DNA replication stress underlies cysts renal phenotypes in CEP290-associated Joubert syndrome

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Introduction
Mutations in CEP290 are known to cause the entire spectrum of ciliopathies, including childhood renal failure due to nephronophthisis (NPHP) (1), Joubert syndrome (JS; MIM 610188), the lethal Meckel Grüber syndrome (MKS; MIM 611134), Bardet-Biedl syndrome (BBS) (MIM 209900), Senior-Løken syndrome (SLS) (MIM 610189), and up to 25% of Leber congenital amaurosis (LCA; MIM 611755) cases, although no precise genotype-phenotype correlations have been found (2). Around 50% of patients with JS in which there is a cerebello-oculo-renal phenotype have mutations in CEP290 (3). Progressive kidney damage, secondary to NPHP, leads to end-stage renal disease (ESRD) in affected patients and occurs at a mean age of 14 years (4). NPHP is the most frequent monogenic cause of ESRD in the first 3 decades of life (5) and accounts for 5% to 10% of all children with ESRD. Disease mechanisms underlying NPHP, which is characterized by a tubulointerstitial fibrosis, tubular atrophy, and corticomedullary cyst formation, implicate abnormal ciliary and centrosomal proteins (5).

CEP290 is a large multidomain centrosomal protein (6); identified binding partners of CEP290 include centrosomal proteins CEP131 and CCDC13; scaffold proteins pericentrin and PCM1; transcription factors, including ATF4; and proteins that are implicated in the DNA damage response (DDR), for example, ataxia telangietasia and RAD3-related (ATR) (1, 7–9). The localization of CEP290 to the centrosome is dynamic, depending on the stage of cell cycle and expression of the primary cilium (1). The primary cilium is expressed in G0 after exit from the cell cycle, when the mother cilium is docked to the plasma membrane. Cells disassemble their cilium at the end of G1 in order to duplicate their centrosome for mitotic spindle formation (10). CEP290 localizes to the transition zone at the base of the primary cilium as well as at the centrosomes in a complex with other centrosomal proteins: NPHP1, INVS (also known as NPHP2), NPHP4, IQCB1 (also known as NPHP5), RPGRIP1L (also known as NPHP8), and NEK8 (also known as NPHP9) (11–13); mutations in any of these proteins can cause one or more ciliopathy syndromes as well. CEP290 also localizes to the nucleus, although its function is entirely unknown (1). One possibility is that CEP290 acts in a manner similar that of the other ciliary proteins mutated in renal ciliopathies (CEP164, ZNF423, SDCCAG8, NEK8), which have been associated with enhanced DDR signaling (14–16). A single study examining the events leading to DNA damage in this setting recently established a role for NEK8 in the ATR-regulated replication stress response and in the regulation S-phase cyclin-dependent kinase (CDK) activity (16). However, only three families with mutations in NEK8 have been described (17), making these data less clinically relevant.

We set out to extend this correlation to a broader clinical base and investigate the role of CEP290 loss in DDR signaling and replication stress. To confirm that defects in DDR signaling underlie progressive renal disease seen in NPHP would allow a novel rationale for therapeutic interventions in these patients. Our findings support the overall hypothesis that NPHP-related ciliopathies...
(NPHP-RC) are initially caused by DNA damage and replication stress during early stages of development (18). Here, we used primary cells isolated from kidneys of Cep290LacZ/LacZ mice with JS symptoms and their WT littermates (19) to investigate DNA damage signaling and the replication stress response. We found enhanced DNA damage signaling and concomitant DNA breaks in Cep290LacZ/LacZ cells, in addition to supernumerary centrioles. Decreased replication fork velocity and fork asymmetry underlie the DNA damage. Additionally, application of CDK inhibitors (CDKi) rescues the DNA damage phenotypes and restores the ability of Cep290LacZ/LacZ cells to ciliate. These findings provide insight into the disease mechanism of NPHP-RC and will help to refine treatment strategies.

Results

CEP290 depletion causes DNA damage ex vivo, in vitro, and in vivo. Recent studies have implicated DDR and sensitivity to replication stress in the development of ciliopathies. To explore the breadth of molecular effects in different human ciliopathies, we tested siRNA targeting of Ofd1 and Mks1, which is characteristic of orofaciodigiodal syndrome (MIM 311200) and MKS, respectively. Increased levels of DDR signaling were observed for all siRNAs tested (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI80657DS1). To test the effect of CEP290 mutations in patient material, we isolated urine-derived renal epithelial cells (URECs) from a patient with JS with compound heterozygous mutations in CEP290, p.Q950Pfs*6, and p.K939N and observed increased γH2AX levels and decreased CEP290 levels compared with those of a healthy donor by Western blot (Figure 1A and Supplemental Figure 1C). We next lowered endogenous levels of CEP290 in URECs from two healthy donors and determined siRNA efficiency to be about 70% in the single donor tested (due to limited material, n = 1; P < 0.01; Supplemental Figure 1A). Western blots from both donors revealed increased DNA damage signaling levels (γH2AX) after 48 hours of siCEP290 transfection (Figure 1B and Supplemental Figure 1B). Similar results were observed upon siRNA knockdown of CEP290 in mIMCD3 and hTERT-RPE cell lines, which suggests that increased DNA damage signaling is not restricted to renal cells alone (n = 3; Figure 1C and Supplemental Figure 1, A and B). Immunofluorescence showed increased γH2AX foci in URECs from the patient with JS as well as in RPE and mIMCD3 cells after siRNA knockdown of CEP290, compared with that in controls (n = 3; P < 0.01 and P < 0.001 Figure 1D). Next, we examined whether DDR signaling would be induced after a cep290 ATG-targeting morpholino (mo) was injected in zebrafish (1). Western blot of 3.5 ng or 4 ng morpholino-injected fish targeting cep290 showed increased γH2AX 48 hours after fertilization, compared with that in controls (n = 3; Figure 1E and Supplemental Figure 1, B and D). Furthermore, γH2AX levels increased with the age of mouse Cep290LacZ/LacZ kidneys as compared with those in WT littermates, suggesting a role for DNA damage or DNA damage signaling in the pathophysiology of NPHP-RC (n = 13, P < 0.05; Figure 1, F and G).

Results from our Western blot analyses support the presence of enhanced DNA damage signaling (γH2AX) in primary mouse collecting duct cells isolated from Cep290LacZ/LacZ kidneys (19) as opposed to their WT littermates (Figure 2A). Immunofluorescence confirmed higher intensities of γH2AX expression per nucleus in the Cep290LacZ/LacZ cells, which was further enhanced by incubation with the replication stress-inducing agent aphidicolin (APH) (20) (400 nM, 18 hours; n = 3; P < 0.05; Figure 2, B and C). To differentiate between actual DNA fragmentation and simple increased DDR signaling, we assessed whether DNA double-strand breaks were formed using neutral comet assays. Increased comet tail moments were observed in Cep290LacZ/LacZ cells and exacerbated by APH incubation (400 nM, 18 hour; n = 5; P < 0.05; Figure 2, D and E). We concluded that loss of CEP290 generates double-stranded breaks in DNA. Because CEP290 is a centrosomal protein, we examined centriole numbers. Supernumerary centrioles were observed in the Cep290LacZ/LacZ cells (Figure 2, F and G), which might contribute to genome instability. We therefore performed DNA content analysis by FACS (DAPI and BrdU); Cep290LacZ/LacZ cells demonstrated changes in DNA content as compared with the WT control cells. WT cells had a normal G1 (2 N DNA) and G2 (4 N DNA) cell cycle profile, whereas Cep290LacZ/LacZ cells had an aberrant DNA content profile that did not match 2 N or 4 N DNA (Figure 2H). To ensure that this result was not confounded by contamination or clonal drift, we grew Cep290LacZ/LacZ cells clonally and observed similar data in all 7 clones tested (Supplemental Figure 2, B and C). Our data support a role for CEP290 in genome stability.

CEP290 protects cells from replication stress. The formation of DNA breaks is frequently a consequence of perturbed DNA replication, a phenomenon referred to as replication stress (21). Clonogenic survival assays using the WT and Cep290LacZ/LacZ mouse primary kidney cells incubated with different doses of APH for 18 hours suggested that Cep290LacZ/LacZ cells are more sensitive to replication stress (n = 3; P < 0.001; Figure 3A). To investigate the underlying molecular mechanism, we next performed single-molecule DNA fiber analysis of replication fork progression in the WT and Cep290LacZ/LacZ mouse primary kidney cells. To label newly synthesized DNA, cells were pulsed with iododeoxyuridine (IdU) for 15 minutes and then with chlorodeoxyuridine (CldU) for 15 minutes. The double labeling with IdU and CldU allows the assessment of fork symmetry and velocity as well as origin firing (ref. 22; Figure 3, B and E). We first asked whether CEP290 modulated the fork velocity, also referred to as the rate of DNA synthesis, by measuring the lengths of the CldU tracts. We found that the replication fork velocity was decreased (n = 4; P < 0.001; Figure 3, B–D) in the Cep290LacZ/LacZ cells. After origin firing, forks moved bidirectionally in a relatively synchronous manner, unless there was a problem with fork progression or restart. Thus, we also analyzed the symmetry of sister forks emanating from a common origin, as fork asymmetry is an indicator of fork stalling. To do so, we measured the lengths of the CldU tracts for each pair of sister forks and plotted each pair as the right length versus the left length. From this analysis, we found that Cep290LacZ/LacZ cells had an increase in asymmetric forks, indicative of an inherent loss of fork stability in the absence of CEP290 (n = 2; P < 0.001; Figure 3, E–G). Importantly, sister fork asymmetry and reduced fork velocity are both indicators of replication stress and suggest that DNA break formation in Cep290LacZ/LacZ cells occurs as a result of increased replication stress. To examine cell cycle S-phase progression defects, which are potentially induced by replication
CEP290 is stabilized upon DNA damage induction. To examine CEP290 expression throughout the cell cycle, WT primary kidney cells were synchronized with thymidine or nocodazole and released for 5 hours. We found that CEP290 levels varied, and the highest levels of CEP290 were observed in S-phase (n = 4; Figure 4, A and B, and Supplemental Figure 2A). Since loss of CEP290 causes replication stress, we examined whether CEP290 precipitates with chromatin. Standard chromatin fractionation assays convincingly showed endogenous CEP290 to be enriched in the chromatin cell fraction (n = 2; Figure 4C). Immunofluorescence revealed increased γH2AX levels in WT and JS URECs and RPE and mIMCD3 cells depleted of CEP290 by siRNA and quantification of staining intensity per nucleus (scale bar: 10 μm; n = 3; 100 cells scored per condition; t test, **P < 0.01, ***P < 0.001). Western blot of zebrafish injected with 3.5 or 4 ng cep290 ATG-targeting mo. Loss of CEP290 protein expression and increased γH2AX levels were detected in cep290 mo-injected zebrafish lysates 48 hours after fertilization. ACTIN was used as loading control (n = 3); samples were run on parallel gels contemporaneously. More γH2AX (brown) staining in kidneys of homozygote Cep290LacZ/LacZ gene trap mice compared with WT mice, which increased with age (n = 13; between 5,000–7,000 cells scored per animal) (linear model, goodness-of-fit test R² = 0.86; *P < 0.05). Example of WT mouse kidney and Cep290LacZ/LacZ gene trap mouse kidney at age 3 months stained for γH2AX (brown). Insets show high-magnification images of kidneys. Scale bars: 100 μm; original magnification ×2 (inset). Quantification of Western blots is shown in Supplemental Figure 1.

stress, we quantified cells in early S-phase 18 hours after BrdU pulse labeling of cells in S-phase. IMCD3 cells had a higher proportion of cells in early S-phase after CEP290 siRNA treatment compared with controls (n = 3; P < 0.01; Supplemental Figure 3A), suggesting that S-phase progression is slower due to replication fork defects. As these results mirror those seen in Nek8−/− cells, it seems likely that defective DNA replication dynamics are a common feature of NPHP-RC.
CDKi rescue DNA damage and cilia. The DNA fiber results suggest that CEP290 has an important function during DNA replication to ensure efficient fork progression and stability. It is known that elevated cyclin A–associated CDK1/2 activity can cause similar defects on replication fork dynamics. Furthermore, we have previously shown that partial inhibition of CDK1/2 activity can largely suppress the DNA damage resulting from NEK8 loss (16). We therefore hypothesized that enhanced CDK1/2 activity in Cep290LacZ/LacZ primary kidney cells could be responsible for the increased DNA damage signaling and DNA breaks during disturbed DNA replication (23, 24). Indeed, total protein levels of CDK1 and CDK2 as well as cyclin A and cyclin B are increased in Cep290LacZ/LacZ primary kidney cells (25, 26), suggesting that CDK1/2 activity is upregulated in these cells. To test this hypothesis, we used a small interfering RNA (siRNA) approach to reduce CDK1 and CDK2 expression in Cep290LacZ/LacZ primary kidney cells. Western blot analysis showed a significant reduction in CDK1 and CDK2 protein levels upon siRNA treatment (Figure 3A). Furthermore, flow cytometry analysis revealed a decrease in the percentage of cells in S phase, consistent with the inhibition of DNA replication (Figure 3B). These results support the hypothesis that enhanced CDK1/2 activity contributes to the increased DNA damage signaling and DNA breaks observed in Cep290LacZ/LacZ primary kidney cells.

Figure 2. DNA damage is enhanced in CEP290-depleted cells. (A) Western blot of Cep290LacZ/LacZ and WT primary mouse kidney cell lysates. Increased H2AX phosphorylation was detected in the CEP290-depleted cells. H2AX was used as loading control. (B) Immunofluorescent staining of γH2AX in control cells treated with DMSO and in cells treated with 400 nM APH for 18 hours. Scale bar: 10 μm. (C) Quantification of γH2AX staining intensity per nucleus in control cells treated with DMSO and in cells treated with 400 nM APH for 18 hours (n = 3; 100 cells scored per condition; 2-way ANOVA, *P < 0.05). (D) Quantification of comet tail moments of cells treated with 400 nM APH for 18 hours normalized to control DMSO-treated WT cells (n = 3; 50 cells scored per condition; 2-way ANOVA, *P < 0.05). (E) Images of SYBR Gold–stained DNA in comet tail assays. Scale bar: 10 μm. (F) Immunofluorescent staining of WT and Cep290LacZ/LacZ cells for pericentrin (white) reveals supernumerary centrioles (arrows) in Cep290LacZ/LacZ cells. The inset shows multipolar (>2) spindles. Scale bars: 10 μm; original magnification ×2 (inset). (G) Quantification of centriole number in WT and Cep290LacZ/LacZ cells (84 cells scored per cell line; n = 3). (H) BrdU FACS of WT and Cep290LacZ/LacZ cells shows normal DNA content and cell cycle for WT cells; however, Cep290LacZ/LacZ cells have irregular DNA content (10,000 events measured; n = 3).
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3661

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Ciliary frequency data, we measured cell cycle distribution; after CDK1/2 inhibition the G1 cell population decreased from 60% to 31%, whereas the G2 cell population increased from 12% to 45% (200 nM, 18 hours, n = 3; Figure 5H). This control experiment indicates that the gain of cilia was not caused by accumulation of cells in G1. Likewise, we wanted to exclude the idea that rescue of the DNA damage and cilia phenotypes by CDK1/2 inhibition was caused by apoptosis. FACS revealed no enhanced apoptosis after CDK1/2 inhibition (18 hours, 200 nM; n = 3; Supplemental Figure 3C). Furthermore, centriole numbers in Cep290 LacZ/LacZ mouse primary kidney cells were normalized after treatment with CDK1/2i (18 hours, 200 nM; Supplemental Figure 3B).

Discussion

CEP290 loss causes enhanced DNA damage signaling, DNA breaks, replication stress, and supernumerary centrioles. These data describe the first function to our knowledge for CEP290 in the nucleus, in which the protein cofractionates with chromatin. We show that CEP290 levels increase after DNA damage. Our data indicate a causal relationship of enhanced DDR to a broad clinical range of CEP290-related ciliopathies. Accumulation of DNA damage can be attributed to disturbed replication forks.

B levels were higher in Cep290LacZ/LacZ cells (Figure 5A). In vitro CDK1 and CDK2 kinase assays revealed a trend toward enhanced cyclin A–associated CDK activity (+26%) and cyclin B–associated CDK activity (+7%) in Cep290LacZ/LacZ cells (n = 4; Figure 5B and Supplemental Figure 4A), although the intraexperimental variation was high. In addition, the single kinase assay of WT URECs and URECs from a patient with JS revealed modestly increased cyclin A–associated CDK activity (+7%) and cyclin B–associated CDK activity (+14%) as well (n = 1; Figure 5C and Supplemental Figure 4B). We followed up by testing whether the increase in DNA damage signaling (using phosphorylated KAP1, γH2AX, and phosphorylated CHK1 as markers; n = 3) and the increase in DNA breaks, as measured by neutral comet assays (n = 2), could be rescued in Cep290LacZ/LacZ cells by treatment with a CDK1/2 inhibitor (CDK1/2i) (200 nM, 18 hours; Figure 5, D and E). Both phenotypes could be rescued by CDK1/2 inhibition. To investigate whether reduced cilia frequency could be similarly rescued by CDK1/2 inhibition, we used a 3D spheroid culture assay and compared Cep290LacZ/LacZ mouse primary kidney cells to WT littermate cells. We observed a rescue of ciliation by CDK1/2i (18 hours, 200 nM; Supplemental Figure 3B).
These insights into the pathogenesis of CEP290 loss are reminiscent of the molecular functions of another ciliopathy protein, NEK8 (16). Furthermore, increased DNA damage signaling was seen in CEP164-, ZNF423-, and SDCCAG8-associated NPHP (14, 15) and hints at a general disease mechanism. It is interesting to note that CEP290 loss affects centriole duplication outside the nucleus. The centriolar satellite complex, including CEP290, has been previously reported to function in maintaining genome stability (7, 8). A systematic analysis of all ciliopathy genes is required to confirm the breadth and depth of replication stress and centriole duplication defects. Furthermore, we demonstrate the use of disease modeling by siRNA-mediated gene depletion in URECs. Potential interventions as well as modeling molecular/cellular temporal-spatial events leading to the pathophysiology of renal ciliopathies from URECs will help tailor personalized treatments.

It is interesting to note that both NEK8- and CEP290-depleted cells demonstrate strikingly similar sensitivity to replication stress, decreased fork velocity, and fork asymmetry (16). In Nek8−/− cells, enhanced cyclin A-associated CDK activity was observed and cyclin B-associated CDK activity was unchanged (16). Both Nek8−/− and Cep290<sup>−/−<sup> and Cep290<sup>−/−<sup> phenotypes are equally rescued by the pan CDKI, suggesting that the pathophysiology is conserved. We believe that the functional consequences of CEP290 depletion beyond cilia and ciliary signaling are novel. The hallmarks of the ciliopathy-associated NPHP are corticomedullary cyst formation and interstitial fibrosis. The renal ciliopathy field has been dominated by studies of ciliary dysfunction and renal cysts; however, the more clinically relevant aspect of renal fibrosis has been largely ignored. We have obtained functional insight into the NPHP-RC pathogenesis and investigated the effect of CDKI on loss of function of CEP290. Use of CDKI as a potential treatment strategy has already shown some success in vivo (25, 26), and our data confirm that changes in CDK protein levels may be widespread in patients with NPHP, justifying this approach. We would like to extend this knowledge to other forms of chronic kidney disease (27) and renal fibrosis, which are associated with DNA damage signaling.

**Methods**

**Cell culture.** mIMCD3 and hTERT-RPE cells (ATCC) were grown in DMEM/F12 (1:1) medium with 10% FCS, 2 mM l-glutamine, and penicillin/streptomycin in 5% CO<sub>2</sub> at 37°C. Cep290<sup>−/−<sup> H-2Kb-tsa58<sup>−/−<sup> and Cep290<sup>−/−<sup> H-2Kb-tsa58<sup>−/−<sup> collecting duct cells were isolated from kidneys of 1-month-old transgenic mice (19). Cells were incubated at 33°C in 5% CO<sub>2</sub>. CCD media was used for cell culture as previously described (28). URECs were obtained from a patient with JS and a healthy gender- and age-matched control and from additional healthy individuals. URECs were derived as previously described (19, 29). All cells were tested for mycoplasma every 2 weeks and were negative.
Figure 5. CDK1/2i rescues DNA damage and primary cilia. (A) Western blot of Cep290
LacZ/LacZ and WT cell lysates showing increased levels of CDK1, CDK2, cyclin A, and cyclin B1 in CEP290-depleted cells. GAPDH was used as loading control; samples were run on parallel gels contemporaneously. (B) In vitro CDK kinase assay of Cep290
LacZ/LacZ and WT cell lysates after precipitation of cyclin A–CDK and cyclin B1–CDK complexes (n = 4). (C) CDK kinase assay of WT URECs and URECs from a patient with JS (n = 1). Histone 1 represents substrate level control. (D) DNA breaks are rescued by CDK1 in comet tail moments of cells treated with 200 nM CDK1/2i (18 hours) normalized to control DMSO-treated WT cells (n = 3; 50 cells scored per condition). (E) Western blot of Cep290
LacZ/LacZ and WT cells treated with 400 nM APH and/or 200 nM CDKi (18 hours). Higher phosphorylation levels of KAP1, H2AX, and CHK1 in Cep290
LacZ/LacZ cells with or without APH treatment, rescued by CDK1/2i. Unphosphorylated protein and GAPDH are loading controls; samples were run on parallel gels contemporaneously. (F) 3D spheroids of WT and Cep290
LacZ/LacZ cells, stained for γH2AX (red) and ciliation (acetylated tubulin; green), treated with 400 nM APH and/or 200 nM CDKi (18 hours). Scale bars: 5 μm. (G) CDKi rescues ciliation in Cep290
LacZ/LacZ and APH-treated spheroids. (35 spheroids scored per condition) (n = 3; data shown for single experiment; 3-way ANOVA, *P < 0.05, **P < 0.01). (H) Cell cycle distribution of WT cells treated with DMSO or 200 nM CDKi (18 hours) reveals increased cell number in G2 (black) and decreased cell number in G1 (gray) after CDKi treatment (10,000 events measured; n = 3).

Antibodies and reagents. Antibodies to phospho S139-H2AX and phospho S345-CHK1 (Cell signaling, 9718S and 2348L, respectively); phospho S824-KAP1 (Bethyl Laboratory, A300-767A); CHK1 (G-4), cyclin A (C-19), cyclin B1 (H433), CDK1 (Cdc2 p34; ref. 17), CDK2 (D-12), and PCNA (Santa Cruz Biotechnologies, 8408, 596, 752, 54, 6248, and 56, respectively); KAP1 (Transduction Lab, K57620); pericentrin, H2AX, and GAPDH (Abcam, Ab4448, Ab11175, and Ab8245, respectively); α-tubulin, acetylated α-tubulin, and β-actin (Sigma-Aldrich, T6199, T6793, and A5441, respectively); actin (C4, ImmunO, 691001); CEP290 (Novus Biologicals, NB100-86991) are all commercially available. The ON-TARGETplus siRNA SMARTpools (Thermo Scientific Dharmacon) for nontargeting pool UGGUUUAACUGGACUUA/UGGGUUAACUGUUGUGA/UGGGUUAACAGGUU-UUCUGA/UGGGUUAACAGGUUUCCUA (D-001810-10) and the
siRNAs targeting human CEP290, GGAUUGGAAUGAAAGAAA/GGAUUGACUACUGUGGAU/GAAAGUUAUGCGAAAUG/GAAGUGACCCCGCAA (L-014590-00), and mouse Cep290, GCGAAUACAGAGGAU/AGACUACCCGAGGGAUC/GUCCAAACUCGCAAGAU/ACUUGAGCUGUGAAGCA (L-052034-00), were purchased from Thermofisher. All cell transfecions were performed using 20 nM siRNA with Lipofectamine RNAiMax (Invitrogen, 13778-150) following the manufacturer’s protocols. The CDK1/2i (217714) was purchased from Millipore, and APH was purchased from Serva (13696).

**Immunostaining.** For immunostaining, cells were grown on coverslips and fixed for 15 minutes in 4% paraformaldehyde or 5 minutes in ice-cold methanol, followed by a 15-minute permeabilization step in 0.1% Triton X-100/1% BSA/PBS. Primary antibody incubations were performed overnight at 4°C in 1% BSA/PBS blocking buffer. Alexa Fluor 488, 568, and 647 secondary antibodies (Invitrogen, dilution 1:500) and DAPI incubations were performed for 1 hour at room temperature. Coverslips were mounted in Fluormount G (Cell Lab, Beckman Coulter). Confocal imaging was performed using a Zeiss confocal laser microscope, and images were processed with the ZEN 2011 software.

**Western blot.** Protein lysates were prepared using RIPA lysis buffer and sonicated. To correct for protein content, BCA protein assay (Pierce) was performed. After blotting, the PVDF membranes were blocked in 5% BSA or 5% dried skim milk in TBS with 0.5% Tween. The primary antibodies were incubated overnight at 4°C. Coomassie blue staining of the gel was performed as additional loading control. The secondary antibody was incubated for 1 hour at room temperature. The ECL Chemiluminescent Peroxidase Substrate Kit (Sigma-Aldrich, CPS1120-1KT) was used for development. Scans of the blots were made with the Bio-Rad ChemiDoc XRS+ device, and Image Lab software 4.0 was used for quantification.

**Comet tail assay.** Comet assays were performed with the Electrophoresis Kit ( Trevigen) according to the manufacturer’s protocols. DNA was stained with SYBR Gold (Invitrogen, SI1494, 1:30,000), and comet tail moments were calculated by counting 50 cells for each sample and analyzed with CometScore software (TriTek Corp).

**In vitro kinase assay.** 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 cyclin A (Santa Cruz Biotechnologies) or cyclin B1 (Santa Cruz Biotechnologies) antibody-bound protein A-Sepharose beads (GE Healthcare) for 2 hours at 4°C. Precipitates were washed 3 times with high-salt buffer and once with kinase buffer (20 mM HEPES, pH 7.5, 5 mM MnCl₂, 5 mM MgCl₂, 5 mM β-glycerophosphate, 1 mM NaF, 1 mM DTT, 1 mM NaVO₄). Kinase reactions were carried out by incubating immunoprecipitated cyclin A/B1 with 50 μl of kinase buffer with 10 μM ATP, 5 μM γ23P-ATP, and 5 μg of substrate histone H1 for 30 minutes at 30°C with and without 2 μM CDKI and stopped by adding SDS sample buffer. Samples were boiled in Laemmli buffer and analyzed by SDS-PAGE. Ponceau S or Coomassie blue staining was used to validate equal substrate loading.

**Chromatin fractionation.** Chromatin fractionation was performed as previously described (16, 31). Approximately 10⁷ cells were used, and fractions were resuspended in Laemmli sample buffer and analyzed by Western blotting. The chromatin fraction was sheared by sonication. Equal loading of fractions was verified by Ponceau S staining and immunoblotting against α-tubulin and GAPDH (cytosolic fraction), PCNA (nuclear and chromatin fractions), and H2AX (nuclear fraction).

**Clonogenic survival.** Cep290<sup>+/-</sup> and Cep290<sup>+/+</sup> cells were treated with APH at the concentrations indicated in figure legends for 18 hours. Subsequently, cells were seeded onto 6-cm plates at a density of 1,000 cells. After 11 days, cells were fixed in 1% formaldehyde in PBS, stained with 0.5% Crystal Violet (Sigma-Aldrich) for 10 minutes, and rinsed with tap water. Resulting colonies were manually counted. Each experiment represents three experimental replicates.

**DNA fiber analysis.** Fiber analysis was performed, and data were processed, as previously described (22). Briefly, Cep290<sup>+/-</sup> and WT cells were pulsed labeled with IdU (Sigma-Aldrich) for 30 minutes, quickly washed with PBS, and pulse labeled with CldU (Sigma-Aldrich) for 30 minutes. DNA fibers were visualized using primary antibodies specific for IdU and CldU (BD Biosciences [347580, 1:50] and Abcam [Ab6326, 1:100]), respectively, and stained with Alexa Fluor 488- or 594-conjugated (1:500) secondary antibodies (Molecular Probes). The DNA fibers were captured with fluorescence microscopy using a 40x objective with a QICAM L2bits CCD camera, such that 1 pixel was equivalent to 4.65 μm. ImageJ was used for pulse labeling analysis.

**RT-qPCR.** Cells were lysed, and total RNA was isolated (RNeasy Mini Kit, Qiagen, 74106) and measured (NanoDrop Spectrophotometer ND-1000, Thermo Fisher Scientific Inc.). cDNA was synthesized from a 1,000-ng RNA template using the iScript cDNA Synthesis Kit (Bio-Rad, 170-8891) according to the supplier’s protocol. Dilutions were made for RT-qPCR analysis to determine mRNA expression levels, which were normalized against a reference gene. The iQ SYBR Green Supermix (Bio-Rad, 170-8880) was used to multiply and measure the cDNA with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). All samples were run in triplicate in 20 μl reactions. The following PCR program was used: 95°C for 3 minutes; followed by 40 cycles of 10 seconds at 95°C, 30 seconds at the annealing temperature indicated with the primer sequence, and 30 seconds at 72°C; and then 10 seconds at 95°C, followed by a melt of the product from 65°C to 95°C. The primer sequences (Sigma-Aldrich) used and concomitant annealing temperatures are as follows: RPLP0 forward 5′-TGGACAATGGCCAGCATCTAC, RPLP0 reverse 5′-ATCCGTCCTCCACAGACAGGG, 58°C, CEP290 for-
ward 5′-TGACTGCTAAGTACAGGAGACACATTG, CEP290 reverse
5′-AGGAGAGTTTTCACCTCCAGGT, 65°C, Rpl27 forward
5′-CGCCCTCTTTTCTTCTGC, Rpl27 reverse 5′-GGTGCATC-
GTCATGTTTCTTC, 53°C, Cep290 forward 5′-GACAAGTACAT-
CGATCACAGT, Cep290 reverse 5′-CGCCTTTATCGTTG-
TACTCA, 64°C. The ΔACT method was used for statistical analysis
to determine gene expression levels.

Histology. Immunocytochemistry was performed as previously
described (27). Mouse kidney tissue sections (n = 15) embedded in
paraffin were deparaffinized, treated with peroxidase block for 15
minutes, and incubated at 100°C in citrate-HCl buffer (pH 6) for 20
minutes. The sections were stained with rabbit antibody to γH2AX
(1:200) overnight at 4°C. Samples were incubated with BrightVision
Anti-Rabbit Poly HRP for 1 hour at room temperature. The Nova RED
Substrate Kit for Peroxidase (Vector, SK-4800) was used, and samples
were counterstained with hematoxylin. Analysis was performed using
Aperio ImageScope software. Five random tubular fields (approx-
imately 1,000 cells per field) were analyzed for positively stained
nuclei using an in-house ImageJ macro. Linear model analysis was
used for statistical analysis.

FACS. To quantify cell cycle phase distribution, WT and
Cep290+/+neo/neo mice were injected with 10 μM BrdU for 30 minutes
and fixed in ice-cold 70% EtOH. To quantify S-phase progression,
IMCD3 cells (48 hours after siRNA) were injected with 10 μM BrdU
for 30 minutes, cultured for 18 hours, and fixed in ice-cold 70% EtOH.
Samples were stained for FACS analysis with BrdU mouse mAb Alexa
Fluor 647 conjugate (1:200; Invitrogen) in 0.1% BSA-PBS-T for 1 hour
on ice and stained with DAPI in PBS. To quantify apoptosis, unfixed
cells were stained with 7-AAD and Hoechst as described previously
(32). 10,000 events were measured with a BD FACS Canto II Flow
Cytometer and analyzed using BD FACS Diva Software.

Zebrafish. WT embryos at the 1- to 2-cell stage were injected with
3.5 ng or 4 ng of a mouse oligonucleotide targeting cep290 ATG codon
sequence (Gene-Tools) (1) in pure water with 0.1% phenol red using
a Nanoject2000 Microinjector (World Precision Instruments). The
sequence of the standard control mo was as follows: 5′-CCTCT-
TACCTCAGTTAAATTATA-3′. For Western blot, 18 embryos were
pooled in 36 μl Trition X-100 lysis buffer and were sonicated.

Statistics. P values were calculated for normally distributed data
sets using a 2-tailed Student’s t test, 1-way ANOVA with Dunnett’s
post-hoc test, 2-way ANOVA and Bonferroni post-hoc tests, or as otherwise indicated. Statistical analyses
represent the mean of at least 3 independent experiments or as otherwise indicated. Error bars represent SEM. P values of less than 0.05 were
considered significant.

Study approval. URECs were obtained from a 3-year-old boy
with clinical JS with a retinal, renal, and cerebellar phenotype, with
compound heterozygous mutations in CEP290, p.Q950Pfs*6 het,
and p.K939N het. Ethical approval was obtained from the National
Research Ethics Service (NRES) Committee North East (14/NE/1076).
URECs were obtained from healthy controls that have been included in the Aetiologic research into Genetic and Occupational/environment-
mental Risk factors for Anomalies in children (AGORA) biobank project.
The Regional Committee on Research involving Human Subjects
(Commissie Mensenbeponden Onderzoek [CMO] Arnhem/Nijmegen)
approved the study protocol. Written informed consent was obtained
from the parents. All mouse work was performed under licenses granted
by the Home Office (United Kingdom) in accordance with the
guidelines and regulations for the care and use of laboratory ani-
mals outlined by the Animals (Scientific Procedures) Act 1986 and
with the approval of the Newcastle University Ethical Review Com-
mitee. Mice with a Cep290+/neo/neo hypomorphic mutation bred on a
pure 129/Ola genetic background (f′, heterozygotes were backcrossed
with C57b6 for 6 generations) were used as previously described (19).
Male and female mice were sacrificed at various ages and geno-
typed. All zebrafish experiments were conducted in accordance with
the Dutch guidelines for the care and use of laboratory animals, with
the approval of the Animal Experimentation Committee (DEC) of the
Royal Netherlands Academy of Arts and Sciences (KNAW) and were
approved by the Animal Care Committee of the University Medical
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