Mutations in multiple components of the nuclear pore complex cause nephrotic syndrome

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Steroid-resistant nephrotic syndrome (SRNS) almost invariably progresses to end-stage renal disease. Although more than 50 monogenic causes of SRNS have been described, a large proportion of SRNS remains unexplained. Recently, it was discovered that mutations of *NUP93* and *NUP205*, encoding 2 proteins of the inner ring subunit of the nuclear pore complex (NPC), cause SRNS. Here, we describe mutations in genes encoding 4 components of the outer rings of the NPC, namely *NUP107*, *NUP85*, *NUP133*, and *NUP160*, in 13 families with SRNS. Using coimmunoprecipitation experiments, we showed that certain pathogenic alleles weakened the interaction between neighboring NPC subunits. We demonstrated that morpholino knockdown of *nup107*, *nup85*, or *nup133* in *Xenopus* disrupted glomerulogenesis. Re-expression of WT mRNA, but not of mRNA reflecting mutations from SRNS patients, mitigated this phenotype. We furthermore found that CRISPR/Cas9 knockout of *NUP107*, *NUP85*, or *NUP133* in podocytes activated Cdc42, an important effector of SRNS pathogenesis. CRISPR/Cas9 knockout of *nup107* or *nup85* in zebrafish caused developmental anomalies and early lethality. In contrast, an in-frame mutation of *nup107* did not affect survival, thus mimicking the allelic effects seen in humans. In conclusion, we discovered here that mutations in 4 genes encoding components of the outer ring subunits of the NPC cause SRNS and thereby provide further evidence that specific hypomorphic mutations in these essential genes cause a distinct, […]

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Mutations in multiple components of the nuclear pore complex cause nephrotic syndrome

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Introduction

Steroid-resistant nephrotic syndrome (SRNS), a disease of the renal glomerular filter, is characterized by proteinuria, edema, and hypoalbuminemia. In contrast to other forms of nephrotic syndrome, SRNS does not respond to drug treatment and requires different therapeutic approaches.

Steroid-resistant nephrotic syndrome (SRNS) almost invariably progresses to end-stage renal disease. Although more than 50 monogenic causes of SRNS have been described, a large proportion of SRNS remains unexplained. Recently, it was discovered that mutations of NUP93 and NUP205, encoding 2 proteins of the inner ring subunit of the nuclear pore complex (NPC), cause SRNS. Here, we describe mutations in genes encoding 4 components of the outer rings of the NPC, namely NUP107, NUP85, NUP133, and NUP160, in 13 families with SRNS. Using coimmunoprecipitation experiments, we showed that certain pathogenic alleles weakened the interaction between neighboring NPC subunits. We demonstrated that morpholino knockdown of nup107, nup85, or nup133 in Xenopus disrupted glomerulogenesis. Re-expression of WT mRNA, but not of mRNA reflecting mutations from SRNS patients, mitigated this phenotype. We furthermore found that CRISPR/Cas9 knockout of NUP107, NUP85, or NUP133 in podocytes activated Cdr42, an important effector of SRNS pathogenesis. CRISPR/Cas9 knockout of nup107 or nup85 in zebrafish caused developmental anomalies and early lethality. In contrast, an in-frame mutation of nup107 did not affect survival, thus mimicking the allelic effects seen in humans. In conclusion, we discovered here that mutations in 4 genes encoding components of the outer ring subunits of the NPC cause SRNS and thereby provide further evidence that specific hypomorphic mutations in these essential genes cause a distinct, organ-specific phenotype.
mutation can be identified (7–9). That in about 30% of patients with SRNS a causative single-gene SRNS (3). Our group and others have recently demonstrated multiple signaling pathways in the molecular pathogenesis of SRNS (3). Genetic findings have revealed podocytes, specialized epithelial cells of the glomerular filter, as the critical site of injury in SRNS (5, 6). Furthermore, identification of disease genes has implicated multiple signaling pathways in the molecular pathogenesis of SRNS (3). Our group and others have recently demonstrated that in about 30% of patients with SRNS a causative single-gene mutation can be identified (7–9).

The nuclear pore complex (NPC) is a large (100 MDa) macromolecular assembly that spans the nuclear envelope and forms a selective barrier between the cytoplasm and the nucleoplasm (10). The central channel of the NPC is filled with intrinsically disordered proteins, which are rich in phenylalanine-glycine (FG) amino acid residues. These FG-rich nucleoporins (NUPs) establish a size-selective diffusion barrier to macromolecules greater than approximately 40 kDa while providing binding sites for nuclear transport receptors (karyopherins, importins, and exportins) that ferry signal-bearing cargo across the NPC (10). The central channel is supported by a structural scaffold built from the inner and outer ring complexes, which are formed by repeating modular units of the NUP93 subcomplex and the NUP107-160 complex, or “Y complex,” respectively (11, 12). The Y complex is the major subunit of the outer rings of the NPC. The atomic structures of the major domains of all 7 members of the Y complex (either alone or in complex), including NUPs 43, 85, 96, 107, 133, and 160, have been solved and modeled into electron cryomicroscopic maps of the human and Xenopus NPC (10).

Interestingly, while NUPs are best understood in their roles at the NPC, they also participate in additional functions in other subcellular locations (13–15). Studies in vertebrate model organisms additionally suggest that NUPs may have important regulatory functions during development and for tissue-specific progenitor cell maturation (16, 17). Genetic depletion of NUP transcripts caused severe developmental defects in zebrafish (18), mice (16), and frogs (15). Interestingly, some NUP-encoding genes show differential expression in different tissues and in different developmental stages (15, 19). NUPs studied in this article (i.e., nup107, nup85, nup133, and nup160) were shown to be expressed in both the rostral portion of the embryo (relevant to CNS development) and the intermediate mesoderm (important for pronephric development) (20).

Recently, we identified mutations in NUP93 and NUP205, encoding 2 components of the inner ring subunit of the NPC, in patients with nonsyndromic SRNS (21). Intriguingly, patients with mutations in NUP107, encoding a component of the Y complex, also developed SRNS (22–24). These observations suggest that a specific pathogenic link between SRNS development and alterations in different NUPs exists.

Performing whole exome sequencing and targeted exon sequencing, we here identified mutations in 4 genes encoding components of the outer ring subunits of the NPC, namely NUP107, NUP85, NUP133, and NUP160, in 29 individuals from 13 families with SRNS. Furthermore, we detected a homozygous mutation in NUP37 in a family with primary microcephaly, a phenotype that was also present in some families with NUP107 mutations. We use different in vivo and in vitro models to elucidate parts of the related pathogenesis.

Results

Hypomorphic mutations in 4 different genes encoding components of the outer ring subunits of the NPC cause nephrotic syndrome. To identify additional genes that cause SRNS if mutated, we performed whole exome sequencing (25) in 160 families with SRNS. After excluding mutations in known SRNS genes, we used homozygosity mapping (26) in consanguineous families to identify potentially novel candidate loci for SRNS. This approach yielded a homozygous missense mutation (c.303G>A, p.Met101Ile) in the gene NUP107 (NM_020401.3) (Table 1 and Figure 1, B–D) in 5 consanguineous families (A1830, A3825, F797, FA, and PN-1), the NUP107 locus was positioned within a homozygous peak region, and we identified the same mutation in NUP107 (Figure 1, B–D, Table 1, Supplemental Table 1, Supplemental Figure 1A, and Supplemental Figures 2 and 3; supplemental material available online with this article; https://doi.org/10.1172/JCI98688DS1). The p.Met101Ile mutation represents a South Asian founder allele that was reported previously in patients with the combined phenotype of SRNS and primary microcephaly (23, 27). Prior studies demonstrated that the p.Met101Ile mutation of NUP107 results in aberrant splicing and causes a reduction in the NUP107 protein level (23). NUP107 is an essential protein for NPC assembly (28, 29). In consanguineous family F797 with nonsyndromic SRNS, we found a homozygous missense mutation (c.2922T>G, p.Ser974Arg) in the gene NUP133 (NM_018230.2), which was located within a homozygous peak region on chromosome 1 (Figure 1, H–J).

Because NUP133 and NUP107 encode 2 interacting components of the outer ring subunits of the NPC, we hypothesized that alterations in other nuclear pore proteins may also cause monogenic SRNS. We therefore performed targeted sequencing of all exons of 19 genes that encode other NUPs in a worldwide cohort of 2,164 families with SRNS using a multiplex PCR–based high-throughput sequencing strategy (7, 30, 31) or using whole exome sequencing. As a result, we detected a homozygous missense mutation of NUP107 (c.2666A>G, p.Tyr889Cys) in family A3825 and 2 compound heterozygous alleles (c.1021dup, p.Glu341Glyfs*3, and c.2129_2131delAAG, p.Glu710del) of NUP107 in family A1830. None of these alleles were reported previously (Table 1 and Figure 1, A–D). All 5 families who carried the p.Met101Ile allele and family A1830 displayed the combined phenotype of SRNS with microcephaly and intellectual disability. In contrast, family A3825 and 9 previously reported families with NUP107 alleles other than p.Met101Ile did not have a neurodevelopmental phenotype (22, 24). We speculate that this allelism may be explained by the fact that only the homozygous p.Met101Ile allele resulted in a protein-truncating mutation (23), while all other families carried at least 1 recessive missense allele. This observation suggests that, as seen in other monogenic dis-
Table 1. Phenotypes in patients with SRNS and mutations in NUP107, NUP85, NUP133, and NUP160 and in 1 family with a mutation in NUP37 and microcephaly

<table>
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<tr>
<th>Family-Individual</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Exon (zygosity, segregation)</th>
<th>PPH2 score</th>
<th>SIFT</th>
<th>MT</th>
<th>Amino acid conservation to species</th>
<th>gnomAD</th>
<th>Ethic origin</th>
<th>Parental consanguinity</th>
<th>Age of onset (yr)</th>
<th>Extrarenal manifestations</th>
<th>Biopsy</th>
<th>Renal phenotype and therapy (ESRD at yr)</th>
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<td>Cleft lip, cleft palate</td>
<td>DMS</td>
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<td>A5195-22a</td>
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The following NCBI GenBank annotations were used: NUP85 (NM_024844.4); NUP107 (NM_020401.3); NUP133 (NM_018230.2); NUP160 (NM_015231.1); NUP37 (NM_024057.3). *Older sister had similar clinical phenotype, but was deceased so that no DNA sample could be obtained. **Chemically conserved (Arg or Lys) to Drosophila melanogaster. ***Chemically conserved (Glu or Gln) to Ciona intestinalis. CD, clindamycy; C. intestinalis, Ciona intestinalis; DC, disease causing; DCM, dilated cardiomyopathy; DD, developmental delay; Del, deleterious; D. melanogaster, Drosophila melanogaster; D. rerio, Danio rerio; DMS, diffuse mesangial sclerosis; ESRD, end-stage renal disease; F, female; FD, facial dysmorphism; FSGS, focal segmental glomerulosclerosis; GH, growth hormone; gnomAD, Genome Aggregation Database (http://gnomad.broadinstitute.org); het, heterozygous; Hom, homozygous; HTN, arterial hypertension; ID, intellectual disability; M, male; MC, microcephaly; MT, Mutation Taster (prediction software); N, no; NA, not applicable; ND, no data; NP, not present; PPH2, PolyPhen-2 (prediction software); SIFT, Sorting Intolerant From Tolerant (prediction software); SRNS, steroid-resistant nephrotic syndrome; SS, short stature; TOL, tolerant; VSD, ventricular septum defect; X. tropicalis, Xenopus tropicalis; Y, yes.
Figure 1. Homozygosity mapping and whole exome sequencing identify recessive mutations of NUP107, NUP85, and NUP133 in 12 families with steroid-resistant nephrotic syndrome. (A) Renal histology of individual A3825-21 (NUP107 mutation) shows diffuse mesangial sclerosis on light microscopy. (B, F, and I) Exon structure of human cDNAs. Positions of start codons and of stop codon are indicated. For protein domain structures, α-helices are depicted as red zigzag lines and β-turns as purple arrows. Arrows indicate positions of pathogenic mutations detected in families with SRNS. H, homozygous; h, heterozygous. (B) Exon structure, protein domain structure, and human mutations of NUP107. (C) Homozygosity mapping identifies 3 recessive candidate loci (red circles) in patient A4649-21. Nonparametric lod (NPL) scores and SNP positions (Affymetrix 250K Sty array) are plotted on human chromosomes concatenated from p-ter (left) to q-ter (right). Genetic distance is given in centimorgans (cM). Genetic distances are calculated on a physical map of chromosome 12 (arrowhead). (D, G, and K) Evolutionary conservation of amino acid residues that are altered in patients with SRNS. (D) Altered amino acid residues of NUP107 (p.Met101Ile) that is positioned within the maximum NPL peak on chromosome 1 (arrowhead). (E) Altered amino acid residues of NUP107 (p.Met101Ile, p.Tyr889Cys). (E) Renal histology of individual A3759-21 (NUP85 mutation) showing podocyte foot process effacement on transmission electron microscopy (TEM) (arrowheads). (F) Exon structure, protein domain structure, and human mutations of NUP85. (G) Altered amino acid residues of NUP85 (p.Ala477Val, p.Ala581Pro, p.Arg645Trp). (H) Renal histology of individual F797-21 (NUP133 mutation) showing podocyte foot process effacement on TEM (arrowheads). (I) Exon structure, protein domain structure, and human mutations of NUP133. (J) Homozygosity mapping in individual F797-21 identifies regions of homozygosity as recessive candidate loci. Within the maximum NPL peak on chromosome 1 (arrowhead), we identified a homozygous mutation in NUP133 (p.Ser974Arg). (K) Altered amino acid residues of NUP133 (p.Arg231Gly, p.Ser974Arg, p.Leu1055Ser).

In a consanguineous Pakistani family with 3 affected children with congenital microcephaly, we discovered a homozygous nonsense mutation (c.916C>T, p.Arg306*) in the gene NUP37 (NM_024057.3) (Table 1 and Supplemental Figure 6). NUP37 encodes an essential component of the Y complex of the NPC. Segregation analysis was performed when possible, and was compatible with a recessive mode of inheritance (Supplemental Figure 6). All 3 patients showed congenital microcephaly (~5 to ~8 standard deviations [SD]), mild intellectual disability, cerebellar vermis hypoplasia, and clinodactyly of the fifth finger (Table 1). Proteinuria was not present in these patients, and renal ultrasound examination did not reveal any abnormalities (Table 1).

Mutations from SRNS patients impair intermolecular interactions between NPC components. NUP107, NUP85, NUP133, and NUP160 are components of the Y complex, whose molecular structure has recently been modeled into the outer ring scaffold of the NPC (11) (Figure 2A). To investigate the pathogenicity of alleles that we discovered in SRNS patients, we gleaned from available structural data (Protein Data Bank, pdb: 5A9Q) (11) that 1 mutation of NUP133, p.Ser974Arg, and 1 mutation of NUP107, p.Tyr889Cys, were located in the interacting α-helix between the 2 proteins (Figure 2A). NUP85 mutations were positioned within a region for which no molecular structure of the human Y complex is available yet (Figure 2A).

We used cDNA constructs reflecting the mutations identified in patients with SRNS (Table 1) and performed half-endogenous coimmunoprecipitation (coIP) experiments in HEK293T cells. These experiments demonstrated that, as predicted based on the structural data, the alleles p.Tyr889Cys of NUP107 and p.Ser974Arg of NUP133 weakened the interaction between NUP107 and its direct binding partner NUP133 (Figure 2, B and C). Other missense alleles of NUP107 or NUP133 did not...
Figure 2. Mutations of NUP107, NUP85, and NUP133 weaken protein-protein interactions within the NPC. (A) The published structure of the Y complex of the NPC (11) was used to determine the localization of amino acid residues that we found altered in individuals with SRNS. Left: The 3D structure of the Y complex (pdb: 5A9Q): NUP133 (dark blue), NUP107 (light green), NUP96 (light blue), SEC13 (orange), SEH1L (violet), NUP85 (light gray), NUP43 (dark green), NUP160 (yellow), and NUP37 (red). Inset on right: The 3D structure of the interface region between NUP107 and NUP133 (pdb: 3CQC). The C-terminal part of NUP85 could not be fully resolved experimentally; a gray area indicates its predicted position. Residues Ala477, Ala581, and Arg645 of NUP85 (gray) and Glu803 of NUP160 (yellow) are located within incompletely resolved areas, and their positions are estimated. Note that amino acid residues Tyr889 of NUP107 (light green) and Ser974 of NUP133 (dark blue) point toward the interaction interface. (B) N-terminally FLAG-tagged NUP107 wild-type (WT) or mutant cDNA was overexpressed in HEK293T cells. Coimmunoprecipitation (coIP) demonstrates that the missense mutation Tyr889Cys weakens the interaction with endogenous NUP133. (C) A coIP experiment using N-terminally FLAG-tagged WT or mutant NUP133 cDNA demonstrates that the missense mutation Ser974Arg weakens the interaction with endogenous NUP107. As expected based on structural data, the 2 other missense mutations (Arg231Gly and Leu1055Ser) do not alter the NUP107-NUP133 interaction. (D) N-terminally Myc-tagged WT or mutant NUP85 cDNA was overexpressed in HEK293T cells. CoIP using an antibody against endogenous NUP160 shows that the missense mutations Ala581Pro and Arg645Trp of NUP85 weaken the interaction between the 2 proteins. FL, full-length; MOCK, empty vector. CoIP experiments in B–D were confirmed in 3 independent experiments.
 interfere with the interaction between the 2 proteins. We furthermore show that the missense mutations p.Ala581Pro and p.Arg645Trp of NUP85 weakened the interaction with its binding partner NUP160 (Figure 2D). The obligatory splice site mutation c.405+1G>A of NUP85 is predicted to cause skipping of exon 5, thus resulting in a frameshift with premature truncating of the encoded protein. We modeled this mutation in a cDNA construct and found that it completely abrogated the interaction between NUP85 and NUP160 (Figure 2D). Confirmatory experiments for all colPs using differently tagged fusion proteins are shown in Supplemental Figure 7. Next, we aimed to investigate the impact of an isolated depletion of SRNS-relevant NUPs on other components of the NPC. To address this question, we generated immortalized human podocyte cell lines with doxycycline-inducible CRISPR/Cas9 mediated knockout of NUP107, NUP85, or NUP133 (Supplemental Figures 8–10 demonstrate successful depletion of targeted proteins). We used an inducible system because sustained knockout of any of the 3 investigated NUP genes had strong effects on cell viability, which we feared would mask any specific effects resulting from the knockout. Using the inducible CRISPR/Cas9 system, we could instead initiate depletion of the specific gene of interest shortly before the experiment and thereby study its direct effect. In this setting, we did not observe significant cell death. Interestingly, we found that short-term depletion of NUP85 or NUP133 had very little effect on the protein levels of other components of the NPC (Supplemental Figure 11).

Morpholino knockdown of Xenopus nup107, nup85, or nup133 causes defects in glomerulogenesis. At the 2-cell stage, Xenopus embryos were injected with a morpholino oligonucleotide targeting nup85, nup107, or nup133. In Xenopus, cell divisions are holoblastic (complete); therefore, we can do lateralized injections of 1 cell at the 2-cell stage such that only half of the animal is affected during development. In this setting, the uninjected side can act as an internal control. In contrast, the initial cleavages of zebrafish embryos are mesoblastic (incomplete); as a consequence, injected mRNAs cannot be localized to just 1 cell. For this reason, Xenopus is an ideal system in which to test rescue especially of lateralized structures such as the kidney (Figure 3A). At stage 35–37, we detected the pronephros using atp1al as a marker in whole-mount in situ hybridization experiments. At this stage, the proximal portion of the pronephros develops a convoluted shape that subsequently straightens out as it extends to the posterior portion of the embryo.

We categorized defects in pronephros morphology based on their severity (Figure 3B). Compared with controls (Figure 3, C and D), morpholino knockdown resulted in abnormal pronephros morphology in 65% of nup85 morphants (Figure 3, E vs. F, and K), 38% of nup107 morphants (Figure 3, G vs. H, and K), and 86% of nup133 morphants (Figure 3, I vs. J, and K). Morpholino knockdown of pax8, which encodes an essential transcription factor in renal development, was used as a positive control to validate this approach (Supplemental Figure 12A). We used the gene NUP155 as a negative control, because the phenotype caused by NUP155 mutations, monogenic atrial fibrillation (33), does not involve the kidney. As expected, knockdown of nup155 did not result in a renal phenotype in the Xenopus model (Supplemental Figure 12B). These findings suggest that the nucleoporins Nup85, Nup107, and Nup133 are required for renal development in Xenopus.

In order to test the specificity of the knockdown experiment and to assess the pathogenicity of human mutations, we used rescue experiments (Supplemental Figure 13). We first injected nup85, nup107, or nup133 morpholinos at the 1-cell stage to deplete these proteins throughout the embryo. At the 2-cell stage, we then injected 1 cell of these morphants with WT or mutant human mRNA of NUP85, NUP107, or NUP133, respectively (Supplemental Figure 13A). While WT mRNA rescued the abnormal kidney morphology seen in morphant kidneys, mutant mRNA reflecting the human SRNS mutations resulted in an impaired restoration of renal morphology (Supplemental Figure 13, B–D). Only the p.Al477Val variant of NUP85 did not show a significantly reduced rescue efficiency. Based on this result, we would predict that this allele may have some residual function, and that it may represent a relatively mild allele as compared with the others. Interestingly, the phenotype in those patients was not notably different.

CRISPR/Cas9 knockout of NUP107, NUP85, or NUP133 increases the level of active Cdc42. Dysregulation of the Rho-like small GTPases RhoA, Rac1, and Cdc42, resulting in impaired actin dynamics, plays an important role in the pathogenesis of monogenic SRNS (34–39). Interestingly, we observed that CRISPR/Cas9–mediated knockdown of NUP107, NUP85, or NUP133 increased the formation of filopodia in immortalized human podocytes (Figure 4, A and B), suggesting that loss of function of these genes alters the podocyte’s cytoskeleton. Because Cdc42 is known to induce filopodia formation, if active (40), we performed the colorimetric Cdc42 G-LISA Activation Assay to determine the cellular level of active Cdc42. We found that in comparison with control cells (Cas9 expression but no guide RNA [gRNA]), CRISPR/Cas9 knockout of any of the 3 genes significantly increased Cdc42 activity (Figure 4C). However, when using the IncuCyte system to assess the migration rate of podocytes, we did not find a significant difference between control and knockout cells (Supplemental Figure 14).

Truncating mutations of nup107 or nup85 in zebrafish induce early lethality, while an in-frame mutation of nup107 does not affect survival. We designed gRNAs targeting exon 2 of nup107, the zebrafish ortholog (NM_001030167.1). Using CRISPR/Cas9 technology, we then generated 2 different stable zebrafish lines, one with a protein-truncating “null” allele (c.137_139del3, p.Ala46delAla) and one with an in-frame mutation, most likely acting as a hypomorphic allele (c.137_139del3, p.Ala46delAla). After het × het in-crossing, we monitored survival twice daily and generated Kaplan-Meier survival curves. Zebrafish with a homozygous truncating mutation of nup107 showed early lethality at 5 days postfertilization (dpf) (Figure 5A) and demonstrated severe developmental malformations, including small eyes, ventral body axis curvature, periorbital edema, and total body edema (Figure 5, C–G, and Table 2). These phenotypes developed at 4 dpf before death on 5 dpf. Zebrafish were born at Mendelian ratios. None of the 29 larvae homozygous for the null allele p.Thr81Argfs*74 were phenotypically normal. Conversely, none of the 42 heterozygous larvae and none of the 34 WT larvae developed any overt phenotype (Table 2). In contrast to the truncating allele, the hypomorphic mutation of nup107 did not affect survival of zebrafish larvae (Figure 5B) and did not result in any overt phenotype (data not shown).

We then used CRISPR/Cas9 technology and a gRNA targeting exon 4 of nup85 (NM_001003625.1) to generate a
Figure 3. Morpholino knockdown of *nup85*, *nup107*, or *nup133* in *Xenopus* embryos causes defects in glomerulogenesis. *Xenopus* embryos were injected with morpholino oligonucleotides (MOs) targeting *nup85*, *nup107*, or *nup133* at the 2-cell stage. Abnormalities in pronephric development, specifically improper formation of the convoluted pronephric duct, were scored at stages 35–37. The pronephros was detected using whole-mount in situ hybridization and *atp1a1* as a marker. (A) Schematic of the experimental setup, in which injection of MO into 1 cell of a 2-cell embryo allows for 1 side of the embryo to develop normally, while the other half serves as an internal control for developmental phenotypes. (B) Based on the severity of the phenotype, morphants were sorted into 4 groups: normal, mild phenotype (delayed or decreased convolution of the pronephric duct, top panel), moderate phenotype (loss of the characteristic pronephric architecture, middle panel), and severe phenotype (pronephros entirely absent, bottom panel). Shown here are examples for each category. (C–J) Left panels display the uninjected control side. Right panels display the injected side. (C and D) Control embryo (injected with nontargeting MO) displaying appropriate pronephric morphology for this stage (see B). (C) Uninjected. (D) Injected. (E and F) MO knockdown of *nup85* results in abnormalities of pronephric development. (E) Uninjected. (F) Injected. (G and H) MO knockdown of *nup107* causes developmental defects of the pronephros. Note the developmental delay and simplification of the convoluted pronephric duct. (G) Uninjected. (H) Injected. (I and J) Morpholino knockdown of *nup133* causes pronephric developmental abnormalities. (I) Uninjected. (J) Injected. (K) Phenotypes in *nup85*, *nup107*, *nup133*, or *nup155* morphants were categorized into the 4 groups. Note that as compared with uninjected controls (UC, *n* = 32), *nup85* (65% abnormal, *n* = 34) and *nup133* (86% abnormal, *n* = 28) morphants more frequently display severe phenotypes, while *nup107* (38% abnormal, *n* = 34) morphants tend to show milder phenotypes. All experiments were performed at least twice.
To test for renal anomalies and for a kidney-specific phenotype, we used transmission electron microscopy (TEM) to investigate the ultrastructure of the renal glomerulus and used H&E staining to study renal histology. For the 2 null alleles of nup107 and nup85, we investigated 2 homozygous versus 2 heterozygous fish by TEM and 8 homozygous versus 8 heterozygous fish by renal histology for each gene. We detected significant tissue decay within the glomerular region, but also globally throughout the zebrafish line carrying the truncating mutation p.Arg107Cysfs*15 of nup85, the zebrafish ortholog of human NUP85. As observed with nup107 knockout, homozygous nup85-knockout fish showed early lethality and died at day 8 dpf (Supplemental Figure 15A). The phenotypic features of nup85-knockout fish resembled those of nup107-knockout fish, including small eyes, body axis curvature, and edema (Supplemental Figure 15, B–E, and Supplemental Table 2).

**Figure 4.** CRISPR/Cas9–mediated knockout of NUP107, NUP85, or NUP133 induces filopodia formation and increases active Cdc42 in human podocytes. Immortalized human podocytes underwent lentiviral transduction with a plasmid expressing a Cas9-GFP fusion construct under the control of a doxycycline-inducible promoter and a single gRNA. For each gene, 2 different cell lines were generated expressing gRNAs against NUP107 (targeting exon 4 or 11), NUP85 (targeting exon 1 or 15), or NUP133 (targeting exon 1 or 5), respectively. Experiments were performed 72 hours after induction of Cas9 expression with doxycycline (1 μg/ml). (A) We stained immortalized human podocytes expressing empty vector (MOCK) or individual gRNAs targeting NUP107, NUP85, or NUP133 with phalloidin to detect F-actin fibers. Podocytes that had either 3 actin-based protrusions or 1 filamentous protrusion of more than one-quarter of the cell body were quantified as “filopodia positive.” Representative images showing “filopodia-negative” control cells and NUP107-, NUP85-, or NUP133-knockout podocytes that exhibited filopodia (arrowheads). Scale bars: 25 μm. The result was confirmed in 3 independent experiments. (B) Quantification of approximately 50 cells for each condition resulted in 22% of MOCK-expressing cells with filopodia (11/50), in contrast to 58% (29/50) for NUP107 gRNA exon 4 (ex4) and 46% (23/50) for NUP85 gRNA ex11; 50% (25/50) for NUP85 gRNA ex1 and 54% (30/56) for NUP85 gRNA ex15; and 44% (22/50) for NUP133 gRNA ex1 and 38% (19/50) for NUP133 gRNA ex5. Note that knockout podocytes show increased filopodia formation. (C) Using the colorimetric G-Lisa Cdc42 Activation Assay Biochem Kit (Cytoskeleton), we demonstrate an increase in the active state of Cdc42 following CRISPR/Cas9–mediated knockout of NUP107, NUP85, or NUP133 in human podocytes. Data points represent 3 independent experiments (highlighted in different colors) and are displayed with mean and SD. P values calculated by 1-way ANOVA are indicated in the figure as *P < 0.05; **P < 0.01.
lavra. We are therefore hesitant to interpret these findings as a specific podocyte phenotype, but rather assume that the loss of an essential protein induces injury in all cell types.

**Primary patient cell lines carrying protein-truncating mutations of NUP37 and NUP107 display changes in NPC composition and nuclear morphology.** We obtained primary cell lines from 2 patients with microcephaly and with mutations of NUP37 and NUP107, respectively. In dermal fibroblasts from a patient carrying the p.Arg306* allele of NUP37, we demonstrated that at the mRNA (Supplemental Figure 6E) and protein level (Figure 6, A and D) NUP37 was reduced in comparison with control cells. Reduced NUP37 protein levels resulted in codepletion of other components of the Y complex, such as NUP107 and NUP160 (Figure 6, A–D). A similar effect was observed in a lymphoblastoid cell line (LCL) from a patient with the p.Met101Ile mutation of NUP107 (Supplemental Figure 16, A–C). These findings suggest that, as previously described (28, 29), proteins of the Y complex depend on each other for protein stability. Interestingly, in both cell lines residual amounts of the mutant protein were present.

When investigating the consequences of reduced NUP37 amounts for NPC density, we found a reduced intensity of the mAb414 antibody signal, suggesting a lower number of nuclear pores in mutant fibroblasts as compared with control cells (Figure 6, E and F). Using antibodies detecting heterochromatin (anti-HP1β; Figure 6G) and nucleoli (anti-fibrillarin; Figure 6, H and I), we demonstrated an alteration of chromatin organization and nuclear morphology in NUP37 mutant fibroblasts. TEM confirmed this observation (Figure 6, J–M, arrowheads). Additionally, we found
the perinuclear space to be widened and irregular (Figure 6, L and M, arrows), and we detected bulbous invasions of the nuclear envelope (Figure 6M, star) in NUP37 mutant fibroblasts. Notably, the cell proliferation rate of NUP37-mutant as compared with control fibroblasts was reduced (Supplemental Figure 6F). We admit that, as mutations in NUP genes predominantly manifest in postmitotic, epithelial tissues, proliferating fibroblasts have important limitations as a model system.

Discussion

In summary, we here discovered recessive mutations in the genes NUP85, NUP133, and NUP160 as 3 monogenic causes of SRNS. Interestingly, all 3 genes and the gene NUP107, in which we found 3 pathogenic alleles, encode proteins that are components of a distinct subunit of the nuclear pore complex (NPC), the so-called Y complex or outer ring subunit of the NPC. In coIP experiments, we demonstrated that 5 human mutations altered protein-protein interaction between direct binding partners in the NPC. The renal phenotype of Xenopus morphants could be rescued by WT, but not mutant, mRNA, thus demonstrating pathogenicity for the alleles that we identified in patients with SRNS. In contrast to all other tested alleles, the p.Ala477Val allele of NUP85 rescued the phenotype, thereby suggesting that in this case the function of the encoded protein was at least partially preserved. This result may be explained by (a) the mild chemical difference between WT and altered amino acid (alanine vs. valine) and (b) the fact that this residue was not conserved between the human and the frog protein. As there was no difference in the severity of the phenotype in affected patients, we assume that the function of the human protein may still be significantly altered by this mutation.

In cell culture studies, we observed that knockout of NUP107, NUP85, or NUP133 in immortalized human podocytes changed the activity state of the Rho-like small GTPase Cdc42, a pathway that is known to be relevant to monogenic forms of SRNS (34–37, 41). When generating stabile, transgenic zebrafish larvae using CRISPR/Cas9 technology, we observed that null alleles of nup107 or nup85 caused severe developmental malformations and early lethality. In contrast, an in-frame mutation of nup107 was compatible with survival and did not cause apparent developmental anomalies, thus reflecting the allelism seen in patients with NUP107 mutations.

Accumulating evidence suggests that apart from their universal function in nucleocytoplasmic transport, certain NUP proteins may have additional, individual functions in cell differentiation and during development (16, 42). By performing morpholino knockdown experiments, we generated the first evidence to our knowledge that Nup107, Nup85, and Nup133 are required for renal development in Xenopus. The observation that knockdown of nup155 did not result in abnormal pronephros morphology suggests that this function is specific to certain NUPs. With one exception, mutant mRNAs, reflecting alleles that we found mutated in SRNS patients, were less efficient than WT mRNA in restoring the pronephros morphology in Xenopus morphants. Based on these observations, we hypothesize that SRNS development in patients with mutations of NUP107, NUP85, or NUP133 partially reflects an alteration of specific functions of those NUPs during renal development.

The Rho-like small GTPases RhoA, Rac1, and Cdc42 are regulators of the actin cytoskeleton (43). Podocytes depend on a highly dynamic and tightly regulated actin cytoskeleton to generate and maintain their actin-based foot processes and the slit membrane of the renal glomerular filter (39). Dysregulation of these Rho-like small GTPases has been observed in several monogenic forms (34–37, 41) and mouse models of SRNS (38, 44). We therefore propose that the observed increase in the level of active Cdc42 upon knockout of NUP85, NUP107, or NUP133 may contribute to the pathogenesis of SRNS in patients with mutations in these genes. Mechanistically, further studies will be needed to elucidate the molecular link between NPC disruption and dysregulation of Cdc42 activity. However, based on currently available data, it seems likely that alterations in NUPs are linked to the activity state of Cdc42 by an indirect effect. This functional link may be mediated, e.g., by nuclear import of specific regulatory elements or by nuclear export of distinct RNA species.

Prominent phenotypic features of nup107- and nup85-knockout zebrafish larvae included edema, ventral body axis curvature, and diminished eye size. This phenotype recapitulated a previously published transcription activator-like effector nuclease-mediated (TALEN-mediated) nup107-knockout zebrafish line (18). Before death, knockout zebrafish showed profound decay in all tissues, including the glomerular area. However, because of its global character, we are hesitant to interpret these findings as a tissue-specific phenotype. We assume that knockout larvae die prematurely as a result of the complete lack of Nup85 and Nup107, two essential proteins, before a specific renal phenotype may develop.

Summarizing all findings, we suggest the following explanation for the observed genotype-phenotype correlation in patients with NUP107 mutations: While missense mutations likely have little impact on protein stability and cellular protein level, the p.Met101Ile allele of NUP107 resulted in a reduced amount of NUP107 protein with consecutive changes in NPC composition and nuclear organization. In conclusion, we propose that 3 types of mutations in essential NUP-encoding genes exist: (a) complete-loss-of-function mutations that cause embryonic lethality and are therefore not observed in human patients; (b) partial-loss-of-function mutations that reduce the amount of the encoded proteins and cause syndromic, developmental phenotypes, but are compatible with survival; and (c) strictly hypomorphic missense mutations that impair only specific aspects of the protein’s functionality without affecting its overall function and therefore result in a distinct, organ-restricted phenotype.

Mutations in the genes NUP93, NUP205, and NUP107, encoding proteins of the NPC, were recently described as causing SRNS (21–24). It was vexing that mutations in these essential genes of high evolutionary conservation and universal relevance gave rise to such a distinct phenotype. By identifying 3 additional genes that encode scaffold proteins of the NPC and cause SRNS, if mutated, we provide further evidence that distinct, hypomorphic mutations in this group of fundamental genes may specifically impact podocytes and may manifest in a cell type–specific manner.

Methods

Study participants. We collected blood samples and pedigree information after informed consent from individuals with SRNS or their guardians.
**Figure 6. Impact of a NUP37 mutation on the composition of the NPC and on nuclear structure.** A primary fibroblast cell line from patient PN-2 with the homozygous truncating mutation p.Arg306* of NUP37 (mutant) was compared with control fibroblasts (WT). DAPI stains DNA (blue). (A–C) Confocal microscopy of immunostaining for NUP37, NUP107, and NUP160 in mutant versus control fibroblasts. (D) Immunoblotting of NUP37, NUP160, and NUP107 in mutant versus control fibroblasts. α-Tubulin serves as a loading control. Note that protein levels are reduced in mutant fibroblasts. (E) Immunostaining with an antibody against several FG-repeat nucleoporins (mAb414) in control (left) versus mutant (right) fibroblasts. (F) Quantification of E demonstrates a significant reduction in the number of NPCs per square micrometer in mutant versus control cells. Data were obtained for 100 cells from 3 different experiments. Error bars denote SEM. \( P = 0.0048 \) (Student’s t test). (G) Immunostaining of HP1β (green), labeling heterochromatin, demonstrates an altered pattern in mutant versus control fibroblasts. (H) Filibarlin (green) was used to stain nucleoli. Note that fibrilarlin staining was more dispersed in mutant fibroblasts. (I) Quantification of 150 cells from 3 independent experiments demonstrates a significantly increased number of nuclei with abnormal nucleoli in mutant versus control cells. Error bars represent SEM. \( P = 0.0018 \) (Student’s t test). Scale bars in A–C, E, G, and H: 5 μm. (J–M) TEM images of control versus mutant fibroblasts. In control (WT) cells, a regular nuclear envelope (arrow outside the nucleus in J) and well-arranged heterochromatin in the proximity of the nuclear envelope (arrow inside the nucleus in J) can be seen. Note that the nuclear architecture of mutant fibroblasts is altered; specifically, (a) there is an abnormal arrangement of heterochromatin and nucleoli (arrowheads, J vs. K); (b) the perinuclear space is widened and irregular (arrows, L vs. M); and (c) bulbous invasions of the nuclear envelope were observed (star, M). Scale bars are defined in each image. Immunofluorescence experiments (A–C, E, G, and H) and immunoblotting results (D) were confirmed in 3 independent experiments. ** \( P < 0.01 \).

**Homozygosity mapping.** Homozygosity mapping was performed using the GeneChip Human Mapping 250k Sty Array, Affymetrix, and the programs GENEHUNTER 2.1 (45, 46) and ALLEGGRO (47) as described previously (48). Alternatively, homozygosity mapping was generated based on whole exome sequencing data using the Genome Analysis Tool Kit (49) and the program Homozygosity Mapper (50). For patients with a homozygous mutation of NUP37, NUP85, or NUP133, we used the open-source program Jmol (an open-source Java viewer for chemical structures in 3D; http://www.jmol.org/) to generate the 3D projections that are shown in Figure 2.

**cDNA cloning and stable CRISPR/Cas9 cell lines.** The following human cDNA constructs were used for this study: NUP107 (NM_020401.3), NUP85 (NM_024844.4), and NUP133 (NM_018230.2). The following expression vectors were used for cell culture experiments: pRKS-N-Myc, pDEST69-N-Flag, and pCDNA6.2-N-GFP. The inducible CRISPR/Cas9 backbone (TLCV2) was a gift from Adam Karpf (Eppley Institute, University of Nebraska Medical Center, Omaha, Nebraska, USA) (Addgene plasmid 87360). This backbone is based on LentivCRISPR-v2 and expresses Cas9-P2A-GFP under the control of a doxycycline-inducible promoter (tight TRE promoter). Additionally, a single gRNA and a puromycin-resistance cassette are expressed constitutively. Stable cell lines were generated using lentiviral transduction and puromycin selection (4 μg/ml) for more than 7 days. Cas9 expression was induced 72 hours before all experiments using doxycycline (1 μg/ml). Western blotting and immunofluorescence experiments confirmed a reduction in the expression level of targeted proteins (Supplemental Figures 8–10).

gRNA sequences are provided in Supplemental Table 3. Antibodies are listed in Supplemental Table 4.

**Cell lines.** Immortalized human podocyte were a gift from Moin Saleem (University of Bristol, Bristol, United Kingdom) and have been extensively characterized in previous publications (56). HEK293T cells were purchased from the ATCC biological resource center and were used for virus production and coIP experiments. Cells were tested monthly for mycoplasma contamination. Primary cell lines, i.e., human dermal fibroblasts or lymphoblastoid cell lines (LCLs) from patients with a homozygous mutation of NUP107 (PN-1) or NUP85 (PN-2), were generated as described previously (57, 58).

**Commmunoprecipitation.** CoIP experiments were performed in HEK293T cells. Thirty-six hours after cDNA transfection, protein lysates were harvested, and 450–600 μg of protein was incubated for 4 hours with (a) EZview Red Anti-c-Myc Affinity Gel (Sigma-Aldrich), (b) EZview Red ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich), or (c) Chromotek-GFP-Trap agarose beads (Allele Biotechnology) depending on the expressed fusion protein and as indicated in the figures. Before elution, beads were washed 5 times with IP lysis buffer (Thermo Fisher Scientific). The result of each coIP study was confirmed in 3 independent experiments and was repeated using differently tagged fusion proteins.

**Phenotyping in Xenopus embryos.** Antisense morpholino oligonucleotides (MOs) or mRNAs were injected either at the 1-cell stage or into 1 cell of the 2-cell embryo as previously described (59). Sequences of MOs are provided in Supplemental Table 3. We generated mRNA of WT and mutated human sequences using a T7 promoter template in the pcDNA6.2-N-GFP backbone. In vitro capped mRNA was gener-
Table 2. Quantification of phenotypes in homozygous nup107-knockout zebrafish larvae (p.Thr81Argfs*74)

<table>
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<th>Phenotype</th>
<th>Small eyes</th>
<th>Ventral curvature</th>
<th>Body edema</th>
<th>Periorbital edema</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homozygous</td>
<td>11</td>
<td>18</td>
<td>7</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>42</td>
</tr>
<tr>
<td>WT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>34</td>
</tr>
</tbody>
</table>

Definitions of assessed phenotypes: Small eyes: assessed by measurement of the area of the retina pigment epithelium in dorsal view images (see yellow drawn circumferences in Figure 5, C and D). Body edema: defined as a clear, fluid-filled space around the torso in dorsal view images (see Figure 5G). Periorbital edema: defined as a clear, fluid-filled space and/or behind the retina pigment epithelium of the eyes in the dorsal view (see Figure 5G). Eye size and body and periorbital edema could not be assessed in ventrally curved fish (see Figure 5F; n = 18).

*Five of seven homozygous p.Thr81Argfs*74 fish with small eyes and body edema also had periorbital edema.

Immunofluorescence, confocal microscopy, and quantification of filopodia. Confocal imaging in human podocytes was performed using the Leica SP8 system with an upright DM6000 microscope, and images were processed with the Leica AF software suite. Experiments for filopodia quantification were performed in human podocytes stably expressing the inducible Cas9-P2A-GFP system with empty vector (MOCK) or single gRNA targeting NUP107, NUP85, or NUP133. Fifty cells for each condition were analyzed. The GFP signal confirmed expression of Cas9-P2A-GFP. Phalloidin stained F-actin fibers and was used to identify filopodia. To score cells as “filopodia positive” or “filopodia negative,” we used the following criteria: (a) cells that displayed at least 1 filamentous protrusion with a length of more than one-quarter of the cell diameter, or (b) cells that had 3 or more actin-based, spike-like protrusions. Cells that fulfilled one or both criteria were scored as filopodia-positive cells.

Images in Figure 6 and Supplemental Figure 16A were taken using a confocal laser scanning microscope (TCS SP8 gSTED, Leica Microsystems) or a confocal microscope (LSM TCS SP5, Leica Microsystems). Nuclear pore density was analyzed using the “particle analysis” tool of ImageJ (version 1.51z; NIH). Samples were prepared for transmission electron microscopy as reported previously (60) and viewed with a transmission electron microscope (JEOL JEM2100PLUS). Quantitative PCR experiments were performed as described previously (53). The cell proliferation assay was performed as described previously (57).

Assessment of the cell migration rate of immortalized human podocytes with CRISPR/Cas9-mediated knockout of NUP85, NUP107, or NUP133. We used the IncuCyte video microscopy system (Essen Biosciences). Podocytes were seeded on a 96-well plate and grown to confluence, and a standardized scratch wound was made using the Woundmaker device according to protocol. Wound closure was recorded by live cell imaging every hour for 20–24 hours. Data analysis was performed using the IncuCyte 96-well Kinetic Cell Migration and Invasion Assay software module. Individual data points are presented as mean ± SD resulting from at least triplicate measurements. Experiments were repeated 3 times independently.

Generation and characterization of stable zebrafish lines with CRISPR/Cas9-mediated introduction of hypomorphic or truncating mutations of nup107 or nup85. We designed single gRNA targeting exon 2 of nup107 and exon 4 of nup85 using the CHOPCHOP online tool (https://chopchop.ucsf.edu) (61). Single gRNA and recombinant Cas9 protein were injected at the 1-cell stage. Successful mutagenesis was determined by a T7 endonuclease assay as described previously (62). Positive clutches were raised to adulthood and outcrossed against WT fish. Founder lines were genotyped using Sanger sequencing. We chose 1 line with a truncating mutation of nup107 (c.50_56del7, p.Thr81Argfs*74), 1 line with an in-frame deletion mutation of nup107 (c.137_139del3, p.Ala46delAla), and 1 line carrying a null allele of nup85 (c.323_332delGAGCCCTGTATinsGCT, p.Arg107Cysfs*15).

Phenotyping in zebrafish larvae. We generated Kaplan-Meier survival curves by comparing WT, heterozygous, and knockout larvae. During the experimental period, we monitored the phenotype twice daily. All dead larvae and all surviving fish were genotyped at the end of the experiment using Sanger sequencing.

Phenotyping was performed at 4 dpf for nup107 fish and 7 dpf for nup85 fish. Zebrafish with ventral body axis curvature were imaged from a lateral view. Therefore, other parameters were not assessed in those fish. Larvae with normal body axis were imaged from a dorsal view. To determine eye size, we measured the surface of the black pigmented epithelium in the retina using the program ImageJ. We defined body edema as the presence of a clear, fluid-filled space around the torso in dorsal view, and periorbital edema as the presence.
of a clear, fluid-filled space before and/or behind the retinal pigment epithelium of the eyes. Statistical analysis of eye size was performed using GraphPad Prism to calculate 1-way ANOVA test with multiple comparisons and a standard confidence interval of 95%. The resulting 2-tailed P values and F values are indicated. Zebrafish experiments were performed in Danio rerio, strain l-fabp; VDBP-GFP (AB). This line was a gift from Weihin Zhou (University of Michigan, Ann Arbor, Michigan, USA). For additional details see Supplemental Methods.

Study approval. Approval for human subjects research was obtained from Institutional Review Boards of the University of Michigan (Ann Arbor, Michigan, USA), Boston Children’s Hospital (Boston, Massachusetts, USA), and the National Institute for Biotechnology and Genetic Engineering, Faisalabad, Pakistan. All national and institutional guidelines for the care and use of laboratory animals were followed. Zebrafish experiments were approved by the Boston Children’s Hospital Institutional Animal Care and Use Committee (IACUC; protocol 15-12-3087R). Xenopus experiments were approved by the Yale IACUC (protocol 2015-11035).

Author contributions
SL, DS, AD, JR, WT, SA, HYG, SS, JAL, JKW, TH, EW, AJM, HH, AM, BB, S Motamemy, JA, S Mane, MSH, PN, RPL, and FH performed whole exome evaluation and mutation analysis. DAB performed coimmunoprecipitation experiments and in vitro experiments in podocytes. WA confirmed the coimmunoprecipitation experiments. RS, A Kolb, TJS, JMS, CAH, TMK, and KE performed zebrafish experiments. JM, CPL, and MKK performed Xenopus experiments. MA, KS, AIAK, AAN, and MSH performed in vitro experiments in primary patient cell lines. SL, DS, AD, DAB, JR, WT, HMF, DFG, LK, SH, KAA, MH, NM, RE, ES, FSA, QS, SX, A Khan, SSW, GA, MU, SMB, SG, CA, and FH recruited patients and gathered detailed clinical information for the study. All authors critically reviewed the paper. FH conceived of and directed the project. DAB and FH wrote the paper.

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