in Tris buffer (20mM Tris pH7.5, 150mM NaCl, 1mM MgCl, 0.5% NP40) and subjected to SDS-PAGE on a 10% acrylamide gel. Imobilon (Millipore) membranes were blocked in 5% milk/TBST and incubated with primary antibodies overnight at 4°C in the following concentrations: NEK8 (1:5000, reference 7), PC2 (1:1000, reference 10), anti-tubulin (1:500, Sigma). Membranes were rinsed and incubated with HRP-conjugated secondary antibodies and detected with the SuperSignal West Femto substrate (Thermo Scientific).

Magnetic Resonance Imaging of mouse embryos

A multi-channel 7.0-T MRI scanner (Varian Inc.) with a 6-cm inner bore diameter insert gradient set was used to acquire anatomical images of E18.5 mouse embryos. Prior to imaging, the samples were immersed in phosphate-buffered saline (PBS) and 2 mM ProHance® (gadoteridol, Bracco Diagnositics Inc.) for a week. The samples were then placed into 13-mm-diameter plastic tubes filled with low-melting-point (LMP) agarose (Fisher) and 2 mM ProHance. Three custom built, 14-mm-diameter solenoid coils with a length of 18.3 cm and over wound ends were used to image three embryos in parallel. Parameters used in the scans were optimized for contrast within the mature mouse embryo: a T2-weighted, 3D fast spin-echo sequence, with TR/TE = 325/30 ms, eight averages, field-of-view 14 x 14 x 25 mm and matrix

size=348 x 348 x 624 giving an image with 40 μm isotropic voxels. Total imaging time was 14.5 h.

Histology and immunofluorescence

Embryos and kidneys were fixed for 7-14 days in Bouin's solution and subjected to vacuum embedding in paraffin. E18.5 WT and *Nek8*-/- thoraces were submitted to the Harvard Medical School Rodent Histopathology Core and 6 micron transverse serial sections were obtained, stained with hematoxylin and eoisin (H&E) and analyzed for structural cardiac defects. Atrial and ventricular septal defects were identified in mutants by this method. Embryonic kidneys fixed in Bouin's were sectioned at each day of development from E15.5 through E18.5 and subjected to H&E to identify developmental defects. For periodic acid Schiff (PAS) staining and immunofluorescence(IF)/lectin procedures, kidneys at E18.5 and P0 were fixed overnight in 4% paraformaldehyde at 4°C, vacuum embedded in paraffin, and 8 micron frontal sections were obtained. Slides were deparaffinized and PAS staining (Sigma) was performed according to the manufacturer's instructions. After deparaffinizing, IF and lectin staining was carried out as follows: slides were rinsed in PBS, subjected to citrate buffer antigen retrieval, incubated with blocking solution (10% goat serum, 1% BSA, 0.1% triton in PBS) for one hour at room temperature, and incubated with anti-acetylated tubulin antibody (Sigma) at a 1:10,000 dilution combined with either FITC-conjugated LTL or DBA (Vector Labs) at 4°C overnight. Slides were rinsed in PBS and incubated with goat anti-mouse AlexaFluor 594 (Invitrogen) for one hour at room temperature, rinsed in PBS and mounted with Vectashield (Vector Labs).

Whole mount IF was performed on E7.75-E8.0 embryos as follows: embryos were fixed in 4% PFA for 30 minutes at room temperature, rinsed in PBS, permeablized in PBT, and

incubated in blocking solution for one hour followed by anti-NEK8 (1:500) or anti-PC2 (1:1000, reference 12) antibodies overnight at 4°C. Embryos were subsequently rinsed in PBT and incubated with anti-acetylated tubulin for one hour at room temperature followed by a combination of goat anti-rabbit AlexaFluor 488 and anti-mouse AlexaFluor 647 secondary antibodies (Invitrogen). Embryos were rinsed in PBT and mounted under individual coverslips with Vectashield (Vector Labs), photographed, removed from the coverslips, and processed for genotyping (described below).

For cilia length analysis, IMCD cells and MEFs were plated on glass coverslips (VWR Scientific) and at approximately 30% confluence the cells were serum-deprived in DMEM/0.2% FBS for 48-hours; cells were then rinsed in PBS, fixed in 4% paraformaldehyde at room temperature for 10 minutes, rinsed in PBS and permeabilized in PBS/0.4% triton. Cells were incubated in blocking solution, mouse anti-acetylated tubulin antibody and goat anti-mouse AlexaFluor 647. Coverslips were subsequently rinsed in PBS and briefly dipped in 100% ethanol, air dried, and mounted with Vectashield (Vector Labs).

To analyze PC2 expression in IMCD cells under shear stress plating conditions (Supplemental Figure S2), cells were incubated with rabbit anti-PC2 antibody 1:100 (sc-25749; Santa Cruz Biotechnology) and mouse anti-acetylated tubulin antibody. Primary antibodies were detected with anti-rabbit Alexa 647 and anti-mouse Alexa 405 secondary antibodies (Invitrogen).

Whole-mount *in situ* hybridization and fixed tissue genotyping

E8.0-E8.5 embryos were harvested and fixed overnight in 4% paraformaldehyde at 4°C, then dehydrated through a graded methanol series (25% MeOH in PBS-tween (PBT) through 100% MeOH) and stored in 100% methanol at -20°C. For hybridization, embryos were

rehydrated through PBT, and taken through the whole-mount *in situ* procedure described by Wilkinson (14). Briefly, embryos were incubated in 10 ug/ml proteinase K for 5-10 minutes, washes and hybridization solutions were made as described, and hybridization was carried out overnight at 65°C. *mPitx2c* (15) and *nodal* (8) probes were synthesized with T3 and T7 polymerases, respectively, using the MAXIscript T7/T3 (Ambion) *in vitro* transcription kit supplemented with digoxygenin (DIG) RNA labeling mix (Roche) and probes were purified with NucAway (Ambion) spin columns. After hybridization, embryos were washed and incubated overnight with anti-DIG antibody (Roche) conjugated with alkaline phosphatase at 4°C and BM purple substrate (Roche) was utilized to visualize the probes. Whole litters were processed simultaneously and after images were obtained embryos were digested with 100ug/ml proteinase K solution overnight, isopropanol-precipitated with glycogen, washed in 70% ethanol, resuspended in water, and genotyping was performed. The forward primer is the same as described above, while reverse primers were designed to efficiently amplify DNA target sequences from fixed tissue (WT 5'-CAGAGCCAGCAGGATCTGCAC-3' and mutant 5'-CGGCTTCGGCCAGTAACGTTAG-3').

Renal explant culture

Renal explant culture was performed as described by Natoli et al. (9). Briefly, kidneys were harvested from E14.5 embryos, transferred to 1.0mm Corning transwell filters in 6-well plates with DMEM/F12/10% FBS, and incubated overnight. At 24-hours media was supplemented with either DMSO or 100uM 8-bromo-cAMP (Sigma). Media was changed daily and images were captured four days after treatment began. Cyst percentage was calculated by obtaining the total surface area of the cysts divided by the surface area of the kidneys, as

determined using ImageJ (http://rsbweb.nih.gov/ij/). Data was analyzed in Prism software. The standard error of the mean (SEM) was calculated from the cyst percentages of pooled data from at least four independent experiments and n=10 kidneys per genotype, and the p values were determined using Bonferroni's multiple comparison test.

Microscopy and imaging

Whole-mount embryos and renal explants were analyzed on a Leica $DM12_5$ dissecting microscope and images were captured with Leica FireCam software. Histological and immunofluorescent experiments were analyzed on a Zeiss Imager.Z1 microscope and images were captured with AxioVision software; IF slides were imaged with the ApoTome engaged for better resolution of cilia and nuclei.

Fluid shear stress experiments and data analysis

IMCD cells stably expressing GCaMP3 were introduced to CO_2 independent HBSS media supplemented with HEPES (25 mM final concentration), non-essential amino acids, sodium pyruvate, glucose and GlutaMAX. Intracellular calcium stores were purged by challenging cells with 2 mM EGTA and 100 μM ATP prior to Ca^{2+} influx measurements. Cells were introduced back to EGTA free HBSS based media and the cell chambers were mounted on the stage of a Nikon Eclipse Ti inverted microscope equipped with a Nikon Plan Fluor 20xA 0.75 NA objective lens and a CoolSnap-HQ (Photometrics). The Nikon FITC cube was used to efficiently reflect 488 nm wavelength and pass the emission wavelengths into the CCD camera detection channel. While fluorescence image time series were acquired, cells were challenged with fluid shear stress via the controlled addition of Ca^{2+} media. IMCD3 cells stably knocked

down for PC2 (m351.2 clone, reference 1) and stably expressing GCaMP3 were utilized as a negative control for PC2-dependant intracellular Ca^{2+} influx measurements. The acquisition settings were kept constant for all samples so that valid comparisons could be made between measurements from different data sets. Acquisition parameters were set within the linear range of the CCD camera detection.

A custom MATLAB (The MathWorks Inc.) subroutine was written to analyze acquired image time series. The mean intensity of the background noise was calculated from empty dark regions in the images and each frame was individually corrected. Regions of interest (ROI) from individual cells were chosen for the subsequent analysis. The mean intensity values measured from the first 30 frames (prior to exposing cells to fluid shear stress) of acquired image time series defined ROIs and used to normalize the values of fluorescence intensity to unity. The standard deviations of the recovered mean values were obtained from the analysis of multiple ROIs in multiple independent experiments.

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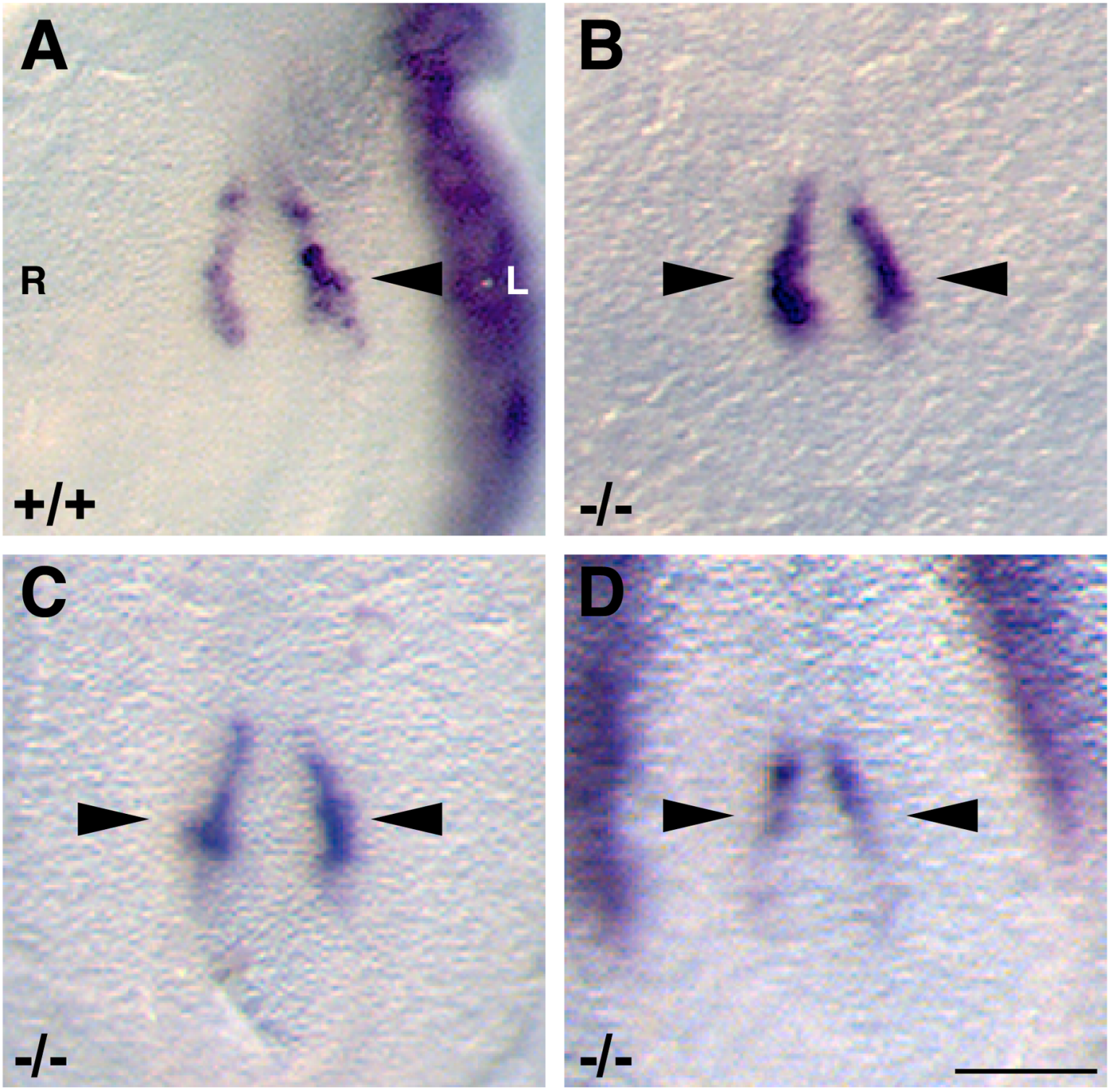
Supplemental Figure Legends

Supplemental Figure S1. *Nek8-/-* embryos maintain symmetrical *Nodal* expression in the node proper

(A-D) *Nodal* is expressed in the node proper in 4-5 somite WT and null embryos.Expression in the (A) WT node is stronger on the left side (arrow; R, right; L, left), but somite-matched mutant embryos (B-D) exhibit symmetrical nodal expression (arrows). Scale bar (A-D) 200μm.

Supplemental Figure S2. PC2 localizes to cilia in WT and NEK8-knockdown IMCD cells under conditions utilized for shear stress assays

(A-C) IF of anti-acetylated tubulin (AT, blue) and anti-PC2 (PC2, red) antibodies on (A) WT, (B) PC2-knockdown (PC2KD) and (C) NEK8-knockdown (N8KD10) IMCD cilia (arrows mark the ends of cilia) confirms PC2 localization in (A)WT and (C) N8KD10 cilia and reduction of PC2 in (B) PC2KD cilia.


A
R
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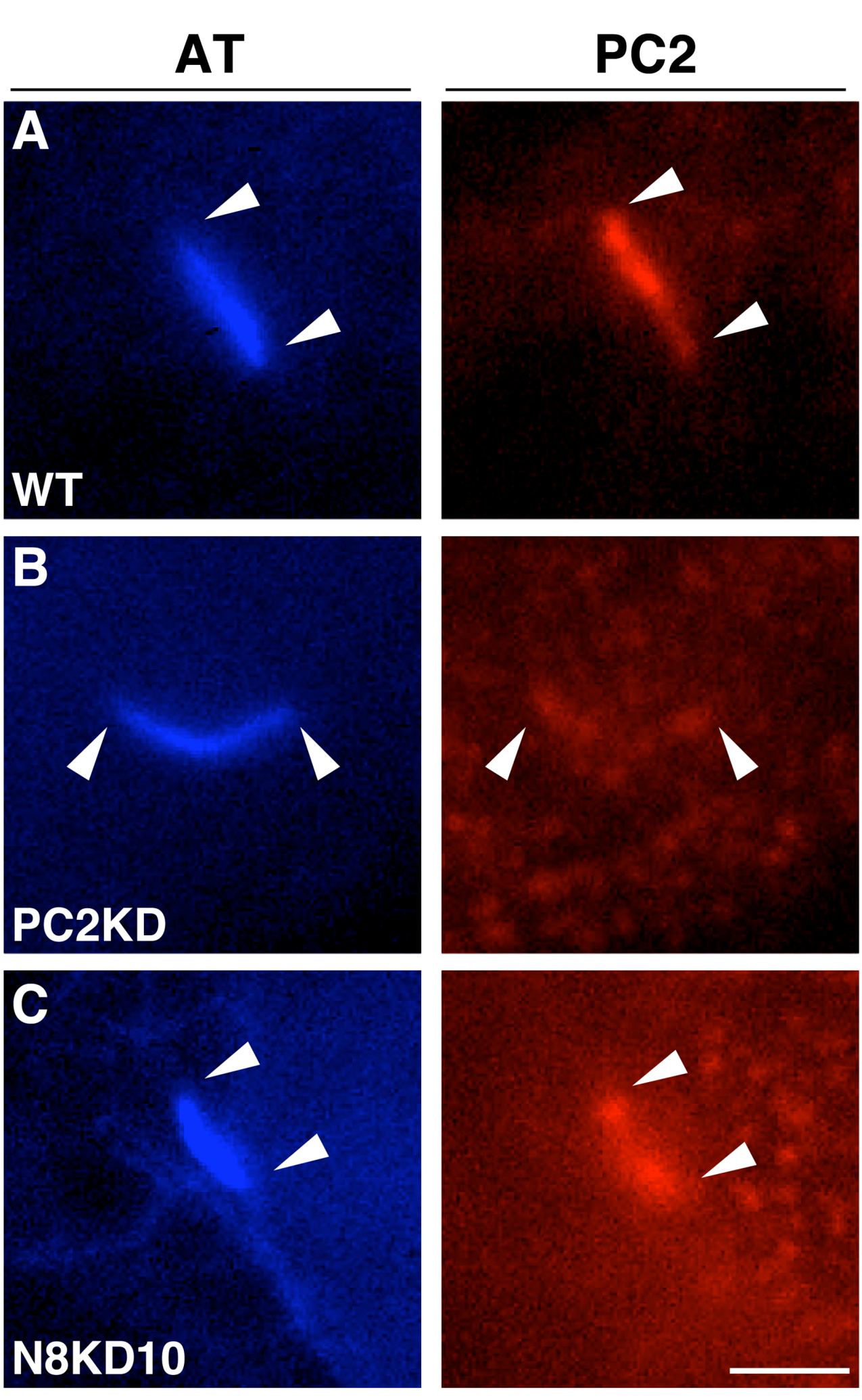
AT
PC2
A
WT
B
PC2KD
C
N8KD10