**LETTERS**

**FAN1 mutations cause karyomegalic interstitial nephritis, linking chronic kidney failure to defective DNA damage repair**

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Chronic kidney disease (CKD) represents a major health burden1. Its central feature of renal fibrosis is not well understood. By exome sequencing, we identified mutations in *FAN1* as a cause of karyomegalic interstitial nephritis (KIN), a disorder that serves as a model for renal fibrosis. Renal histology in KIN is indistinguishable from that of nephronophthisis, except for the presence of enlarged nuclei, as seen in KIN from that associated with NPHP, except for the presence of enlarged nuclei, as seen in KIN from that associated with NPHP, except for the presence of enlarged nuclei, as seen in KIN from that associated with NPHP, except for the presence of enlarged nuclei, as seen in KIN from KIN, serves renal fibrosis.

Nephronophthisis (NPHP)-related ciliopathies are a heterogeneous group of recessive diseases that cause CKD through chronic fibrosis and cyst development in the kidney7. To identify additional causative genes for NPHP, we performed homozygosity mapping8 and exome sequencing9 in two siblings (from family A1170 of Maori descent (Table 1). Renal histology in these individuals was indistinguishable from that associated with NPHP, except for the presence of enlarged nuclei, as seen in KIN10 (Fig. 1). KIN is a kidney disease with renal tubular degeneration of unknown origin that was first described in 1974 (ref. 11). KIN causes CKD through renal histological changes that are characteristic of NPHP2, including tubular basement membrane degeneration, atrophic tubules, tubular microcysts, interstitial infiltrations and pronounced fibrosis (Fig. 1a)12. The only feature that distinguishes KIN from NPHP is the presence of karyomegaly (Fig. 1b,c), which can also be present in the lung, liver and brain13. To date, only 12 families with KIN have been described10,14. They were compatible with an autosomal recessive mode of inheritance.

By homozygosity mapping in family A1170, we defined seven candidate regions of homozygosity by descent (Supplementary Fig. 1a). Exome sequencing identified a homozygous nonsense mutation (coding for p.Trp707*) in FAN1 (which encodes the Fanconi anemia–associated nuclease 1 protein) in both affected siblings (Table 1 and Supplementary Fig. 1b). No additional homozygous truncating mutations were detected in any other genes within the mapped candidate regions in family A1170 (Supplementary Fig. 1a). We then obtained DNA samples from five published families with KIN and five unpublished cases (Table 1). Clinical phenotypes have been published for families A4385 (ref. 15), A4393 (ref. 16), A1170 (ref. 10), A4433 (ref. 13) and A4333 (ref. 14) (Supplementary Table 1). With Sanger sequencing of all FAN1 exons, we found 12 different mutations of FAN1 in 9 of the 10 families with KIN (Table 1 and Supplementary Fig. 1b), detecting both mutated alleles in 9 families (Table 1). Eight of the 12 mutations truncated the conceptual reading frame (Table 1). Three missense mutations (encoding p.Gln929Pro, p.Gly937Asp and p.Asp960Asn) altered amino-acid residues that have been conserved across species (Table 1). Two were absent from 96 healthy controls. They were compatible with an autosomal recessive mode of inheritance.

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FAN1 is considered to be an effecter of the Fanconi anemia pathway, a DDR signaling pathway involved in the repair of ICL damage\(^{27}\). Individuals with Fanconi anemia are characterized by developmental abnormalities, bone marrow failure and predisposition to cancer\(^{18}\). However, no FAN1 mutations have been detected in individuals with Fanconi anemia of unassigned complementation groups (F. P. L. and A. S., unpublished data). The FAN1 protein is recruited to sites of ICL damage by interacting with a monoubiquitinated FANCI-FANCD2 complex through its UBZ domain\(^{3-6}\). In vitro, FAN1 has nuclease activity.

We examined FAN1 expression in fibroblasts and lymphoblastoid cell lines (LCLs) from individuals with KIN (Fig. 2a). As predicted, no FAN1 protein was detected in the three individuals (A1170-22, A4385-23 and A4466-22) who had two truncating mutations in FAN1 (Fig. 2a and Supplementary Fig. 2). Conversely, the protein was detected in the cell line from individual A4486-23 with a missense mutation (encoding p.Asp960Asn) in the nucleotide domain of FAN1 (Fig. 2a).

As depletion of FAN1 sensitizes human cell lines to ICL-inducing agents\(^{4-6}\), we examined FAN1-mutant cells from individuals with KIN for genome instability upon exposure to mitomycin C (MMC) (Fig. 2b and Supplementary Table 2). We observed chromatid breaks and radial chromosomes on metaphase spreads (Fig. 2b), which is consistent with a role for FAN1 in genome maintenance and DDR. The levels of genome instability observed in KIN cell lines were not as high as in Fanconi anemia cell lines that lack FANCA gene function (RA3087 and RA3157) but were above background levels seen in wild-type cells (Supplementary Table 2). Notably, the results of the classic test for Fanconi anemia, diepoxybutane (DEB) breakage\(^{4}\), were negative in all FAN1-mutant cell lines tested but positive in the control FANCA-mutant cell lines (RA3087 and RA3157) (Supplementary Table 2).

Despite the differences in chromosomal instability following MMC and DEB exposure, survival of FAN1-mutant cell lines from affected individuals was severely compromised after exposure to either ICL-inducing agent (Fig. 2c,d). ICL sensitivity was observed in multiple cell lines from affected individuals (Fig. 2c,d). In contrast, cell cycle arrest in late-S/G2 phase, which is characteristic of Fanconi anemia cells, was seen in FAN1-mutant cells only after MMC and not after DEB exposure (Fig. 2e). These observed differences between FANCA and FAN1-mutant cells might be explained by differential engagement of FAN1 versus other Fanconi anemia pathway directed nucleases in the repair of different ICL lesions or by different processing of the same kind of lesion by these distinct nucleases. Unlike the Fanconi anemia pathway defect, the FAN1 deficiency clearly resulted in high ICL sensitivity in the survival assays but did not lead to the profound genomic instability seen in Fanconi anemia cells. These differences in the cellular phenotype of FAN1- and FANCA-deficient cell lines may explain the lack of phenotypic similarity between FAN1-deficient individuals, who present with KIN, and individuals with Fanconi anemia, who have bone marrow failure and cancer predisposition. A different phenotype for KIN is also consistent with our finding that FAN1 is not necessary for activation of the Fanconi anemia pathway, as judged by the presence of normal FANCD2 ubiquitination in FAN1-deficient cells (Supplementary Fig. 3).

To complement the FAN1 defect, we transduced fibroblasts of individual A1170-22 with wild-type FAN1 cDNA or with FAN1 cDNA carrying KIN-associated mutations or mutations known to inhibit nuclease activity (encoding p.Glu975Ala/Lys977Ala) or interaction with FANCD2 (p.Cys44Ala/Cys47Ala)\(^{9}\) (Fig. 3a,b). No FAN1 protein was detected in immunoblotting of fibroblasts expressing the two

### Table 1 Mutations of FAN1 in nine families with KIN

| Family | Individual | Ancestry | Nucleotide alteration\(^{a,b}\) | Deduced protein change | Exon or intron (state) | Continuous amino-acid sequence conservation | Parental consanguinity |
|--------|------------|----------|-----------------------------|------------------------|-----------------------|---------------------------------------|------------------------|
| A4385  | 21 (no DNA) | French   | c.1234+2T>A                  | Splice site            | 2 (het)               | N/A                                   | No                     |
|        | 22          |          | c.2036_7delGA                | p.Arg679Thrfs*5        | 7 (het)               | N/A                                   | No                     |
| A4466  | 21          | French   | c.1234+2T>A                  | Splice site            | 2 (het)               | N/A                                   | No                     |
|        | 22          |          | c.2245C>T                    | p.Arg749*              | 9 (het)               | N/A                                   | No                     |
| A4393  | 21          | French   | c.1375+1G>A                  | Splice site            | 3 (het)               | N/A                                   | No                     |
|        | 23          |          | c.2616delA                   |                        |                      | N/A                                   | No                     |
| A4605  | 21          | German   | c.1606C>T                    | p.Asp873Thrfs*17       | 12 (het)              | N/A                                   | No                     |
|        | 22          |          | c.2786A>C                    | p.Gln929Pro            | 12 (het)              | D. rerio\(^c\)                        | No                     |
| A4486  | 22          | German   | c.1606C>T                    | p.Asp536*              | 5 (het)               | N/A                                   | No                     |
|        | 23          |          | c.2878G>A                    | p.Asp960Asn            | 13 (het)              | S. pombe\(^c\)                       | No                     |
| A1170  | 22          | New Zealand Maori             | c.2120G>A               | p.Trp707*             | 8 (hom)               | N/A                                   | Distant?               |
|        | 25          |          |                              |                        |                      |                                       |                        |

\(^{a}\)Four different European founder mutations are marked in bold. \(^{b}\)FAN1 cDNA mutations are annotated according to human cDNA reference NM_014967.4, where 1 corresponds to the A of the ATG start translation codon. Exon 1 is non-coding. \(^{c}\)Amino-acid residue is continually conserved throughout evolution, including in Danio rerio, Saccharomyces pombe or Ciona intestinalis, as indicated. \(^{d}\)Amino-acid residue is not conserved in evolution but is never substituted by an arginine residue.

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**Figure 1** Renal histology in individuals with KIN. (a-c) Renal histology of individuals with FAN1 mutation shows the characteristic triad of nephronophthisis, with cystic dilation of renal tubules (asterisks), interstitial infiltrations (dotted circle in a), and widespread fibrosis (blue-gray coloring in Masson’s trichrome staining in b). Karyomegaly is observed (white arrowheads in b and c) in tubules that have lost epithelial cells at their circumference (black arrowheads in b and c). The tubular basement membrane is thickened (double arrows in c) as well as attenuated (black arrow in c). a and b are from individual A4393-21; c is from individual A4466-21. Scale bars, 100 µm.
truncating variants (p.Trp707* and p.Arg679Thrfs*5), suggesting that they are unstable, whereas p.Leu925Profs*25 yielded a shortened protein product (Fig. 3b). While wild-type FAN1 did complement the MMC sensitivity defect, none of the cDNAs carrying mutations from individuals with KIN rescued the defect, with the exception of the encoded p.Cys871Arg variant, which partially complemented cell survival upon MMC exposure, suggesting that it represents a hypomorphic allele of FAN1 (Fig. 3a). Notably, the FAN1 variant p.Cys44Ala/Cys47Ala that abolishes FANC2-dependent localization of FAN1 to sites of DNA damage is fully capable of rescuing the MMC resistance defect, strongly suggesting that FAN1 activity can be independent of the Fanconi anemia pathway. The p.Glu975Ala/Lys977Ala mutant lacking nuclease activity could not complement the FAN1 defect.

It was previously shown in DT40 chicken cells that deletion of FAN1 has an effect on ICL sensitivity additive to the effect seen upon deletion of the Fanconi anemia–associated genes FANCC and FANCJ. To test whether inhibition of the Fanconi anemia pathway in the FAN1-mutant cells led to increased ICL sensitivity, we depleted transcripts of FANCD2, SLX4 and the SLX4-associated nuclease XPF and MUS81 (Fig. 3c,d and Supplementary Fig. 4). Depletion gave rise to profound MMC sensitivity that was greater than in cells with

Figure 2 Phenotypes of FAN1-mutant cells. (a) Protein blot analysis with antibody raised against the N-terminal 90 aa of FAN1. Specificity of the antibody was confirmed by the abrogation of signal after transduction of BJ fibroblasts with small hairpin RNAs (shRNAs) against FAN1 and in individuals with protein-truncating mutations in FAN1. FIB, fibroblasts; LCL, lymphoblastoid cell line; luc, luciferase. (b) Examples of metaphase spreads of the indicated cell lines after exposure to 50 nM MMC. Arrows, radial chromosomes; arrowheads, chromatid breaks. FA, Fanconi anemia. (c,d) Sensitivity to ICL of the indicated FAN1-mutant (mut) cell lines in comparison to FANCA-mutant and wild-type cell lines. Primary fibroblasts (c) or LCLs (d) were treated in triplicate with increasing concentrations of MMC (left) or DEB (right). After 6 or 8 d, cell numbers were determined. Total cell numbers at each dose were divided by the number of cells in the initial untreated sample to give percent survival. Error bars, s.d. (e) Cell cycle analysis of the indicated fibroblast cell lines after treatment with 100 nM MMC or 0.1 μg/ml of DEB. Untreated samples were analyzed in parallel.

Figure 3 Complementation of FAN1-mutant cells with FAN1 cDNA and epistasis analysis with genes implicated in ICL resistance. (a) Complementation of MMC sensitivity in fibroblasts of individual A1170-22 with KIN. Fibroblasts stably transduced with empty vector (vector control) or with vector expressing wild-type FAN1 or FAN1 mutants were exposed to different levels of MMC. After 8 d, cell numbers were determined. Total cell numbers at each dose were divided by the number of cells in the initial untreated sample to give percent survival. Error bars, s.d. (b) Immunoblot showing expression of FAN1 mutants in A1170-22 fibroblasts used in the MMC sensitivity assay in a. Note that FAN1 p.Trp707* and p.Arg679Thrfs*5 result in lack of FAN1 protein, and FAN1 p.Leu925Profs*25 results in a shortened protein. (c) MMC sensitivity in A1170-22 fibroblasts transfected with the indicated small interfering RNAs (siRNAs). Error bars, s.d. (d) Immunoblots of expression of the indicated proteins after siRNA-mediated knockdown in A1170-22 fibroblasts from the experiment in c.
FAN1 deficiency alone, suggesting that FAN1 can work independently of the Fanconi anemia pathway to repair ICL damage. The mechanisms that produce the fibrotic and cystic kidney phenotypes observed in NPHP-related ciliopathies are still mostly unknown. Because most DDR pathways as well as NPHP-related ciliopathy phenotypes are conserved in zebrafish, we evaluated whether loss of FAN1 function through morpholino oligonucleotide knockdown of fan1 in zebrafish embryos would cause both disturbance of DDR signaling and NPHP-like phenotypes. Injection into 1–4 cell stage embryos of a morpholino (fan1D7) that targets the splice-donor site of exon 7 (Supplementary Fig. 5a–c) caused the characteristic NPHP-like phenotype of shortened body axis (Fig. 4a,b) but also the DDR phenotypes of microcephaly, microphthalmia and massive cell death throughout the embryo (Fig. 4c). Cell death was from apoptosis, as shown by greater amounts of activated Caspase-3 (Fig. 4d and Supplementary Fig. 5d,e). DDR signaling was activated, as reflected by an increased signal for γH2AX shown in immunofluorescence analysis (Fig. 4e and Supplementary Fig. 5f,g). These findings are similar to the ones described for knockdown of the Fanconi anemia–associated gene fancd2 in zebrafish. However, in addition, our fan1D7 morphants showed phenotypes characteristic of NPHP-related ciliopathies, including ventral body axis curvature and renal cysts when apoptosis was suppressed through knockdown of p53 function (Fig. 4f–h). Specifically, zebrafish embryos injected with fan1D7 and tp53 morpholinos at 72 h post-fertilization (h.p.f.) showed pronephric kidney cysts (19 ± 3%) and body curvature (data not shown) (45 ± 4%), whereas tp53 morpholino alone did not cause pronephric cysts (Fig. 4g,h) and caused body axis curvature in a significantly smaller fraction of embryos (11 ± 2%) (Fig. 4h). Thus, loss of fan1 function results in ciliopathy-related phenotypes, which are revealed when p53 function is inhibited. Masking of the renal cystic phenotype in the presence of p53 function is most likely due to the fact that fan1 morphants had embryonic deformity (Fig. 4b,c) and highly elevated levels of apoptosis (Fig. 4d and Supplementary Fig. 5d,e), which prevented the observation of kidney cysts that developed in later embryonic stages. Our data show that loss of fan1 function in zebrafish embryos leads to a dual set of phenotypes: one representing activation of DDR and apoptosis (microcephaly and microphthalmia) and the other mimicking NPHP-like ciliopathy phenotypes that are revealed when p53-dependent apoptosis is suppressed. Notably, we found that, whereas knockdown of fancd2 in zebrafish led to increased γH2AX staining, it did not cause kidney cysts or other ciliopathy phenotypes seen following fan1 knockdown (data not shown).

The clinical phenotypes caused by FAN1 and FANCD2 mutations differ substantially. The former causes KIN and histological karyomegaly in the liver and brain, whereas the latter causes Fanconi anemia. To evaluate whether these phenotypic differences can be partially attributed to differential tissue expression, we performed quantitative PCR of 48 different human tissue sources and observed significant differences in expression levels in 25 sources. Notably, FAN1 expression exceeded that of FANCD2 in parenchymatous organs, including the kidney, liver, neuronal tissue and female reproductive organs (Fig. 5a), whereas FANCD2 expression levels far exceeded those of FAN1 in six different lymphatic or bone marrow–derived sources, as well as in skin and testis (Fig. 5b).


c

Figure 4 Phenotype caused by loss of fan1 function in zebrafish. (a–c) Knockdown of fan1 in zebrafish causes developmental abnormalities. A fan1D7 morpholino (MO) that targets the splice-acceptor site of exon 7 (Supplementary Fig. 5) was injected into 1–4 cell stage embryos at 0.1 mM (b) and 0.2 mM (c). (a) Standard control morpholino was injected at 0.2 mM. At 25 h.p.f., morphant embryos showed shortened body axis, ventral body axis curvature and massive cell death (dark gray tissue) throughout the embryo compared to embryos receiving control morpholino. Scale bars, 750 μm at left, 150 μm at right. (d,e) Knockdown of fan1 in zebrafish induces widespread apoptosis. (d) Upon fan1 knockdown using fan1D7 as in a–c, widespread apoptosis was seen in the anterior body and the tail (Supplementary Fig. 5) of zebrafish 27 h.p.f., as detected by an antibody against activated Caspase-3 (CASP3) compared to embryos receiving negative control MO. Images are representative of the 32 embryos evaluated for both control and MO knockdown. (e) Knockdown of fan1 leads to increased staining for γH2AX, indicating increased DDR. Of embryos with knockdown, 24 out of 27 had elevated Caspase-3 staining, and 27 of 32 with γH2AX staining had higher expression of γH2AX (see also Supplementary Fig. 5). Scale bars, 250 μm. DAPI, 4′,6-diamidino-2-phenylindole. (f–h) Knockdown of fan1 on the background of tp53 morphants reveals pronephric cysts. (f) Embryos with tp53 knockdown alone at 72 h.p.f. show normal pronephric tubules (arrows). (g) Zebrafish embryos 72 h.p.f. co-injected with fan1D7 (0.1 mM) and tp53 (0.2 mM) MOs show pronephric kidney cysts (19 ± 3%) (asterisks) and greater body axis curvature. (h) tp53 knockdown alone causes body axis curvature in a significantly smaller fraction. Scale bars, 50 μm.
particularly high in the kidney (Supplementary Fig. 6), suggesting that the kidney might depend on FAN1 for its normal function.

We recently identified two additional genes that have a role in DDR signaling that cause NPHP-related ciliopathies when mutated\(^3\). These findings strongly suggest that DDR may have a significant role in the pathogenesis of CKD in NPHP-related ciliopathies.

We further hypothesized that a defect in DDR signaling might represent a broader pathogenic mechanism that applies also to other forms of CKD. We therefore evaluated renal tissue sections from a standard congenic rat model for progressive chronic renal failure, the fawn-hooded hypertensive (FHH) rat, which has well-defined physiological parameters. Ten animals were selected on the basis of disease progression at 9–10 months of age, as measured by proteinuria\(^3\). To determine the load of DDR in the kidneys of these animals, we applied the widely accepted criterion of increased nuclear $\gamma$H2AX staining in immunohistochemistry (Fig. 5c–e). After staining, whole slides (two to four kidney sections per animal) were digitally scanned, ten randomized cortical fields were analyzed per section for positively stained nuclei using a modified algorithm and results were calibrated for diagnostic pathology. In total, 2,000–3,000 cells were scored per animal by a researcher blinded to the disease status of the animals. A positive correlation ($R^2 = 0.64$, $P < 0.0054$) was found between disease progression and DDR in this assay (Fig. 5c).

Having established a quantitative correlation in a well-defined congenic model for kidney disease, we examined genetically heterogeneous affected humans. A small pilot study using renal transplant biopsies from individuals that were clinically well characterized by a nephropathologist and nephrologist supported the correlation found in the rat model. We examined $\gamma$H2AX immunostaining in a specimen from a transplant kidney 4 months after transplantation (histology report without injury and no proteinuria) (Fig. 5f), a specimen from a transplant kidney 16 years after transplantation (pathology report of chronic damage) (Fig. 5g) and a transplant kidney 10 years after transplantation (pathology report of chronic tissue damage) (Fig. 5h). These data support in human CKD the relationship seen in the FHH rat model between the extent of kidney damage and DNA lesions ($\gamma$H2AX). They suggest that DDR may partially drive renal damage in pediatric NPHP-related ciliopathies and in CKD of adults.

We here identify recessive mutations of FAN1 as the cause of KIN, which leads to CKD with fibrotic degeneration of the kidney. Thus, DDR, which has a known role in cellular senescence\(^3\), might contribute to the premature aging phenotype of renal fibrosis in NPHP-like diseases. A KIN-like phenotype has also been described in humans and animal models that were exposed to ochratoxin A\(^15\), busulfan or pyrrolizidine alkaloids\(^11\), all of which cause ICL. Thus, FAN1 mutation represents the genetic equivalent of environmental genotoxic causes of KIN by the shared pathogenic mechanism of defective ICL repair.

In this context, it is notable that, among the 12 individuals with KIN-associated and FAN1 mutations, CKD presented by a median age of 45 years (Supplementary Table 1) and that 7 of the 12 individuals with KIN carried 2 truncating mutations or missense mutations affecting the nuclease domain of FAN1, which most likely represent null alleles (Fig. 3a and Table 1). This raises the question of whether other individuals who carry hypomorphic alleles rather than two null alleles might develop end-stage kidney disease even later in life. Notably, a high percentage of adult individuals treated within chronic dialysis programs suffer from fibrotic nephropathy of unknown cause. We will therefore follow up on the hypothesis that a subset of cases in this cohort might be caused by two hypomorphic alleles of FAN1. Furthermore, as we show here that recessive mutations in FAN1 confer susceptibility to MMC and DEB, FAN1 mutations may also sensitize to other ICL-causing environmental genotoxins, such as ochratoxin A, which are abundant\(^16\). Because CKD always shows a histological phenotype of chronic interstitial fibrosis similar to the one observed in KIN, it will be important to examine a potential role for ICL-causing genotoxins in the absolute and relative increase in end-stage kidney disease that has been observed over the last 20 years in developed countries\(^1\).

URLs. University of Michigan Affymetrix & Microarray Core, http://www.umich.edu/~caparray/; BLAT, http://genome.ucsc.edu/cgi-bin/
The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome.

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Auerbach, A.D. Fanconi anemia and its diagnosis. 

Am. J. Pathol. 15, 113–170 (1949).

Nature Genetics, University of Michigan, Ann Arbor, Michigan, USA.

Hildebrandt, F., Benzing, T. & Katsanis, N. Ciliopathies. 

Cell 131, 147–150 (2007).

Auerbach, A.D. Fanconi anaemia and its diagnosis. 

Mutat. Res. 668, 4–10 (2009).

Auerbach, A.D. & Wolman, S.R. Susceptibility of Fanconi’s anaemia fibroblasts to carcinogenic DNA damage by carcinogens. 

Nature 263, 494–496 (1979).

Mihatsch, M.J. et al. Systemic karyomel演艺 associated with chronic intestinal nephropathy. 

A new disease entity? 

Clin. Nephrol. 12, 54–62 (1979).

Kratz, K. et al. Deficient expression of FANCC-associated protein (FANCC-AIP) sensitizes cells to interstrand cross-linking agents. 

Cell 142, 77–88 (2010).

Liu, T., Ghosal, G., Yuan, J., Chen, J. & Huang, J. 

FA13 acts with FANCI-FANCD2 to promote DNA interstrand cross-link repair. 

Science 329, 693–696 (2010).

Mihatsch, M.J. et al. Identification of nephrocystin-6 (NPHP6, FANCI) as a kidney tumor suppressor gene recruited to DNA damage by monoubiquitinated FANCD2. 

Cell 142, 65–74 (2010).

Smogorzewska, A. et al. A genetic screen identifies FAN1, a Fanconi anemia-associated nuclease necessary for DNA interstrand crosslink repair. 

Mol. Cell 39, 36–47 (2010).

Hildebrandt, F., Benzing, T. & Katsanis, N. Ciliopathies. 

Nat. Med. 16, 1533–1543 (2011).

Hildebrandt, F. et al. A systematic approach to mapping recessive disease genes in individuals from outbred populations. PLoS Genet. 5, e1000353 (2009).

Otto, E.A. et al. Candidate exome capture identifies mutation of SIDD9C2 as the cause of a renal-retinal ciliopathy. 

Nat. Genet. 42, 840–850 (2010).

Palmer, D., Lalou, S., Matheson, P., Bethwaite, P. & Tomson, K. 

Ciliogenic intestinal nephropathy: a pitfall in urine cytology. 

Diagn. Cytopathol. 35, 179–182 (2007).

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1. Coresh, J., Astor, B.C., Greene, T., Eknayan, G. & Levey, A.S. Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Examination Survey. 

Ann. J. Kidney Dis. 41, 1–12 (2003).

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ONLINE METHODS

Research subjects. We obtained blood samples and pedigrees, after obtaining informed consent, from individuals with KIN. Approval for research on human subjects was obtained from the University of Michigan Institutional Review Board and the other institutions involved. Diagnosis with NPHP-related ciliopathy was based on published clinical criteria. The clinical features of many individuals with KIN have been reported (Table 1)10,13–16,32.

Homozygosity mapping. For genome-wide homozygosity mapping8, the Human Mapping 250k Sty1 array and the Genome-wide Human SNP 6.0 Array from Affymetrix were used. Genomic DNA samples were hybridized and scanned using the manufacturer’s standard protocol at the University of Michigan Core Facility. Non-parametric logarithm of odds (LOD) scores were calculated using a modified version of the GENEHUNTER 2.1 program33,34 through stepwise use of a sliding window with sets of 110 SNPs using ALLEGRID5. Genetic regions of homozygosity by descent (homozygous peaks) were plotted across the genome as candidate regions for recessive disease-causing genes (Fig. 1a) as described45. Disease allele frequency was set at 0.0001, and European or Asian ancestry marker allele frequencies were used.

Whole-exome sequencing. Exome enrichment was conducted following the manufacturer’s protocol for NimbleGen SeqCap EZ Exome v2 beads (Roche NimbleGen). The kit interrogates a total of approximately 30,000 genes (~330,000 Collaborative Consensus Coding Sequence (CCDS) exons). Massively parallel sequencing was performed as described46.

Mutation calling. Following exome sequencing, mutation calling was performed using CLC Genomics Workbench software. The minimum length fraction with which a read had to match the reference sequence was set to 90%. For SNP detection, the minimum quality score of the central base and the minimum average quality score of surrounding bases were kept at default values (20 and 15, respectively). Quality assessment was performed within a window of 11 bases. Only reads that uniquely aligned to the reference genome were used for variant SNP or deletion/insertion polymorphism (DIP) calling. In individuals with evidence of homozygosity by descent, the threshold for the number of reads (minor allele frequency) was set to >55%.

Filtering of variants from normal reference sequence (VRS). For DIPs and SNPs, we used the following a priori criteria to restrict the high number of VRS (average of 53,272 for DIPs and 315,372 for SNPs). (i) We retained exonic variants (missense, nonsense and indels) and obligatory splice-site variants only. (ii) We included only VRSs that were not listed in the SNP129 database of innocuous polymorphisms. (iii) We evaluated exonic changes only within genomic regions in which homozygosity mapping showed linkage for both affected siblings (repeating on average 38 for DIPs and 169 for SNPs). (iv) Variants were analyzed using the BLAT program at the UCSC human genome Bioinformatics Browser for the presence of paralogous genes, pseudogenes, misalignments at ends of sequence reads and for whether the variant was a known variant in dbSNP132 with an allele frequency of >1% in populations of European ancestry. In families in whom mapping showed homozygosity by descent, we retained only homozygous variants and examined all of them in the sequence alignments within CLC Genomics Workbench software for the presence of mismatches indicating potential false alignments or poor sequence quality. (v) Sanger sequencing was performed to confirm the remaining variants in original DNA samples and to test for intrafamilial segregation in a recessive mode. (vi) Finally, remaining variants were ranked by whether mutations truncated the conceptual reading frame (nonsense, frameshift and obligatory splice variants), by analysis of evolutionary conservation of missense variants, by using web-based programs predicting the impact of disease candidate variants on the encoded protein and by whether variants were known disease-causing mutations.

Segregation analysis by Sanger sequencing. We applied Sanger dideoxy-terminator sequencing for confirmation and segregation of potential disease-causing variants in the respective affected subjects, their affected siblings and their parents. In affected subjects in whom only one heterozygous mutation was detected by exome capture and massively parallel sequencing, all exons and flanking intronic sequences of the respective gene(s) were analyzed by Sanger sequencing. PCR was performed using a touchdown protocol described previously27. Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730 XL sequencer (Applied Biosystems). Sequence traces were analyzed using Sequencer (version 4.8) software (Gene Codes Corporation).

Web-based variant analysis. Predictions of the possible impact of an amino-acid substitution on chemical change, evolutionary conservation and protein function were obtained using the following web-based programs: Polymorphism Phenotyping (PolyPhen-2, SIFT and MutationTaster). GERP calculation was performed at the SeattleSeq website.

Cell culture. Human fibroblasts were grown in 3% oxygen in DMEM supplemented with 15% FBS, 100 U/ml of penicillin, 0.1 mg/ml streptomycin and nonessential amino acids (all from Invitrogen). BJ cells are normal foreskin fibroblasts and were obtained from the American Type Tissue Collection (ATCC). Fibroblasts were immortalized using pWZlhtERT and/or pMCSVNeo HPV16E6E7 plasmids. LCLs were immortalized using Epstein–Barr virus (EBV) and were grown in RPMI supplemented as above, except with 20% FBS.

DNA damage sensitivity assay. Cells were plated in a 6-well plate in triplicate at a density of 5 × 10⁴ cells per well for primary fibroblasts and LCLs or 2.5 × 10⁴ cells per well for transformed fibroblasts. Immediately after plating for LCLs or 24 h later for fibroblasts, MMC or DEB was added at a final concentration of 0–100 nM for MMC or 0.75–1 µg/ml for DEB. After 6–8 d of culture, cell numbers were determined using a Z2 Coulter Counter (Beckman Coulter). Cell number after MMC or DEB treatment was normalized to cell number in the untreated sample to give the percentage of survival.

RNA interference (RNAi). For siRNA experiments, A1170-22 E6E7/HTERT cells were transfected with a pool of three siRNAs using Lipofecta
cmRNAiMAX (Invitrogen) according to the manufacturer’s instructions, with the final concentration of total siRNA at 25 nM. siRNA sequences are given in Supplementary Table 3.

Mutagenesis. Mutagenesis of FAN1 was performed on a pDONR223 FAN1 DNA construct6 using the QuickChange Multi site-directed mutagenesis kit (Agilent). Primer sequences are given in Supplementary Table 3. Other FAN1 mutants were previously described6.

Antibodies. Antibody to FAN1 (RC394) was raised in a rabbit using GST-FAN1aa1–90 as an antigen and was affinity purified against HIS-FAN1aa1–90. Commercial antibodies were purchased for HA (Covance, MMS-101R), XPF/ERCC4 (Bethyl Laboratory, A301-315A), MUS81 (Sigma, M1445) and FANCD2 (Novus, NB100-182).

Breakage analysis. Cells were exposed to 0.01 or 0.1 µg/ml DEB for 72 h or with 50 nM MMC for 24 h arrested with colcemid (0.167 µg/ml) for 2 h, harvested, incubated for 10 min at 37 °C in 0.075 M KCl and fixed in freshly prepared methanol/glacial acidic acid (3:1). Cells were stored at 4 °C and, when needed, were dropped onto wet slides and air dried at 40 °C for 1 h before staining with Karyomax Giemsa (Invitrogen) Gurr Buffer for 3 min. After rinsing with fresh Gurr Buffer and then with distilled water, slides were fully dried at 40 °C for 1 h and were scanned using the Metasystems Metafer application.

Cell cycle analysis. Cells were left untreated, were treated with 100 nM MMC and grown for 48 h or were treated with 0.1 µg/ml of DEB and grown for 72 h. Collected cells were resuspended in 300 µl of PBS. While vortexing, 700 µl of ice-cold 100% ethanol was added dropwise, and suspensions were stored at −20 °C at least overnight. Thirty minutes before fluorescence-activated cell sorting (FACS), cells were spun down, resuspended in propidium iodine mix (1 ml of PBS, 10 µl of RNase (from a stock solution of 20 µg/ml) and 10 µl of propidium iodine (from a stock solution of 1 mg/ml)) and analyzed
using a FACS Calibur instrument (Becton Dickinson). Cell cycle analysis was performed using FlowJo software (Tree Star).

**Morpholino-mediated knockdown of fan1 in zebrafish.** Morpholino oligonucleotides were obtained from Gene Tools. Morpholinos (fan1D7 at 0.1 mM, standard control morpholino at 0.2 mM and tp53 morpholino at 0.2 mM) were injected into zebrafish embryos at the 1–4 cell stages. Embryos were then fixed at 27 h.p.f. with 4% paraformaldehyde in PBS with 1% DMSO overnight, permeabilized with aceton at −20 °C for 7 min and were stained with antibody against phosphorylated zebrafish γH2AX (1:1,000 dilution; a gift from J. Amatruda, or antibody against cleaved Caspase-3 (1:200 dilution; BD Biosciences, 55082). Alexa568-conjugated secondary antibody to rabbit IgG was used at a 1:2,000 and a 1:1,000 dilution, respectively, for each primary antibody. The immunofluorescence procedure followed a standard protocol. After washing off secondary antibody, embryos were mounted in Vector-Shield Mounting Media and were imaged with a Leica SP5X confocal system. Z stacks were processed to reduce noise and were overlaid to obtain representative images of immunofluorescence for γH2AX and cleaved caspase. DAPI was used to stain nuclei. The sequence of the fan1D7 morpholinos is shown in Supplementary Table 3.

**Quantitative RT-PCR.** cDNA from 48 human tissues was purchased from OriGene (Tissue SCANTM Normal Tissue qPCR Arrays, HMRT502). Quantitative RT-PCR was performed using the TaqMan Gene Expression Assay kit (Applied Biosystems) according to the manufacturer’s instructions. Briefly, 1 μl of cDNA was mixed with 10 μl of 2× TaqMan Universal Master Mix and 1 μl of 20× TaqMan Gene Expression Assay, bringing the total volume to 20 μl with RNase-free water. Target amplification was performed in 96-well plates using the StepOnePlus Real-Time PCR System (Applied Biosystems). TaqMan probes for FAN1 (Hs00429686_m1), FANCD2 (Hs00276992_m1) and GAPDH (Hs02758991_g1) were purchased from Applied Biosystems. PCR thermal cycling conditions included an initial 10-min hold at 95 °C to activate the AmpliTaq Gold DNA polymerase followed by 40 cycles of denaturation (15 s at 95°) and annealing and primer extension (15 s at 60 °C). More than three RT-PCR analyses were executed for each sample, and the obtained threshold cycle values were averaged. Relative RNA expression levels were calculated via a comparative threshold cycle (Ct) method using GAPDH as control, where ΔCt = Ct(FAN1 or FANC D2) – Ct(GAPDH). Fold change in gene expression, normalized to GAPDH and relative to the control sample (FAN1 expression in the kidney), was calculated as 2−ΔΔCt.

**Immunocytochemistry in the FHH rat and in humans with kidney disease.** Kidney tissue sections from FHH rats (n = 10) embedded in paraffin were deparaffinized, treated with peroxidase block for 15 min and incubated at 100 °C in citrate-HCl buffer for 20 min. The sections were stained with mouse antibody to γH2AX (1:200 dilution; Millipore, 05-636) overnight at 4 °C. Samples were incubated with horseradish peroxidase (HRP)-conjugated secondary polyclonal rabbit antibody to mouse (1:100 dilution; Dako, P0260) for 30 min at room temperature. Finally, samples were incubated with BrightVision Poly HRP-Anti Rabbit IgG (Immunologic, DPVR55HRP) for 1 h 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. Ten random tubular fields in the cortex (approximately 270 cells per field) were analyzed for positively stained nuclei using an in-house algorithm macro. GraphPad Prism 5.0 was used to calculate the R² and P values.

**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 differences between cohorts.

**Bioinformatics.** Genetic locations are annotated according to March 2006 Human Genome Browser data.

32. Moh, H., Spöndlin, M., Schmassmann, A. & Mihatsch, M.J. Systemic karyomegaly with chronic interstitial nephritis. Discussion of the disease picture based on an autopsy case. Pathol. Res. Pract. 15, 44–48 (1994).
33. Kruglyak, L., Daly, M.J., Reeve-Daly, M.P. & Lander, E.S. Parametric and nonparametric linkage analysis: a unified multipoint approach. Am. J. Hum. Genet. 58, 1347–1363 (1996).
34. Strauch, K. et al. Parametric and nonparametric multipoint linkage analysis with imputing and two-locus–trait models: application to mite sensitization. Am. J. Hum. Genet. 66, 1945–1957 (2000).
35. Gudbjartsson, D.F., Jonasson, K., Frigge, M.L. & Kong, A. Allegro, a new computer program for multipoint linkage analysis. Nat. Genet. 25, 12–13 (2000).
36. Bentley, D.R. et al. Accurate whole human genome sequencing using reversible terminator chemistry. Nature 456, 53–59 (2008).
37. Otto, E.A. et al. Mutation analysis in nephronophthisis using a combined approach of homozygosity mapping, CEL I endonuclease cleavage, and direct sequencing. Hum. Mutat. 29, 418–426 (2008).
38. Koeners, M.P., Braam, B., van der Giezen, D.M., Goldschmeding, R. & Joles, J.A. Perinatal micronutrient supplements ameliorate hypertension and proteinuria in adult fawn-hooded hypertensive rats. Am. J. Hypertens. 23, 802–808 (2010).