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Exome Capture Reveals *ZNF423* and *CEP164* Mutations, Linking Renal Ciliopathies to DNA Damage Response Signaling

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SUMMARY

Nephronophthisis-related ciliopathies (NPHP-RC) are degenerative recessive diseases that affect kidney, retina, and brain. Genetic defects in *NPHP* gene products that localize to cilia and centrosomes defined them as "ciliopathies." However, disease mechanisms remain poorly understood. Here, we identify by whole-exome resequencing, mutations of *MRE11*, *ZNF423*, and *CEP164* as causing NPHP-RC. All three genes function within the DNA damage response (DDR) pathway. We demonstrate that, upon induced DNA damage, the NPHP-RC proteins ZNF423, CEP164, and NPHP10 colocalize to nuclear foci positive for TIP60, known to activate ATM at sites of DNA damage. We show that knockdown of *CEP164* or *ZNF423* causes sensitivity to DNA damaging agents and that *cep164* knockdown in zebrafish results in dysregulated DDR and an NPHP-RC phenotype. Our findings link degenerative diseases of the kidney and retina, disorders of increasing prevalence, to mechanisms of DDR.

INTRODUCTION

Nephronophthisis (NPHP) is a recessive cystic kidney disease that represents the most frequent genetic cause of end-stage kidney disease in the first three decades of life. NPHP-related ciliopathies (NPHP-RC) are single-gene recessive disorders that affect kidney, retina, brain, and liver by prenatal-onset dysplasia or by organ degeneration and fibrosis in early adulthood. Identification of recessive mutations in more than ten different genes (*NPHP1-NPHP*13) revealed that their gene products share localization at the primary cilia-centrosomes complex and mitotic spindle poles in a cell-cycle-dependent manner, characterizing them as retinal-renal "ciliopathies" (Ansley et al., 2003; Hildebrandt et al., 2011). Multiple signaling pathways downstream of cilia have been implicated in the disease mechanisms of NPHP-RC, including Wnt signaling (Germino, 2005; Simons et al., 2005) and Shh signaling (Huangfu and Anderson, 2005; Huangfu et al., 2003). However, despite convergence of ciliopathy pathogenesis at cilia and centrosomes it remains largely unknown what signaling pathways downstream of cilia and centrosome function operate in the disease mechanisms that generate the NPHP-RC phenotypes.

Centrosomal proteins have been recently implicated in DNA damage response (DDR). Both pericentrin (PCNT), a core centrosomal protein (Doxsey et al., 1994), as well as CEP152, encoding a centrosomal protein required for centriolar duplication (Blachon et al., 2008), are defective in Seckel syndrome, an autosomal-recessive disorder characterized by dwarfism, microcephaly, and mental retardation (Griffith et al., 2008; Kalay et al., 2011; Rauch et al., 2008). *PCNT*- and *CEP152* mutant cells are also defective in ATR-dependent DDR signaling, consistent with the fact that the first mutation identified in Seckel syndrome was in ataxia-telangiectasia mutated and RAD3-related (ATR), a key phosphoinositide 3-kinase-related protein kinase involved in DDR signaling (O'Driscoll et al., 2003), but the mechanism of the signaling defect is not fully understood.

The known *NPHP* genes explain less than 50% of all cases with NPHP-RC, and many of the single-gene causes of NPHP-RC are still unknown (Otto et al., 2011). The finding that some of the recently identified genetic causes of NPHP-RC are exceedingly rare (Attanasio et al., 2007) necessitates the ability to identify novel single-gene causes of NPHP-RC in single affected families. To achieve this goal, we developed a strategy that combines homozygosity mapping (HM) with whole-exome resequencing (WER) (Otto et al., 2010). Because this approach allows identification of multiple different causes of NPHP-RC within a short time frame, it has the potential of delineating pathogenic pathways.

Using this approach, we identify here mutations in three NPHP-RC genes, *MRE11*, *ZNF423*, and *CEP164*, which together suggest involvement of a DDR signaling pathway in NPHP-RC pathogenesis.

RESULTS

Whole-Exome Resequencing Accelerates Discovery of *NPHP-RC* Genes

Identification of monogenic causes of ciliopathies is limited by their rarity (Attanasio et al., 2007), necessitating methods to identify ciliopathy-causing genes in *single* families by using WER. However, WER typically yields hundreds of variants from normal reference sequence (Ng et al., 2009), whereas only a single-gene mutation will represent the disease cause. To overcome this limitation, we here combined WER with HM (Hildebrandt et al., 2009) in sib pairs affected with NPHP-RC and performed functional analysis of the identified genes (Otto et al., 2010).

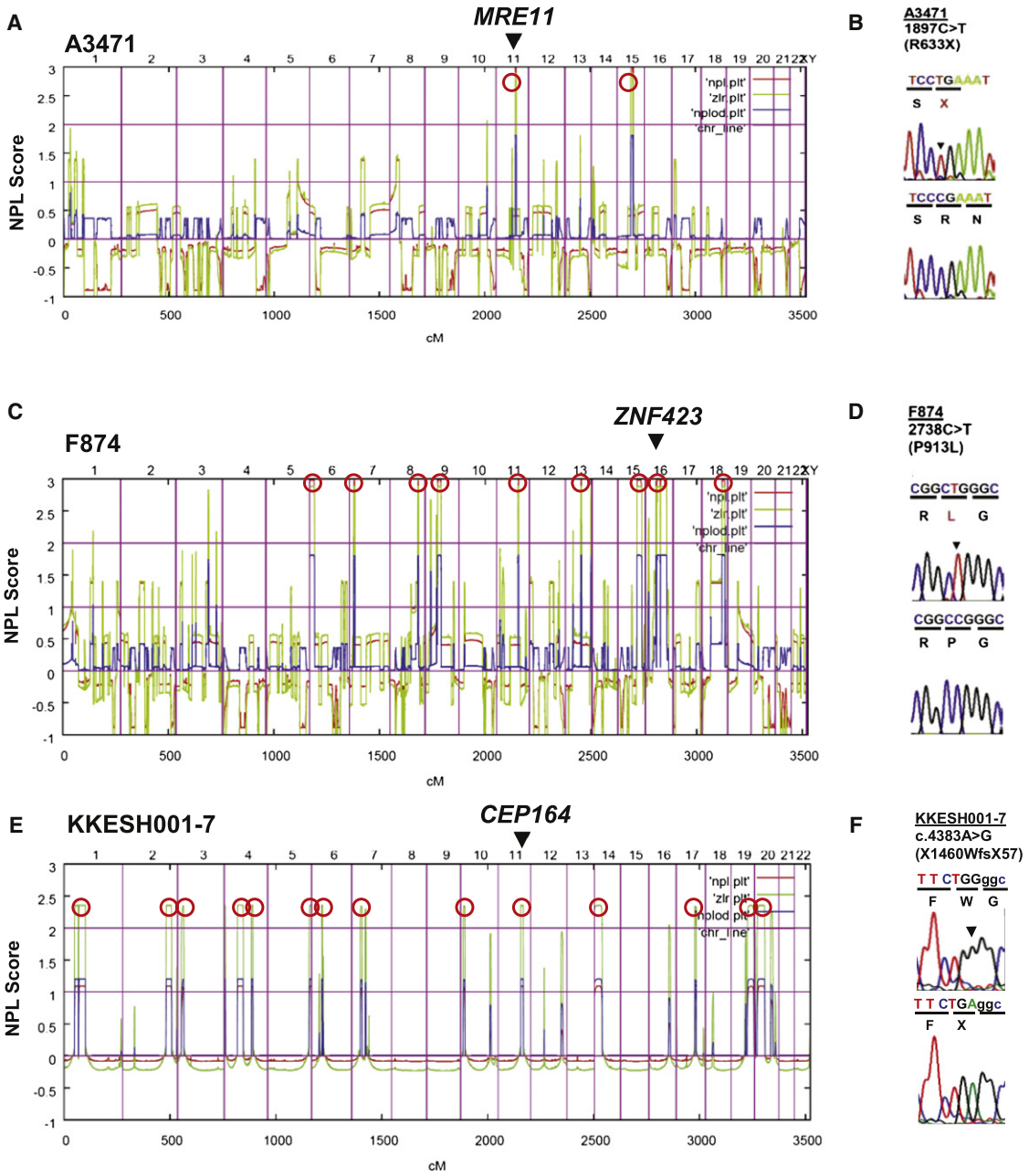

Figure 1. Identification of Recessive Mutations in *MRE11*, *ZNF423*, and *CEP164* in NPHP-RC Using HM and WER

Data regarding HM and mutations are shown for family A3471 with *MRE11* mutation (A and B), family F874 with *ZNF423* mutation (C and D), and family KKESH001 with *CEP164* mutation (E and F). (A, C, and E) Nonparametric lod scores (NPL) are plotted across the human genome in three families (A3471, F874, and KKESH001) with NPHP-RC (see also Table 1). The x axis shows SNP positions on human chromosomes concatenated from *p*-ter (left) to *q*-ter (right). Genetic distance is given in cM. Maximum NPL peaks (Hildebrandt et al., 2009) (red circles) indicate candidate regions of homozygosity by descent. The genes *MRE11*, *ZNF423*, and *CEP164* are positioned (arrow heads) within one of the maximum NPL peaks. (B, D, and F) Homozygous mutations detected in families with NPHP-RC. Family number (underlined), mutation (arrowheads), and predicted translational changes (in parenthesis) are indicated (see also Table 1). Sequence traces are shown for mutations above normal controls. (For additional mutations in other families see also Table 1 and Figure S2).

HM yielded positional candidate regions of homozygosity by descent (Hildebrandt et al., 2009) in families A3471 (two regions), F874 (nine regions), and KKESH001-7 (14 regions) (Figure 1), who had one or more features of NPHP-RC, including NPHP, retinal degeneration, liver fibrosis, or cerebellar degeneration/hypoplasia (Table 1). We then performed WER in one affected individual of each of the three NPHP-RC families (Ng et al., 2009; Otto et al., 2010). Remarkably, each of three NPHP-RC

Table 1. Mutations of *MRE11, ZNF423* and *CEP164* in families with NPHP-RC

Family	Individuals	Ethnic Origin	Nucleotide Alteration[a,b] (Hg19 Position)	Deduced Protein Change	Exon (State)	Continuous Amino Acid Sequence Conservation	Parental Consanguinity	Kidney (Age at ESKF)	Eye (Age at RD)	Other (at Age)
MRE11										
A3471	-21 and -22	Pakistani	c.1897C>T (Chr11: 94,170,372)	p.R633X	16 (hom)	N/A	Yes	No renal failure	Normal -21:	-21: CVA (MRI), ataxia, dysarthria, myoclonus; -22: CVA (MRI), ataxia
ZNF423										
F874	-21 and -22	Turkey	c.2738C>T (Chr16: 49,670,325)	p.P913L	5 (hom)	(*D. rerio*)	Yes	NPHP	ND	CVH Infantile NPHP *Situs inversus*
A106	-21 and -22	Iceland	c.1518delC (Chr16: 49,671,545)	p.P506fsX43	5 (het)	(*X. tropicalis*)	No	PKD	LCA	CVH (Joubert)
A111	-21	?	c.3829C>T (Chr16: 49,525,212)	p.H1277Y	9 (het)	(*D. rerio*)	?	PKD	RD	CVH, NPHP, perinatal breathing abnormality, tongue tumor
CEP164										
F319	-21 and -22	Turkey	c.32A>C (Chr11: 117,209,334)	p.Q11P	3 (hom)	(*Ch. Reinhardtii*)	Yes	NPHP, no Bx; -21: (8 years); -22: (8 years)	-21: RD (11yr, not yet blind); -22: no RP at 8 yrs	-21: obesity? no LF; -22: obesity? LF?
F59	-21, -22, -23	USA (Europe)	c.277C>T, (Chr11: 117,222,588) c.1573C>T (Chr11: 117,252,580)	p.R93W, p.Q525X	5 (het), 13 (het)	(*Ch. Reinhardtii*), N/A	No	NPHP, no Bx; -21: (9 years); -22: (8 years); -23: normal	-21: RD (6 years); -22: LCA (legally blind at 5 months); -23: (2 years)	-22: NY (birth), mild AI; -23: seizures[c], substantial DD, mild ID
NPH505		ND	c.1726C>T (Chr11: 117,257,920)	p.R576X	15 (hom)	N/A	Yes	NPHP, Bx (8 yr)	RD and flat ERG (not blind)	CVH, FD, bilateral PD, bronchiectasis (1 mo), abnormal LFT, obesity
KKESH001-7		Saudi	c.4383A>G (Chr11: 117,282,884)	p.X1460W extX57	33 (hom)	N/A	Yes	normal	(RD) LCA, flat ERG (blind <2 yr)	N/A

AI, aortic insufficiency; Bx, Kidney biopsy; CVH, cerebeller vermis hypoplasia; DD, developmental delay; ERG, electroretinogram; ESKF, end-stage kidney failure; FD, facial dysmorphism; het, heterozygous; hom, homozygous; ID, intellectual disability; LCA, Leber congenital amaurosis; LF, liver fibrosis; LFT, liver function tests; MRI, magnetic resonance imaging; N/A, not applicable; ND, no data; NPHP, nephronophthisis; NPHP-RC, nephronophthisis-related ciliopathies; NY, nystagmus; PD, polydactyly; RD, retinal degeneration; SS, short stature.

[a]All mutations were absent from >270 healthy control individuals and from the ESP Exome Variant Server data base, except the *CEP164* variant p.R576X (allele frequency in European Americans 1/7,019).

[b]cDNA mutation numbering is based on human reference sequences NM_014956.4 for *MRE11*, NM_015069.2 for *ZNF423*, and NP_055771 for *CEP164*, where +1 corresponds to the A of ATG start translation codon.

[c]Seizures were intractable, generalized and/or partial complex.

genes consecutively identified by this approach, *MRE11*, *ZNF423*, and *CEP164*, suggested a functional connection to the DDR pathway (Figure 1; Table 1).

A Mutation of *MRE11* in Progressive Cerebellar Degeneration Suggests Link to DDR

In family A3471, two siblings had cerebellar vermis hypoplasia (CVH), a central feature of NPHP-RC (Table 1). Homozygosity mapping yielded two candidate loci (Figure 1A). WER detected a homozygous truncation mutation (p.R633X) of *MRE11* (Figure 1B; Table 1) previously described for CVH in another Pakistani family (Stewart et al., 1999), suggesting a founder effect for this allele. MRE11 is an essential component of the ATM-Chk2 pathway of DDR (Figure S1 available online), where it recruits ATM (ataxia telangiectasia-mutated) to sites of DNA double-strand breaks (Figure S1A). Rediscovery of this *MRE11* mutation in family A3471 thus generated an unexpected link between NPHP-RC phenotype and the ATM pathway of DDR signaling (Figure S1A).

Patients with the NPHP-RC Joubert Syndrome Have Defects in ZNF423

Another link of NPHP-RC to the ATM pathway of DDR signaling emerged from HM and WER in two siblings (F874) with infantile onset NPHP, CVH, and *situs inversus* (Table 1). SNP mapping yielded nine candidate regions of homozygosity by descent (Figure 1C). We identified in both affected individuals a homozygous missense mutation (p.P913L; conserved in vertebrates) of *ZNF423* (Figure 1D). In addition, when examining 96 additional Joubert syndrome (JS) subjects, we detected two heterozygous-only mutations of *ZNF423*: p.P506fsX43 in family A106 and p.H1277Y in individual A111-21 (Table 1). Mutations of the mouse ortholog *Zfp423* cause reduced proliferation and abnormal development of midline neural progenitors resulting in a loss of the cerebellar vermis (Alcaraz et al., 2006; Cheng et al., 2007) similar to that seen in JS patients with CVH.

ZNF423 encodes a protein with 30 zinc fingers (Figure 2A). Mouse models display phenotypic variability that is subject to modifier genes, environment, and stochastic effects (Alcaraz et al., 2011; Alcaraz et al., 2006), consistent with the variable presentations of NPHP-RC patients. The homozygous mutation p.P913L, located between zinc fingers 21 and 22 (Figure 2A), most likely exerts recessive loss-of-function, analogous to the *Zfp423* mouse models.

We next examined whether the heterozygous-only mutations (Table 1) lead to loss of function via a dominant mechanism, using a proliferation assay in P19 cells (Figures 2B–2D). Mutations were engineered into a FLAG-tagged *ZNF423* cDNA and assayed by a S-phase index, defined as the proportion of transfected cells that incorporate BrdU in 1 hr, 48 hr after transfection. Simple loss-of-function alleles should not interfere with endogenous *Zfp423* activity in this assay. Indeed, overexpression of either wild-type or the homozygous p.P913L allele had no effect (Figure 2D). However, transfection with either the p.P506fsX43 frame-shifting allele, which removes the zinc fingers required for SMAD (similar to mothers against decapentaplegic) and EBF (early B cell factor) interactions, and the H1277Y substitution allele, which destroys the terminal zinc finger required for EBF interaction, reduced the mitotic index to little more than half that of cells transfected with green fluorescent protein (GFP) control vector or other alleles of *ZNF423* (Figures 2B–2D). A dominant mechanism is plausible for the two heterozygous mutations, as each is predicted to interfere selectively with a subset of interaction domains (Figure 2A). Neither subject had siblings, and DNA from parents was not available to determine whether the mutations occurred de novo.

We detected five additional putative mutations in highly conserved (including histidine knuckle) residues of *ZNF423* among JS families (Table S1). Although these mutations have not been confirmed functionally, the high incidence of predicted deleterious mutations found in patients but absent from 270 healthy control individuals, dbSNP, and 1000 Genomes Project data further support identification of *ZNF423* as a causal gene in NPHP-RC and JS.

ZNF423/OAZ was recently shown to interact with the DNA ds-damage sensor PARP1 (poly-ADP ribosyl polymerase 1) (Ku et al., 2003), which recruits MRE11 and ATM to sites of DNA damage (Figure S1A). This indirectly linked ZNF423 to the ATM pathway of DNA damage signaling (Figure S1A). We therefore tested whether *ZNF423* mutations affect interaction between ZNF423 and PARP1. Coimmunoprecipitation verified the association of ZNF423 and PARP1 in reciprocal assays (Figure 2E). More importantly, the truncating mutation P506fsX43, which we detected in a JS patient (Table 1), abrogates this interaction (Figure 2E), whereas H1277Y inhibits multimerization of ZNF423 (Figure 2E). In addition, depletion of *ZNF423* mRNA caused sensitivity to DNA damaging agents (see below).

Furthermore, we identified ZNF423 as a direct interaction partner of CEP290/NPHP6, which is mutated in NPHP-RC (Sayer et al., 2006; Valente et al., 2006). In a yeast two-hybrid screen of human fetal brain library with a *CEP290* (JAS2; amino acids 1917–2479) "bait" we found three in-frame "prey" sequences corresponding to ZNF423 (amino acids 178-406). This interaction was confirmed (Figures 2F and 2G). CEP290/NPHP6 is known to interact with the NPHP-RC protein NPHP5 (Schäfer et al., 2008) and localizes to the ciliary transition zone (Sang et al., 2011).

Mutations of *CEP164* Cause NPHP-RC

We obtained 14 candidate regions by HM in a Saudi family (KKESH001) of first-cousin parents with a child who had LCA (which can be allelic with NPHP-RC) with nystagmus, hyperopic discs, vascular attenuation, diffuse retinal pigment epithelium atrophy, and nonrecordable ERG (Table 1) (Figure 1E). Using WER we detected a homozygous point mutation in *CEP164* (*centrosomal protein 164 kDa*) that abolished the termination codon, adding 57 amino acid residues to the open reading frame (p.X1460WextX57) (Figure 1F, Table 1). The mutation was absent from 96 Saudi healthy controls and from 224 North American LCA patients who lack mutations in other known LCA genes.

We performed exon-PCR and Sanger sequencing of all 31 coding exons for one affected individual in each of 856 different NPHP-RC families (see Extended Experimental Procedures). We detected both mutated *CEP164* alleles in each of three additional families with NPHP-RC (Table 1; Figure S2). We thereby identified recessive mutations of *CEP164* as an additional cause of NPHP-RC. Because of the significant overlap of phenotypic

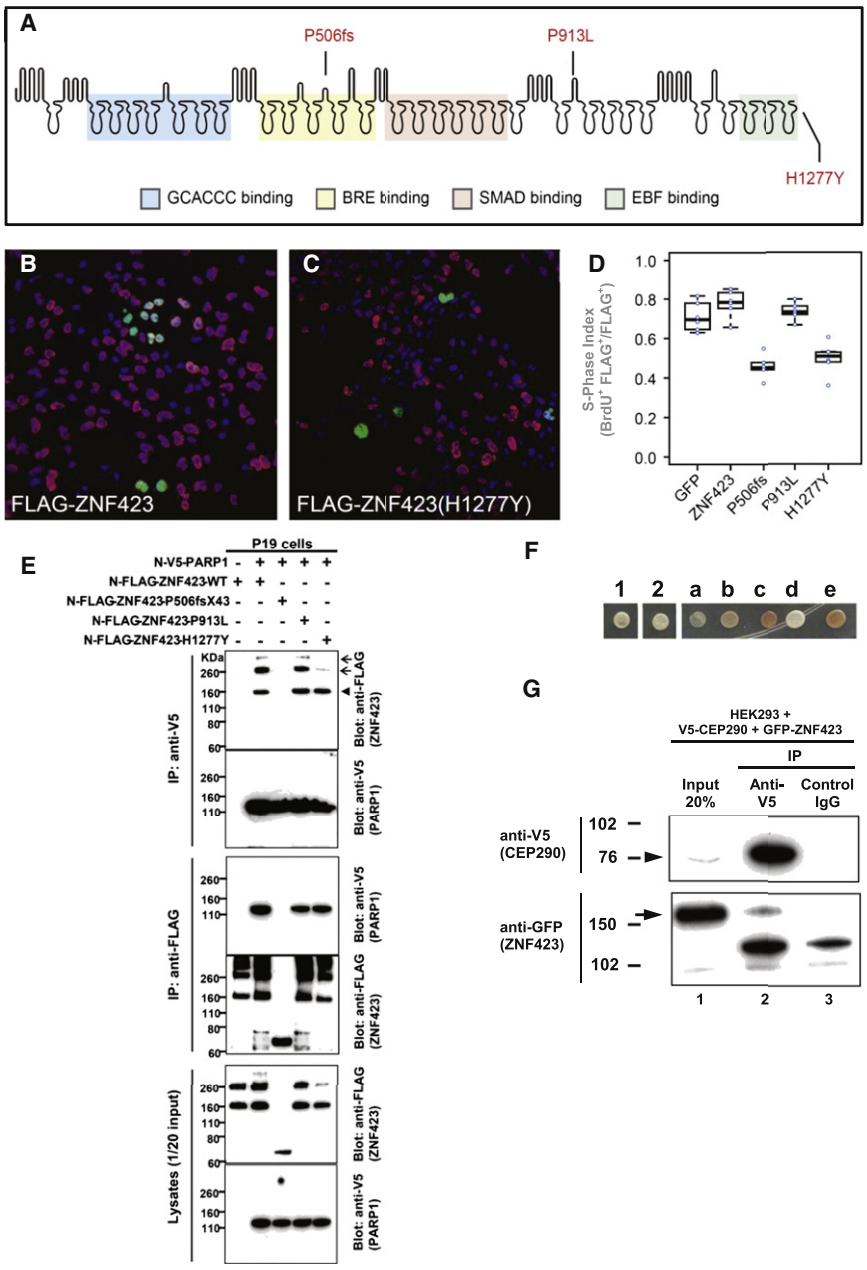

Figure 2. Two *ZNF423* Mutations Have Dominant Negative Characteristics, *ZNF423* Mutation Abrogates Interaction with PARP1, and ZNF423 Directly Interacts with the NPHP-RC Protein CEP290/NPHP6

(A) Amino acid residues altered by NPHP-RC mutations in *ZNF423* are drawn in relation to functional annotation of its 30 Zn-fingers.

(B–D) S-phase index assay (fraction of transfected cells incorporating BrdU) for P19 cells transfected with either wild-type or mutant *ZNF423*. (B) Representative field of cells transfected with wild-type *ZNF423* shows high frequency of $BrdU^+$ $FLAG^+$ double-positive cells. (C) *ZNF423–H1277Y* transfected cells exhibits fewer FLAG–positive cells and a lower proportion that are double positive. (D) S-phase index measured in duplicate transfections for each of three DNA preparations per construct. A GFP construct was used as a nonspecific control. Constructs with *P506fsX43* and *H1277Y* mutations detected in NPHP-RC show significantly reduced S-phase index ($p < 10^{-5}$, ANOVA with post-hoc Tukey honestly significant difference [HSD]).

(E) ZNF423 interacts with PARP1. P19 cells were cotransfected with expression constructs for N terminally FLAG-tagged human full-length ZNF423 and V5-tagged human full length PARP1. Comparable amounts of both proteins were present in all lysates (lower panels). Proteins were precipitated, using anti-V5 (upper panels) and anti-FLAG antibodies (middle panels), respectively. Reciprocal coIP demonstrates interaction between ZNF423 and PARP1. Note that the ZFN423 mutation P506fsX43 abrogates this interaction (arrowhead) and that mutation H1277Y diminishes ZNF423 multimerization (arrow).

(F–G) ZNF423 directly interacts with CEP290/NPHP6. (F) A human fetal brain yeast two-hybrid library screened with human CEP290/NPHP6 (JAS2; aa 1917–2479) fused to the DNA-binding domain of GAL4 (pDEST32) identified ZNF423 as a direct interaction partner of CEP290/NPHP6. The interaction was confirmed using direct yeast two-hybrid assay where 1 and 2 represent colony growth of CEP290 bait with ZNF423 prey. a–e are controls for colony growth on medium deficient in histidine, leucine and tryptophan. (G) HEK293T were cotransfected with human V5-tagged partial human *V5-CEP290* clone and GFP-tagged full-length human *ZNF423* clone. Immunoprecipitation with anti-V5 (lane 2), but not control IgG (lane 3) precipitated both the V5-tagged CEP290 (arrowhead) as well as GFP-tagged ZNF423 (arrow).

features with other forms of NPHP-RC we introduce the alias "NPHP14" for ZNF423 and "NPHP15" for the CEP164 protein.

Although the number of families with *CEP164* mutation is small, our findings support a gradient of genotype-phenotype correlations characteristic of NPHP-RC (Table 1), in which null mutations cause the severe dysplastic phenotypes of Meckel syndrome and JS, whereas hypomorphic alleles cause the milder degenerative phenotypes of NPHP and SLSN (Hildebrandt et al., 2011). *CEP164* is transcribed into three common isoforms (Figures S2A–S2C) and is part of the photoreceptor sensory cilium proteome (Liu et al., 2007). To study subcellular localization of the CEP164 protein, we utilized antibodies against human CEP164 for immunoblotting and immunofluorescence (Figure S3).

Mutation of *CEP164* Interferes with Ciliogenesis

By confocal microscopy of GFP-labeled CEP164 protein with other labels, we show that CEP164 colocalizes in hTERT-RPE cells with the mother centriole, with the mitotic spindle poles, and with the abscission structure in a cell-cycle-dependent way (Figure S4), a feature characteristic of proteins involved in single-gene ciliopathies (Otto et al., 2010; Graser

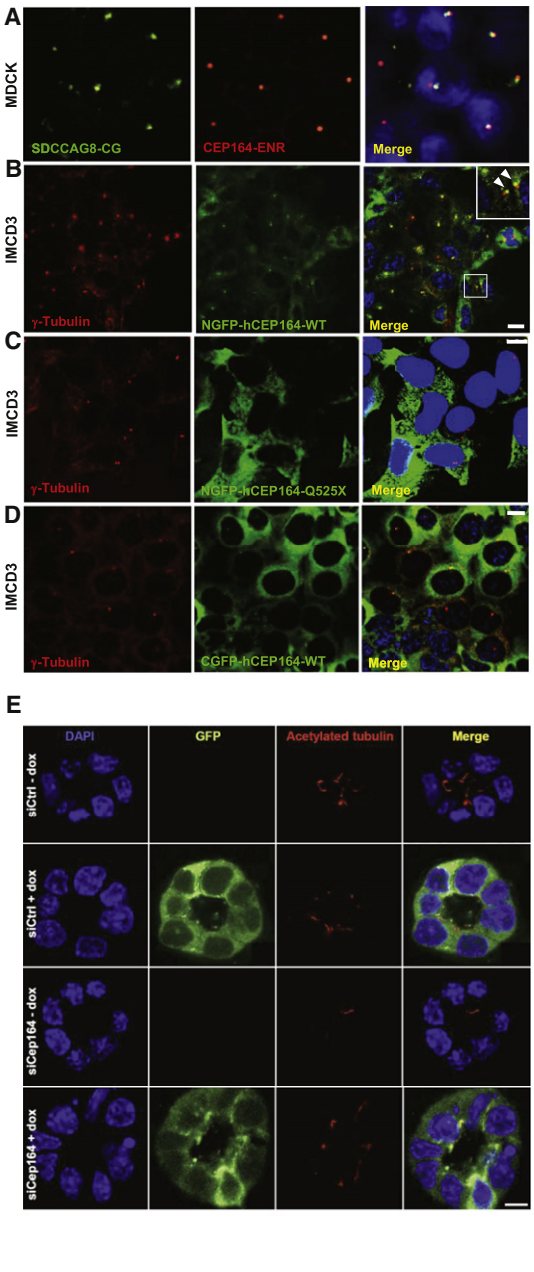

Figure 3. Expression of Mutant *CEP164* in Renal Epithelial Cells Abrogates Localization to Centrosomes

(A) Immunofluorescence using α-SDCCAG8/NPHP10-CG antibody in MDCK cells, labels both centrioles, whereas α-CEP164-ENR antibody demonstrates CEP164 staining at the mother centriole only.

(B) Inducible overexpression of N terminally GFP-tagged human full-length CEP164 isoform 1 (NGFP-CEP164-WT) in IMCD3 cells demonstrates, in addition to a cytoplasmic expression pattern, localization at one of the two centrioles (inset, arrow heads) consistent with selective localization to the mother centriole (Graser et al., 2007). Both centrioles are stained with an anti-γ-tubulin antibody.

(C) In contrast, the centrosomal signal is abrogated upon overexpression of an N terminally GFP-tagged truncated CEP164 construct representing the mutation p.Q525X.

(D) The number of centrosomes positive for CEP164 is reduced upon overexpression of C terminally GFP-tagged human full-length CEP164 isoform 1 (CGFP-hCEP164-WT), which mimics the mutation p.X1460WextX57 that causes a read-through of the stop-codon X1460, adding 57 aberrant amino acid residues to the C terminus of CEP164. Similar data were obtained upon CEP164 expression in hTERT-RPE cells (see also Figures S3B–S3D). IMCD3 cells were stably transfected with the respective *CEP164* constructs in a retroviral vector for doxycyclin-inducible expression (pRetroX-Tight-Pur). Scale bars, 10 μm.

(E–H) Knockdown of *Cep164* disrupts ciliary frequency. (E) Depletion of *Cep164* by siRNA (F) causes a ciliary defect in 3D spheroid growth assays. IMCD3 cells transfected with either siCtrl or *siCep164* were grown to spheroids in 72 hr and immunostained for acetylated tubulin (red). DAPI stains nuclei (blue). Doxycycline induced stably transfected NGFP-hCEP164-WT (green). Space bar represents 5 μm. (G) Nuclei and cilia were scored within a single spheroid to generate ciliary frequencies. si*Cep164* transfected cells manifest lower cilia frequencies (33%) compared to control transfected IMCD3 cells (49%). Induction of NGFP-hCEP164-WT in *siCep164* transfected cells rescues this ciliary defect (57%). 50 spheroids per condition were analyzed in three independent experiments. Error bars represent SEM, n = 3, *p value < 0.0002. (H) Ciliary frequency is not rescued by mutant CEP164. Ciliary frequencies are reduced in si*Cep164* transfected IMCD3 cells (39%) compared to control *siCtrl* transfected IMCD3 cells (54%). Induction of NGFP-hCEP164-Q525X does not rescue this ciliary defect (34%). 50 spheroids per condition were analyzed. Error bars represent SEM, ***p value < 0.0002.

See also Figure S3.

et al., 2007) and that this colocalization is abrogated by mutations (Figure 3, Figures S3C–S3F). We thus demonstrated lack of centrosomal localization for the truncating mutation p.Q525X and for an equivalent of the p.X1460WextX57 mutation.

Loss of function of several genes that cause nephronophthisis in NPHP-RC cause disruption of 3D architecture of renal epithelial cell culture (Otto et al., 2010; Sang et al., 2011). To evaluate CEP164 by this criterion, we transfected murine kidney IMCD3 cells with siRNA oligonucleotides against murine *Cep164*, or random sequences (Ctrl) in 3D spheroid growth assays. Cells transfected with si*Cep164* developed spheroids with overall normal architecture and size, but with markedly reduced frequency of cilia (Figures 3E–3H). We conclude that Cep164 affects ciliogenesis or maintenance but that the overall architecture of renal 3D growths is not as grossly affected as we have previously seen for knockdown of other NPHP-RC genes (Sang et al., 2011).

NPHP-RC Proteins Colocalize with the DDR Protein TIP60 to Nuclear Foci

A noncentrosomal localization for CEP164 was described by demonstrating its translocation to nuclear foci in response to DNA damage (Pan and Lee, 2009; Sivasubramaniam et al., 2008). CEP164 plays a role in DDR signaling where it interacts with the DDR protein ATRIP (Figure S2C), is activated by the DDR proteins ATM and ATR, and is necessary for checkpoint-1 (Chk1) activation. Abrogation of CEP164 function leads to loss of G_2/M cell-cycle checkpoint and aberrant nuclear divisions (Sivasubramaniam et al., 2008).

Localization of SDCCAG8 (alias NPHP10) (Otto et al., 2010), shows nuclear foci in hTERT-RPE cells in addition to its centrosomal localization (Figures 4B–4C). Transient shRNA knockdown confirmed specificity of the signal (Figures S4B–S4D). SDCCAG8/NPHP10 did not colocalize with markers for PLM bodies (Janderová-Rossmeislová et al., 2007) or CENP-C (marking chromosomal centromeres) (Figures S5A and S5B). In contrast, SDCCAG8/NPHP10 fully colocalized with SC35 in hTERT-RPE cells (Figures 4A–4C). SC35, also known as serine/arginine-rich splicing factor 2 (SRSF2), plays a role in DDR by controlling cell fate decisions in response to DNA damaging agents (Edmond et al., 2011; Reinhardt et al., 2011). SC35 marks hubs of enhanced gene expression (Szczerbal and Bridger, 2010), is phosphorylated by topoisomerase I (Elias et al., 2003), and is required for genomic stability during mammalian organogenesis (Xiao et al., 2007). Moreover, ZNF423 also fully colocalizes (Figure 4D), and CEP164 partially colocalizes (Figure 4E) with SC35 in nuclear foci. Consequently, ZNF423 and CEP164 also colocalize with SDCCAG8/NPHP10 in SC35-positive nuclear foci (Figures 4F and 4G).

SC35 functions within a TIP60 complex, in which TIP60 acetylates SC35 on lysine 52 (Figure S1B), modifying the role of SC35 in the promotion of apoptosis and inhibition of G_2/M arrest (Edmond et al., 2011), which is regulated by the checkpoint proteins Chk1 and Chk2 (Figure S1D). Interestingly, the TIP60 protein, together with the heterotrimeric MRN complex (of which MRE11 is a component) constitutes the major activator of ATM within the ATM pathway of DDR signaling (Ciccia and Elledge, 2010) (Figure S1A). In hTERT-RPE cells the ATM activator TIP60 colocalizes to nuclear foci with SC35/SRSF2 (Figure 4H) and partially with the identified NPHP-RC protein CEP164 (Figure 4I). We thus identify a group of NPHP-RC proteins and demonstrate that they colocalize to nuclear foci with the DDR proteins TIP60 and SC35. These gene products include the identified NPHP-RC proteins ZNF423 and CEP164 as well as SDCCAG8/NPHP10. Interestingly, the protein OFD1, which is mutated in the ciliopathy oral-facial-digital syndrome, is part of the TIP60 complex. We recently identified OFD1 as a direct interaction partner of SDCCAG8/NPHP10 (Figure S1B) (Otto et al., 2010).

Cep164 Associates with DDR Proteins and Its Loss Causes DDR Defects

Because one of the central mechanisms controlled by DDR signaling is cell-cycle regulation through phosphorylation of checkpoint-1 (Chk1) and checkpoint-2 (Chk2) proteins (Figure S1D), we tested whether Chk proteins are recruited to SC35/SRSF2-positive nuclear foci. SC35 and p317-Chk1 colocalize to nuclear foci in hTERT-RPE cells (Figure 4J). We then tested whether localization of CEP164 to nuclear foci was inducible by DNA damage. Following irradiation with 20–50 J/m^2 of UV light, CEP164-positive nuclear foci condensed to larger size and colocalized with TIP60 and Chk1 to foci of similar size (Figures 4K–4O). TIP60 and p317-Chk1 colocalized within these foci (Figure 4P). We thus demonstrate that CEP164 translocates in response to DNA damage to nuclear foci that contain the DDR proteins TIP60 and Chk1.

Lagging chromosomes on anaphase spindles (''anaphase lag'') are a hallmark of many mutations that affect mitotic checkpoint integrity. We show that si*Cep164* knockdown in IMCD3 cells increased anaphase lag from 1% in si*Ctrl* controls to 21% in si*Cep164*-treated cells (Figures 5A and 5B, $p = 0.04$). This phenomenon was specific, since doxycycline-inducible expression of *WT-CEP164* during *Cep164* siRNA knockdown reduced the incidence of anaphase lag to just 4% (Figure 5B). These data indicate a requirement for Cep164 at the G_2/M checkpoint.

The DDR pathway can be activated by the CDK inhibitor roscovitine, which also reduces Chk1 expression (Maude and Enders, 2005). Roscovitine reduces the development of kidney cysts in the *Nphp9* mouse model, *Jck* (Bukanov et al., 2006). We therefore tested the influence of roscovitine (targeting CDK2, 5, 7 and 9) on DDR activation in IMCD3 cells. Immunofluorescence shows increased uniform distribution of γH2AX (activated H2AX phosphorylated at Ser139) in the nucleus of IMCD3 cells upon roscovitine treatment in irradiated cells, indicating partial DDR activation (Figure 5C). Second, in cells treated with roscovitine, UV irradiation caused enhanced γH2AX staining with a prominent nuclear foci pattern, characteristic of strong DDR activation (Figure 5D). Immunoblotting showed that roscovitine decreased the amount of CEP164 present in both control and UV-irradiated cells (Figures 5E and 5F). This was most likely due to translocation of CEP164 into the nucleus upon roscovitine treatment, as shown by subcellular fractionation (Figure 5F). As expected, UV radiation increased phosphorylation of Chk1 at Ser317 (p-Chk1) (Figure 5E), and roscovitine decreased Chk1 protein expression and abrogated UV-induced p-Chk1 in both cytoplasm and nucleus (Figures 5G and 5H). These data indicate that CDK inhibition by roscovitine causes nuclear translocation of CEP164 and inhibits Chk1 activation. γH2AX activation by roscovitine may restore cell-cycle control by Chk2 activation instead (Maude and Enders, 2005).

Human Wild-Type *CEP164* but Not Its NPHP-RC Truncation Mutant Rescues IMCD3 Cell Proliferation

In clonally selected IMCD3 cells expressing wild-type human *CEP164* cDNA construct *N-GFP-CEP164-WT* under doxycycline (Dox) control, depletion of endogenous mouse *Cep164* retarded proliferation in comparison to either undepleted control cells or undepleted cells that were Dox-induced to overexpress *N-GFP-CEP164-WT* alone (Figure 5G). Cep164-depleted growth was rescued by Dox-induced expression of human *N-GFP-CEP164-WT* (Figure 5G). Cells expressing truncated cDNA construct *N-GFP-CEP164-Q525X*, modeling the NPHP-RC mutation in family F59, exhibited retarded growth, even when the endogenous Cep164 was present (Figure 5H), consistent with a dominant negative effect. Further depletion of the endogenous

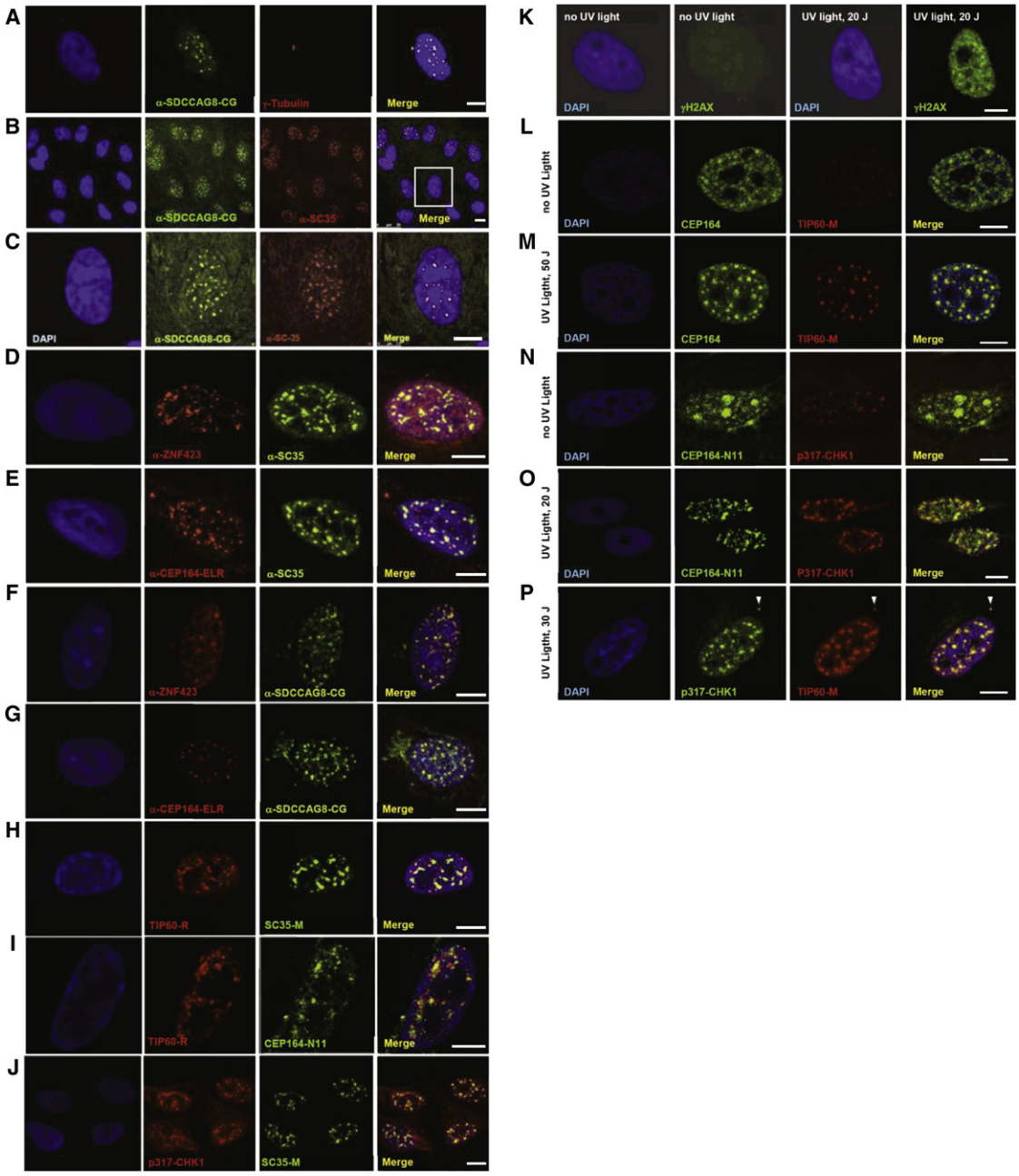

Figure 4. Colocalization upon Immunofluorescence of the NPHP-RC Proteins SDCCAG8/NPHP10, ZNF423 and CEP164 to Nuclear Foci that Are Positive for the DDR Signaling Proteins SC35, TIP60 and Chk1 in hTERT-RPE Cells

(A–G) Colocalization of NPHP-RC proteins with SC35 in nuclear foci. SDCCAG8/NPHP10 (A-C) and ZNF423 (D) fully colocalize to nuclear foci with SC35, and (E) CEP164 partially colocalizes with SC35. SDCCAG8/ NPHP10 also colocalizes with the identified NPHP-RC proteins ZNF423 (F) and CEP164 (G).

(H–J) Colocalization of NPHP-RC proteins with the DDR protein TIP60 and Chk1 to nuclear foci. (H) TIP60 fully colocalizes with SC35. (I) TIP60 partially colocalizes with CEP164. (J) Chk1 fully colocalizes with SC35/ SRSF2. DNA is stained in blue with DAPI. Scale bars, 5 μm.

(K–P) Colocalization of DDR and NPHP proteins upon induction of DDR by UV radiation in HeLa cells. (K) Following irradiation of HeLa cells with UV light at 20 J/m^2 a strong immunofluorescence signal of an anti-γH2AX antibody indicates activation of DDR. (L–M) Upon irradiation with UV light, CEP164-positive nuclear foci condense and colocalize with TIP60 foci of similar size. (N–O) In untreated cells (N) a pattern of broad CEP164 speckles, which are Chk1-negative and locate to DAPI-negative domains, changes to a pattern of multiple smaller foci (O) that are double positive for both CEP164-N11 and Chk1. (P) p317-Chk1 fully colocalizes with TIP60 to nuclear foci and to the centrosome (arrowhead).

See also Figures S4, S5A, and S5B.

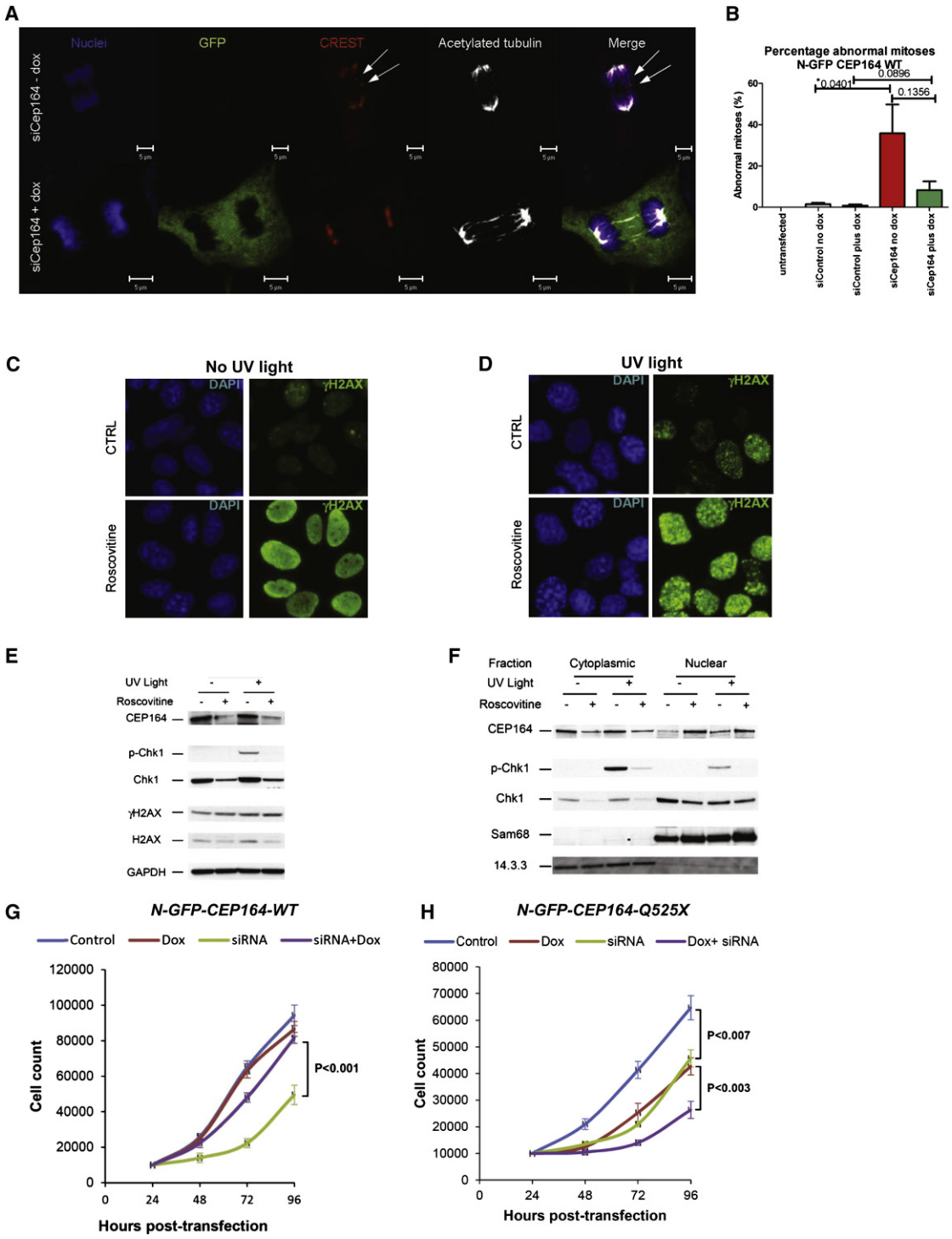

Figure 5. Knockdown of *Cep164* Causes Anaphase Lag and Retarded Cell Growth

(A and B) Knockdown of *CEP164* causes anaphase lag. si*Cep164* knockdown in IMCD3 cells increased anaphase lag incidence from 1% after si*Ctrl* to 21% after si*Cep164*-treated cells (n > 250 anaphases, five independent experiments). CREST antiserum (red) and DAPI (blue) confirmed the presence of incomplete mitotic congression and unattached kinetochores during late anaphase (white arrows). Doxycycline-inducible expression of *WT-CEP164* during *Cep164* siRNA knockdown reduced the incidence of anaphase lag to 4%, whereas untransfected IMCD3 cells had no detectable anaphase lag (0%) (B). Bars represent SEM, p values (Student's t test) are indicated above the bar graph.

(C–F) The effect of roscovitine on UV-induced DDR. Cells were UV irradiated with 30 J/m^2 and analyzed 1 hr after UV irradiation. Where indicated, cells were preincubated for 24 hr with the CDK inhibitor roscovitine (80 μM). (C and D) Immunofluorescence analysis showed that roscovitine triggered uniform nuclear

Cep164 in *N-GFP-CEP164-Q525X*-expressing cells showed an additive effect on growth retardation, confirming the dominant negative effect of *N-GFP-CEP164-Q525X* in this experimental system (Figure 5H).

CEP164 Directly Interacts with CCDC92 and TTBK2

NPHP-RC proteins are known to interact with other NPHP-RC proteins in the dynamic "NPHP-JS-MKS interaction network" (Sang et al., 2011).To identify novel direct interaction partners of CEP164, we performed yeast two-hybrid screening. We identified CCDC92 and TTBK2 as direct interactors of CEP164 (Figures S5C–S5J). Interactions between CEP164 and both partners were validated by GST pull-down (Figure S5D) and coimmunoprecipitation (Figures S5E–S5H). Immunofluorescence showed that CCDC92 fully colocalizes with CEP164 at the mother centriole (Figures S5I and S5J).

CEP164 also interacted with NPHP3 and weakly with NPHP4 (Figures S6A–S6B), demonstrating that CEP164 is in a complex with other known NPHP-RC proteins (Figures S1A and S1B). The DDR protein DDB1 interacted with NPHP2 (Figures S6C and S6D). The disheveled protein (Dvl), which is a central component of the Wnt pathway, interacts with NPHP2/inversin targeting Dvl for proteasomal degradation, thereby triggering a switch from canonical to noncanonical Wnt signaling (Germino, 2005; Simons et al., 2005). We identify interaction between Dvl3 and CEP164 (Figures S6E–S6H). Immunocytochemistry reveals that endogenous Dvl3 and CEP164 share centrosomal localization (Figure S6E). We demonstrate that GST-CEP164 (aa 2–195) is sufficient to pull down endogenous Dvl3 from the cellular lysate (Figure S6F). Domain mapping for Dvl3 suggests that CEP164 interacts with the proline-rich region of Dvl3, because only mutants containing this sequence efficiently coimmunoprecipitate with CEP164-GFP (Figure S6G). Interestingly, only wild-type CEP164-mCherryRFP but not the NPHP-RC causing mutant CEP164-Q525X detected in family F59 (Table 1) can be efficiently immunoprecipitated with Dvl3 (Figure S6H), further supporting its pathogenic role.

cep164 Loss of Function Causes NPHP-RC and DDR Activation in Zebrafish

To test in a vertebrate model whether loss of *cep164* function results in both, an NPHP-RC phenotype as well as DDR activation, we performed *cep164* knockdown in zebrafish embryos using morpholino-oligonucleotides (MOs) (Figure 6). A *p53* MO was injected to reduce off-target MO effects (Robu et al., 2007). At 28 hr postfertilization (hpf) we observed the ciliopathy phenotypes of ventral body axis curvature and cell death (Figure 6A–6C). Embryos showed increased expression of phosphorylated γH2AX (Figures 6D and 6E). At 48 hpf, *cep164* morphants displayed the typical ciliopathy phenotype of abnormal heart looping (Figures 6F–6I). At 72 hpf, embryos developed further NPHP-RC phenotypes, including pronephric tubule cysts (Figures 6J and 6K), hydrocephalus, and retinal dysplasia (Figures 6L–6M).

Depletion of CEP164 or *ZNF423(Zfp423)* Causes Sensitivity to DNA Damaging Agents

To assess whether depletion of *CEP164* causes sensitivity to DNA damage, *Cep164* expression was stably suppressed in the mouse renal cell line IMCD3 (Figures 6P and 6Q). *Cep164* knockdown resulted in a dose-dependent increase of γH2AX intensity levels in a FACS analysis, signifying increased radiation sensitivity to IR and perturbed DDR. Cellular sensitivity to IR was also seen in cells depleted of *CEP164* using a multicolor competition assay (MCA) (Smogorzewska et al., 2007) (Figures S7A and S7B).

To test whether *ZNF423*(*Zfp423*) affects DDR, we examined P19 cells, which express high levels of endogenous *Zfp423* (Figures 6R–6T). Replicate cultures infected with lentivirus expressing either scrambled control or *Zfp423*-targeted shRNA were exposed to 0–10 Gy of X-irradiation and imaged for Zfp423 and nuclear γH2AX foci (Figure 6R). Quantification showed significantly increased γH2AX intensities in *Zfp423*-depleted cells at lower (0.5 and 1.0 Gy) exposures (Figure 6S), but the effect was nonsignificant when corrected for the number of exposures. To determine whether sensitivity to lower dose is reproducible, we exposed 32 additional cultures at 1.0 Gy (Figure 6T). Normalized γH2AX fluorescence in *Zfp423* knockdown had both higher mean (9.6 versus 4.7) and median (6.6 versus 5.2) values than control (Figure 6T). These data replicate the radiation sensitivity with high significance ($p = 0.018$, Mann-Whitney U test, 2 tails), indicating that P19 cells require Zfp423 for quantitatively normal DDR. These results are further confirmed by multicolor competition assays Figure S7C.

DISCUSSION

Disease Gene Identification Implicates NPHP-RC Proteins in DDR

Many DDR signaling proteins localize to nuclear foci and to centrosomes. In addition, dual localization of proteins at

distribution of γH2AX (activated H2AX phosphorylated at Ser139) in non-UV irradiated cells suggesting partial DDR activation (C). UV radiation caused enhanced γH2AX staining with a prominent nuclear foci pattern, characteristic of strong DDR activation (D). (C and D) Error bars denote SEM.

(E and F) The effect of roscovitine on UV-triggered subcellular localization of CEP164 and Chk1. CEP164 and Chk1 proteins, along with nuclear marker Sam68 and cytoplasmic marker 14.3.3 were analyzed by Western blot. Roscovitine decreased the amount of CEP164 present in control and UV-irradiated cells (E). This was most likely due to translocation of CEP164 into the nucleus upon roscovitine treatment as shown by subcellular fractionation (F). As expected, UV radiation increased phosphorylation of Chk1 at Ser317 (p-Chk1) (E), and roscovitine decreased Chk1 protein expression and abrogated UV-induced p-Chk1 in both cytoplasm and nucleus (E-F). Proteins 14.3.3 and Sam68 serve as controls for cytoplasmic versus nuclear fraction, respectively. See also Figure S6.

(G and H) Transient knockdown of *Cep164* inhibits proliferation, which is rescued by wild-type but not mutant CEP164. In clonally selected and doxycycline (Dox)-inducible mouse IMCD3 cells siRNA knockdown was performed. (G) IMCD3 cells depleted of murine *Cep164* grew more slowly (siRNA, green line) than nondepleted cells (control, blue line) or the nondepleted cells induced to express human wild-type CEP164 (Dox, red line). Expression of WT *Cep164* in siRNA-depleted cells rescued the slow growth phenotype of *Cep164* depletion (siRNA$^+$dox, purple line). (H) As in (G), except mutant *Cep164* cDNA (*CEP164-Q525X*) was expressed under doxycyclin control. Expression of this allele itself had a negative impact on cell growth (green line), suggesting a dominant negative effect. An even greater negative effect was seen when the endogenous Cep164 was depleted in cells expressing *CEP164-Q525X* (siRNA$^+$dox, purple line). The average counts are plotted with standard deviations. Asterisks indicate significant differences by unpaired Student's t test ($p < 0.05$).

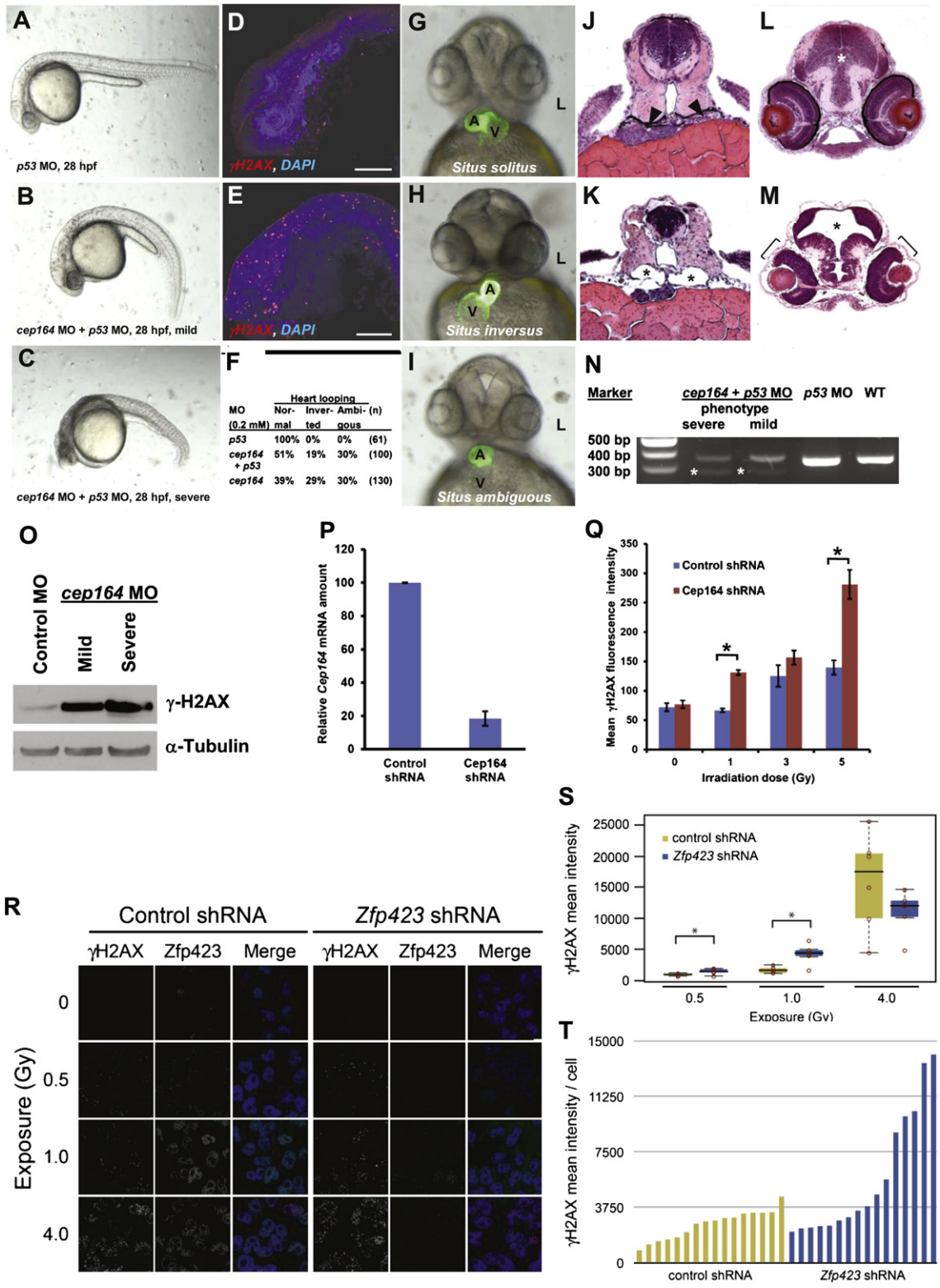

Figure 6. Knockdown of *cep164* in Zebrafish Embryos Results in Ciliopathy Phenotypes, and Knockdown of *Cep164* or *Zfp423(Znf423)* Causes Sensitivity to DNA Damage

A morpholino-oligonucleotide (*cep164* MO) targeting the exon 7 splice donor site of zebrafish *cep164* was injected into fertilized eggs at the one to four-cells stage together with *p53* MO (0.2 mM) to minimize nonspecific MO effects.

centrosomes and at nuclear foci has been demonstrated for multiple known DDR proteins related to ataxia or CVH. Individuals with mutations in the three NPHP-RC-causing genes that we identify here, *MRE11*, *ZNF423*, and *CEP164*, share the NPHP-RC phenotypes of CVH and ataxia. The first protein that strongly linked DDR signaling to the ataxia phenotype was the protein ATM (ataxia telangiectasia mutated) (Savitsky et al., 1995). Interestingly, we identify here in individuals with NPHP-RC, CVH and ataxia, mutations in proteins that colocalize to nuclear foci with TIP60 and/or its interaction partner SC35. These proteins are ZNF423, CEP164, and the previously identified NPHP-RC protein SDCCAG8/NPHP10 (Otto et al., 2010).

Our findings support the notion that many products of genes, which if mutated cause NPHP-RC and/or ataxia, play a role in DDR and are part of a dynamic protein complex.

A DDR-Based Pathogenic Hypothesis of Dysplasia and Degeneration in NPHP-RC

We here generate evidence that NPHP-RC proteins exhibit dual localization at centrosomes and in nuclear foci and that they play a role in DDR. We also demonstrate the parallel occurrence of DDR defects with an NPHP-RC phenotype upon *cep164* knockdown in zebrafish. We therefore propose that defects in DDR may participate in the pathogenesis of NPHP-RC. Whereas multiple signaling pathways have been implicated in the pathogenesis of NPHP-RC (Hildebrandt et al., 2011), including noncanonical (Simons et al., 2005) and canonical Wnt signaling (Yu et al., 2009), Shh signaling (Huangfu et al., 2003), and mitotic spindle orientation (Fischer et al., 2006), none of them consistently explains the phenotypes observed. In particular, none of these mechanisms provides a model for the dichotomy of dysplasia phenotypes resulting from null-alleles of *NPHP-RC* genes versus degenerative phenotypes resulting from hypomorphic alleles of the same *NPHP-RC* genes. Based on our findings we here propose a pathogenic hypothesis for NPHP-RC that implicates DDR signaling as a relevant disease mechanism. Within this hypothesis, loss of function of NPHP-RC proteins with a dual role in DDR and centrosomal signaling, would cause disturbance of cell-cycle checkpoint control, which is particularly detrimental for embryonic and adult progenitor cell survival. This notion is in accordance with the orthologous mouse model for *ZNF423* loss of function, the *Zfp423*$^{-/-}$ mouse, in which CVH with ataxia is caused by defective granule progenitor proliferation in the cerebellum (Alcaraz et al., 2006).

Within this pathogenic hypothesis for NPHP-RC, a DDR signaling defect would lead to impairment of cell-cycle checkpoint control, which in turn would cause lack of progenitor cells. This hypothesis could lend a possible explanation to the following persisting conundrum of NPHP-RC pathogenesis: in certain NPHP genes (e.g., NPHP3, 6, or 8) *null mutations* cause severe, congenital-onset phenotypes of dysplasia and malformation in kidney, eye, CVH, and liver, whereas *hypomorphic mutations* in the same gene cause only late-onset degenerative phenotypes such as renal tubular degeneration and fibrosis (nephronophthisis), retinal degeneration (Senior-Loken syndrome), and liver fibrosis. However, the disease mechanisms of neither the degenerative nor the dysplastic phenotypes are understood. These findings suggest that null mutations act during morphogenesis in embryonic development causing dysplasia and malformation, whereas hypomorphic mutations act during the "chronic" processes of tissue maintenance and repair, which are spread out over months or years of the life of an organism. Because DDR signaling is in high demand under conditions of high proliferation during development (morphogenesis), causing high "replication stress" to progenitor cells, tissue dysplasia would be an expected pathogenic outcome. Conversely, during tissue maintenance, low replication stress would be expected, but persistent DDR impairment would allow

(A–E) Whereas *p53* MO injection (n = 67) did not produce any phenotype (A), coinjection of *cep164* MO at 28 hpf caused the mild ciliopathy phenotype of ventral body axis curvature in 48% of embryos (60/125) (B). 50% of embryos (62/125) showed severe cell death throughout the body as judged by gray-appearing cells in the head region (C). Embryos with severe cell death also showed increased expression of phosphorylated γH2AX (D) compared to *p53* MO control (E). Most embryos with massive cell death did not survive beyond 48 hpf.

(F–I) At 48 hpf, surviving *cep164* morphants displayed the ciliopathy phenotype of laterality defects. Whereas *p53* MO did not cause any abnormal heart looping (F and G), *cep164* MO caused inverted heart looping (H) or ambiguous heart looping (I). (A, atrium; L, left; V, ventricle).

(J–M) At 72 hpf, *cep164* morphant embryos developed further ciliopathy phenotypes. When compared to *p53* MO controls (J), pronephric tubules (arrow heads) exhibited cystic dilation (K), asterisks) in 25% (7/28) of embryos, compared to *p53* MO controls (J and L), 0% (0/67) of which showed kidney cysts, hydrocephalus (asterisk), or retinal dysplasia (brackets) (M).

(N) At 0.2 mM, *cep164* MO knockdown effectively altered mRNA processing as revealed by RT-PCR. The wild-type (WT) mRNA product is 339 bp. A shorter aberrantly spliced mRNA product appeared in *cep164* morphants (asterisks), and the normal mRNA product was significantly reduced. *p53* MO alone did not affect *cep164* mRNA processing.

(O) Quantification of γ-H2AX levels in *cep164* MO morphants. Whole-fish lysates were prepared from morphants injected with control MO (p53 0.2 mM) or *cep164* MO (p53 0.2 mM, *cep164* 0.2 mM). Injection of *cep164*-targeting MO causes upregulation of γ-H2AX in *cep164* morphant embryos signifying perturbed DDR. γ-H2AX levels correlate with the phenotypic severity of the *cep164* morphants (see A–C). Anti-α-tubulin antibody was used to show equal loading.

(P and Q) *Cep164*-deficient IMCD3 cells exhibit radiation sensitivity. In IMCD3 cells transduced with shRNA retrovirus, *Cep164* expression was suppressed by shRNA knockdown to about 20% of control as judged by qPCR (P). *Cep164* knockdown resulted in a dose-dependent increase of γH2AX-positive cells in a FACS assay, signifying increased radiation sensitivity to IR and perturbed DDR. See also Figure S7. In (Q) the level of significance of two-tailed t test ($p < 0.001$) is indicated by an asterisk. Error bars denote SEM.

(R–T) *Zfp423(Znf423)*-deficient P19 cells exhibit radiation sensitivity. P19 cells transduced with shRNA lentivirus were exposed to the indicated level X-irradiation. Zfp423 and γH2AX immunofluorescence was quantified in matched replicate cultures for each virus 2 hr after irradiation. (R) Representative images illustrate dose-responsiveness of γH2AX and effective knockdown of *Zfp423* expression. (S) γH2AX intensity normalized to DAPI$^+$ nuclei is increased following IR at 0.5 and 1.0 Gy, signifying increased IR sensitivity and perturbed DDR (2 fields from each of 6 replicate cultures per condition). Asterisks, uncorrected pair-wise $p < 0.05$, Mann-Whitney U test, 2 tails. (T) Histogram shows average γH2AX intensity per cell in 16 additional replicate cultures for each shRNA at 1.0 Gy exposure. $p = 0.018$, Mann-Whitney U test, 2 tails. See also Figure S7. Box plots delimit quartiles in (S).

slow accumulation of DNA damage with a phenotype of tissue degeneration.

At least two related findings support this model: (1) In a mouse conditional knockout model of the cystic kidney disease gene *Pkhd1*, knockout of the gene before 2 weeks of postnatal life, up to which a high proliferation state prevailed, caused (dysplastic) kidney cysts, whereas knockdown after 2 weeks of postnatal life, when proliferation rate was shown to be dramatically reduced, only caused occasional cysts, the number of which increased when tissue injury was induced (Piontek et al., 2007). This phenomenon could be explained by different degrees of replication stress, and thereby DDR activation, under different proliferation rates. (2) In Seckel syndrome (primordial dwarfism), a progeria syndrome with CVH caused by mutation of the centrosomal and DDR proteins ATR, CEP152, or pericentrin, the degree of cerebellar impairment is dependent on cell proliferation state (Kalay et al., 2011; Murga et al., 2009; Rauch et al., 2008).

A DDR-Based Pathogenic Hypothesis Might Explain Specific Organ Involvement in NPHP-RC

Regarding the question why organ degeneration occurs in specific organs and at characteristic sites, it is tempting to speculate that the specific tissue regions or cell types affected in NPHP-RC are more strongly exposed to genotoxins. In the kidney, the distal convoluted tubule segment, around which most fibrotic changes occur, is more strongly exposed to genotoxins such as hydroxyurea. Retinal degeneration could be caused by postnatal accumulation of UV light-induced DNA damage. Most strikingly, bile duct-surrounding cholangiocytes in the liver are the one mammalian cell type that is most strongly exposed to genotoxins that are generated by the liver for excretion in bile.

In summary, a testable pathogenic hypothesis of NPHP-RC that implicates DDR signaling, impaired cell-cycle checkpoint control with lack of progenitor cells might potentially explain some of the ill understood features of ciliopathies:

(1) It might provide a mechanism for the dual phenotypes of degeneration/dysplasia seen in NPHP-RC in kidney, eye, cerebellum and liver.
(2) It would implicate in the NPHP-RC pathogenesis, lack of response to replication stress-sensing as a functional basis for understanding the dualism of dysplasia that occurs in high-proliferation states during development/morphogenesis or repair versus degeneration, which occurs during the low proliferation state of tissue maintenance.
(3) It would characterize the degenerative phenotypes as diseases of "organ-specific premature aging," thereby pointing in new directions for identification of small compounds for therapy including cyclin inhibitors.

EXPERIMENTAL PROCEDURES

Research Subjects

We obtained human samples following informed consent from individuals with NPHP-RC. Approval for human subjects research was obtained from the University of Michigan Institutional Review Board and the other institutions involved. The diagnosis of NPHP-RC was based on published clinical criteria (Chaki et al., 2011).

Linkage Analysis

For genome-wide HM the GeneChip Human Mapping 250k *Sty*I Array from Affymetrix was used. Nonparametric LOD scores were calculated using a modified version of the programs GENEHUNTER 2.1 (Kruglyak et al., 1996; Strauch et al., 2000) and ALLEGRO (Gudbjartsson et al., 2000) in order to identify regions of homozygosity as described (Hildebrandt et al., 2009; Sayer et al., 2006).

Bioinformatics

Genetic location is according to the February 2009 Human Genome Browser data (http://www.genome.ucsc.eduH).

Immunoblotting and Immunoprecipitation

Immunoblotting and immunoprecipitation were performed as previously described (Bryja et al., 2007). HEK293 cells were transfected with the indicated constructs and lysed 48 hr later. Samples were analyzed using SDS-PAGE and western blotting, or subjected to immunoprecipitation. The antibodies used for immunoprecipitation are described in Extended Experimental Procedures.

cep164 Zebrafish Morpholino Oligo-Mediated Knockdown

MOs were obtained from Gene Tools, LLC (Philomath, OR). MOs (*cep164* at 0.1 mM, standard control at 0.2 mM, and *p53* MO at 0.2 mM) were injected into zebrafish embryos at 1–4 cell stages. Embryos were fixed at 27 hpf with 4% PFA/PBS +1% DMSO overnight, permeablized with acetone at −20°C for 7 min, and stained with antibody against phosphorylated zebrafish γH2AX (1:1,000, gift from Amatruda lab at UT Southwestern) or antibody against cleaved Caspase-3 (1:200, BD Biosciences). Alex568-anti rabbit IgG was used at 1:2,000 and 1:1,000 respectively. The IF procedure followed standard protocol. Morpholinos were: cep164 MO: 50-TATATGCTCTTCTCCATC ACCTCAT; p53 MO: 50-GCGCCATTGCTTTGCAAGAATTG. For histological analyses, embryos were fixed at 72 hpf with 4% PFA/PBS and embedded in JB-4 resin (PolySciences) following the manufacturer's protocol. Six millimeter sections were obtained using a Leica R2265 microtome and stained with hematoxylin-eosin following published procedures (Zhou et al., 2010).

Statistical Analysis

Student's two-tailed nonpaired t tests and normal distribution two-tailed z tests were carried out using pooled standard error and S.D. values to determine the statistical significance of different cohorts.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.06.028.

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