Mutations in ANKS6 Cause a Nephronophthisis-Like Phenotype with ESRD

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Abstract

Nephronophthisis (NPHP) is one of the most common genetic causes of CKD; however, the underlying genetic abnormalities have been established in <50% of patients. We performed genome-wide analysis followed by targeted resequencing in a Turkish consanguineous multiplex family and identified a canonical splice site mutation in ANKS6 associated with an NPHP-like phenotype. Furthermore, we identified four additional ANKS6 variants in a cohort of 56 unrelated patients diagnosed with CKD due to nephronophthisis, chronic GN, interstitial nephritis, or unknown etiology. Immunohistochemistry in human embryonic kidney tissue demonstrated that the expression patterns of ANKS6 change substantially during development. Furthermore, we detected increased levels of both total and active β-catenin in precystic tubuli in Han:SPRD Cy/+ rats. Overall, these data indicate the importance of ANKS6 in human kidney development and suggest a mechanism by which mutations in ANKS6 may contribute to an NPHP-like phenotype in humans.


CKD is a progressive and irreversible condition with significant health and economic effects. The incidence of CKD in children varies between countries, ranging between 3 and 12 cases per million.1–3 Whereas most cases of pediatric CKD are related to hereditary and congenital abnormalities of suspected genetic origin, monogenic disease causes have been identified in only a fraction of patients to date (http://omim.org/statistics/entries). Nephronophthisis (NPHP), an autosomal recessive cystic kidney disease, is one of the most frequent genetic causes of CKD requiring RRT in the first 3 decades of life.4 Clinical symptoms include polyuria, polydipsia with regular fluid intake at nighttime, secondary nocturnal enuresis, and anemia. Ultrasonography shows increased echogenicity and cyst formation at the corticomedullary junction in small or normal-sized kidneys. Renal histology shows a characteristic triad of tubular basement membrane disruption, tubulointerstitial nephropathy, and corticomedullary cysts.5 NPHP is a genetically heterogeneous disorder. To date, more than a dozen genes causing NPHP have been identified, all of which affect cilia/centrosome function.6–18 Furthermore, an NPHP-like phenotype was recently associated with defects in a magnesium transporter, thus expanding the spectrum of the disease.19 However, all NPHP genes identified to date account for <50% of patients with an NPHP-like phenotype,20 indicating that a substantial number of disease-causing genes are still waiting to be discovered.

In our large cohort of patients with CKD, we identified a Turkish multiplex pedigree with no apparent etiology that comprised six patients with CKD/ESRD over four generations (Figure 1A, Table 1; see the Supplemental Appendix for a detailed description of the clinical history). Participants with a diagnosis of NPHP or CKD between 3 and 12 cases per million.1
of unknown etiology were ascertained and enrolled in the study after providing informed consent, in accordance with human subject research protocols approved by the Ethical Committee of Hacettepe University in Ankara, Turkey (HEK 12/112-13).

The index patient (V-1) with a biopsy-proven NPHP (Supplemental Figure 1), her apparently healthy sibling (V-2), and their parents (IV-5 and III-11) as well as four relatives with CKD/ESRD (III-5, III-6, III-7, and III-10) were genotyped using a 250K Affymetrix single-nucleotide polymorphism (SNP) array followed by homozygosity mapping (Figure 1, A and B). We identified only one uninterrupted homozygous region across the genome, which was located on chromosome 9q (Figure 1B). This result was also confirmed by multipoint parametric linkage analysis using MERLIN (version 1.1.2; http://www.sph.umich.edu/csg/abecasis/MERLIN/) (Figure 1C, Supplemental Figure 2). Within the critical region on chromosome 9q22, haplotypes residing between SNP markers rs12347549 and rs1933796 were identical in all affected individuals and in the apparently healthy 6-year-old sibling (V-2) (Supplemental Figure 3). This region (approximately 6 megabases) contains approximately 600 exons in 63 known and predicted genes. We therefore performed next-generation sequencing of the target region (Supplemental Table 1). A homozygous acceptor splice site variation segregating with the disease was detected in ANKS6 (c.2512-2A>C) and could subsequently be confirmed by Sanger sequencing. All individuals with CKD/ESRD as well as the apparently healthy younger sibling were homozygous for this variant and the parents were heterozygous (Figure 1D, Table 2). The variant was not detected among 180 healthy Turkish individuals and was not present in public databases.

We analyzed the effects of the identified variant on RNA splicing. Sequencing of cDNA showed that the c.2512-2A>C variation causes retention of intron 14 and loss of exon 15, which encodes 34 evolutionarily conserved amino acids of the C-terminal part of the protein (Figure 1, E and F, Table 2). The retained intron was predicted to introduce 31 different amino acids, which are not found in the wild-type protein (Supplemental Figure 4). The expression of mutant protein was demonstrated via immunohistochemistry with an antibody recognizing amino acid residues between 726 and 855 of human ANKS6, which showed minimal glomerular but strong tubular staining (Figure 2A). In the absence of published information regarding ANKS6 expression in the human fetal kidney, we performed immunohistochemistry in first-, second-, and third-trimester kidney tissues. Consistent with published animal data,21 we found strong staining in S-shaped bodies and the Bowman’s capsule but moderate staining in glomeruli of fetuses in the first and second trimesters of pregnancy. As the fetus develops, ANKS6 expression is restricted to the tubular epithelium and disappears from the glomeruli (Figure 2, B–E). This expression pattern suggests a critical role of ANKS6 in tubular and glomerular development and later on in tubular maintenance.

Recent in vitro studies demonstrated the interaction of two sterile alpha motif domain-containing proteins, Anks6 and Bicc1, which are involved in murine polycystic kidney disease (PKD).22 Furthermore, an Anks6 (p.R823W) mutation was recently found to cause the cystic phenotype in the PKD/Mhm/cy/+ rat, a widely used animal model of human PKD.23

The role of Wnt signaling is well established in cystic kidney diseases.24–26 The canonical β-catenin pathway and the planar cell polarity pathway recently received attention for their roles in multiple cellular processes within the kidney.27 It has been demonstrated that both overactivation and inactivation of the canonical Wnt pathway lead to cystic renal phenotypes,28–30 suggesting that the Wnt/β-catenin system must be delicately balanced in the kidney to prevent cyst formation. Bicc1 is known to regulate Dishevelled signaling29 and mutations in BICC1 were reported to cause cystic renal dysplasia in humans that is associated with hyperactivation of the Wnt pathway.30 Hoff et al.18 recently reported that ANKS6 affects the function of NPHP2/inversin (INVS), another regulator of Dishevelled and Wnt signaling.31 For these reasons, we tested whether the Wnt pathway was activated in a biopsy tissue from the index patient (PN25, V-1) and in the kidneys of 12-week-old Cy/+ rats. Semiquantitative evaluation showed that tubular β-catenin staining was higher in the patient than those seen in minimal change disease but was comparable with PKD (Figure 2F, Supplemental Figure 5). In Cy/+ rats, we clearly showed that both total and active β-catenin were increased in pre cystic tubuli, indicating strong activation of the Wnt pathway (Figure 2G). Taken together, these findings support the involvement of the Wnt/β-catenin pathway in the pathogenesis of this disease and may offer a mechanistic explanation for the NPHP phenotype observed in our patient.

This study was expanded to other patients with CKD caused by NPHP, chronic GN, interstitial nephritis, or unknown etiology in order to identify additional families. Screening of 23 patients from our own cohort and 33 patients from the DNA biobank of the international 4C study (http://www.4C-Study.org) yielded two missense, one splice site, and one non-sense heterozygous variations in ANKS6 (Table 2, Supplemental Figure 6; see the Supplemental Appendix for detailed clinical histories).

Although heterozygous ANKS6 mutations identified in those patients are less likely to fully explain the phenotype, oligogenic inheritance can be hypothesized and has been described in NPHP.32 Therefore, we screened all known NPHP genes for further mutations via targeted next-generation sequencing (Table 2). Interestingly, in patient PNS21, we found an additional heterozygous c.1838G>A (p.Arg613Gln) variation in NPHP2/INVS, which was recently shown to be connected to NEK8/NPHP9 by ANKS6.18 In silico analyses predict that it might be a benign variation (Table 2). However, this variation is not present in existing databases, including our in-house database that has been generated by whole genome/exome sequencing of 275 individuals. In addition, the patient’s healthy dizygotic twin showed the wild-type sequence for both changes in ANKS6 and NPHP2/INVS, which may support
Figure 1. ANKS6 mutation was identified in a Turkish multiplex family using homozygosity mapping and targeted resequencing. (A) Pedigree of the index family. Squares indicate male participants and circles female participants. Solid symbols indicate affected individuals. Double-horizontal bars indicate consanguinity. (B) The schematic representation of homozygous chromosome region captured from VIGENOS. Homozygous genotypes identical to the genotype data obtained from the index case (V-1) are shown in blue, whereas heterozygous SNPs.
the notion that the variations might be related to the phenotype. These observations might corroborate our oligogenicity hypothesis in this subtype of NPHP and might point to the pathogenic role of ANKS6 in the disease. Another notable finding was the detection of a homozygous NPHP1 deletion in one of the patients (patient 4Cis3009) harboring a heterozygous ANKS6 mutation. Oligogenic inheritance has been described in patients with a homozygous NPHP1 deletion as well.32 One could argue that the NPHP1 deletion would explain the phenotype on its own; however, proteinuria was present in this and other patients (PN25 V-1, PN306, PN516, and PN521; see Table 1 and the Supplemental Appendix), which is a rather unexpected finding in patients with NPHP. We demonstrated ANKS6 expression in both the developing glomerulus (Figure 2) and in differentiated and undifferentiated human podocyte cell lines (data not shown). In addition, in neonatal PKD/Mhm(cy+) rats, distinct Anks6(e.R823W) mRNA expression has been found in the tubular epithelium and in podocytes.21 Glomerular cysts have been reported in homozygous Nek8 knockout mice and in men harboring homozygous truncating NPHP3 mutations.33,34 Moreover, the homozygous Pro209Leu mutation in TTC21B (encoding the cilia protein IFT139), which has previously been associated with isolated NPHP, was unexpectedly found to cause nephrotic syndrome/FSGS in seven families.35 Thus, the presence of proteinuria in our patients might result from an additive effect of the ANKS6 mutation and may point to a critical function of this gene not only in tubular epithelial cells but also in podocytes. This might also provide an explanation of the early onset of the disease in the index patient (PN25, V-1) who is also carrying a heterozygous nephrin mutation. Hoff et al.18 recently attempted to identify interacting proteins of never in mitosis A (NEK8; which is mutated in NPHP) in HEK293T cells and described ANKS6 as a new NPHP family member connecting NEK8 to INVS. Mutation analysis of their NPHP cohort identified eight individuals from six families. Of these six families, a c.2512-2A>C mutation has been described in a Turkish family with PKD and aortic stenosis. We also identified the same mutation in a family with NPHP with a different approach. However, the families described in these two studies were not related to each other as confirmed by different pedigrees, indicating that this mutation might be a founder in Turkish population. Cardiac defects are very common in humans with mutations of NEK8, NPHP3, and INVS as well as in knockouts of the corresponding genes and are considered a part of a heterotaxy phenotype.34,36 Hoff et al.18 observed structural heart defects, which can result from defective cardiac looping during embryogenesis in zebrafish embryos deficient for Anks6, Nek8, and Nphp3. Similarly, we also observed cardiac involvement, namely mitral and tricuspid insufficiency, mitral stenosis, aortic stenosis, and aortic aneurysm, in four individuals, confirming that ANKS6 is also involved in cardiac development. However, we did not note situs inversus or other heterotaxy findings in any of the analyzed patients.

In summary, we identified mutations in ANKS6 as a cause of both NPHP-related disorders and proteinuria. We also showed that ANKS6 participates in the regulation of Wnt/β-catenin signaling in the kidney, a pathway that is important in both tubular and glomerular development as well as in tubular maintenance later in life.

**CONCISE METHODS**

**Homozgyosity Mapping and Linkage Analyses**

Genomic DNA was extracted from peripheral blood lymphocytes using standard procedures. Genome-wide analysis was performed with GeneChip mapping 250K NspI SNP arrays from Affymetrix according to the manufacturer’s recommendations. VIGENOS (Hemosoft) and MERLIN softwares were used to carry on homozgyosity mapping and parametric linkage analysis, respectively. Autosomal recessive inheritance with 99% penetrance and a phenocopy rate of 0.001 were assumed. For the calculations, consanguniuty loops were broken.

**Next-Generation Sequencing**

**Targeted Resequencing**

Biotinylated probes covering the entire homozgyous region were designed by NimbleGen (Roche Applied Science/NimbleGen). Library preparation, target enrichment, and emulsion-based PCR were completed according to the manufacturer’s procedures (Roche Applied Science/NimbleGen). Total beads obtained from emulsion-based PCR were sequenced by both GS-Junior and GS-FLX sequencers, and all sequence reads obtained from these instruments were combined. GS Reference Mapper software was used for bioinformatic analysis (Roche Applied Science).

**Sequencing of Known NPHP Genes**

All exons and adjacent intronic boundaries of NPHP and NPHP-related genes described thus far appear in orange. (C) Graphical representation of the multipoint LOD score using the parametric linkage analysis program (MERLIN), which shows a maximum LOD score in chromosomal region of 9q22 with an interval between 97 and 104 cm. (D) Sanger sequence electropherograms of the ANKS6 mutation (arrowheads) (c.2512A>C). The upper chromatogram reports the heterozygous mutation in one of the parents (III-11). The lower chromatogram shows the homozygous mutation in the proband (V-1). Codons and the corresponding amino acids are displayed above the chromatograms. Lowercase letters indicate the intronic sequence. (E) Sequencing of ANKS6 cDNA of peripheral blood lymphocytes from one healthy individual (top) and the proband (bottom). Splice site mutation causes retention of intron 14. (F) Multiple sequence alignment by Clustal omega. Amino acid residues are given as single letter codes with the following color for residues: red, small hydrophobic or aromatic (AVFPMILWY); blue, acidic (DE); magenta, basic (RHK); and green, hydroxyl, amine, basic, and glutamine (STYHCNGQ). The lowest row indicates conservation among the group of sequences. The asterisk indicates positions that have a single, fully conserved residue, whereas colons indicate conserved substitutions and periods indicate semiconserved substitutions. LOD, logarithm of the odds.
<table>
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<tr>
<th>Family, Individual</th>
<th>Consanguinity</th>
<th>Age at Onset (yr)</th>
<th>Initial Presentation/Renal Phenotype</th>
<th>Proteinuria (mg/mg creatinine)</th>
<th>Serum Albumin (g/dl)</th>
<th>Extrarenal Findings</th>
<th>Age at ESRD (yr)</th>
<th>RRT</th>
<th>Histology</th>
<th>Last eGFRa (ml/min per 1.73 m²)</th>
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<tr>
<td>PN25</td>
<td>Yes</td>
<td>7</td>
<td>Screening</td>
<td>0.47</td>
<td>4.5</td>
<td>None at 15 yr</td>
<td>13</td>
<td>RTx</td>
<td>NPHP-like</td>
<td>114</td>
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<td>V-1</td>
<td>Yes</td>
<td>—</td>
<td>None at 7 yr</td>
<td>None</td>
<td>None at 7 yr</td>
<td>None at 7 yr</td>
<td>None</td>
<td>ND</td>
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<td>III-3</td>
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<td>CKD stage 2</td>
<td>N/A</td>
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<td>None</td>
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<td>31</td>
<td>HD</td>
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<td>Died</td>
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<td>N/A</td>
<td>MS,TI</td>
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<td>HD</td>
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<td>N/A</td>
<td>MS,AS</td>
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<td>Accidentally</td>
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<td>N/A</td>
<td>MI, aortic aneurysm</td>
<td>None at 52 yr</td>
<td>None</td>
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<td>PN306</td>
<td>No</td>
<td>6</td>
<td>Nephrotic syndrome</td>
<td>15</td>
<td>1.6</td>
<td>Diastolic dysfunction</td>
<td>18</td>
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<td>NDb</td>
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<td>49</td>
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<td>2</td>
<td>RTx</td>
<td>GS, IF, TA</td>
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<tr>
<td>II-1</td>
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<td>RD, ARF</td>
<td>2</td>
<td>6</td>
<td>ASD, MMR</td>
<td>1.5</td>
<td>CAPD</td>
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<td>II-2(DT)</td>
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<td>None</td>
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<td>110</td>
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<tr>
<td>4Cis3009</td>
<td>Yes</td>
<td>6</td>
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<td>3.8</td>
<td>None</td>
<td>12</td>
<td>RTx</td>
<td>NPHP</td>
<td>30</td>
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</tbody>
</table>

Patients reported by Hoff et al.18

| A3121:21 | Yes | N/A | PKD | N/A | None | 6 | N/A | Died |
| B7397    | N/A | N/A | PKD | N/A | None | Early childhood | N/A | N/A |
| A3114:21 | Yes | N/A | PKD | N/A | AS, PS, periportal LF | 2 | RTx | N/A |
| B6794    | N/A | N/A | PKD | N/A | AS, periportal LF, MM | 1 | N/A | N/A |
| A649:21  | No  | N/A | PKD | N/A | AS, HO, CM, PDA, cholestatic hepato osg, SIT | At birth | N/A | Died |
| NPH316:21| Yes | N/A | PKD | N/A | None | 25 | RTx | N/A |
| .22      | N/A | N/A | PKD | N/A | None | 16 | N/A | N/A |
| .23      | N/A | N/A | PKD | N/A | AS | 12 | N/A | N/A |

RTx, renal transplantation; ND, not done; N/A, not available; MI, mitral insufficiency; HD, hemodialysis; MS, mitral stenosis; TI, tricuspid insufficiency; AS, aortic stenosis; GS, glomerular sclerosis; IF, interstitial fibrosis; TA, tubular atrophy; RD, respiratory distress; ARF, acute renal failure; ASD, atrial septal defect; MMR, motor and mental retardation; CAPD, continuous ambulatory peritoneal dialysis; DT, dizygotic twin; PS, pulmonary stenosis; LF, liver fibrosis; HO, hypertrophic obstructive cardiomyopathy; PDA, patent ductus arteriosus; SIT, situs inversus totalis.

*eGFR was calculated according to the Schwartz formula.

bA renal biopsy could not be performed because the patient had a solitary kidney with function that was severely impaired.
The patients described in the present article were analyzed with an average coverage of 120-fold. Bioinformatic analysis was performed using the SeqPilot SeqNext module (v3.5.2; JSI Medical Systems) and in-house bioinformatic pipelines established at the Center for Human Genetics at Bioscientia. All mutations were confirmed by Sanger sequencing. The homozygous NPHP1 deletion found in patient 4Cis3009 was confirmed by multiplex ligation probe amplification.

**Sanger Sequencing**

Sanger sequencing was performed using BigDye terminator chemistry 3.1 on the 3130 Genetic Analyzer (Applied Biosystems). Primer pairs for exons of ANKS6 are listed in the Supplemental Table 2. We also performed Sanger sequencing for protein encoding exons that are not covered by targeted resequencing (primer pairs are listed in Supplemental Table 2).

**cDNA Analyses of ANKS6**

Total RNA was isolated from peripheral blood lymphocytes using TRIZol reagent (Invitrogen) according to the manufacturer’s protocol and was purified with ethanol precipitation by standard procedure. The QuantiTect cDNA synthesis kit (Qiagen) was used to synthesize cDNA from each sample. The housekeeping gene ACTB (β-actin) was used as a positive control of cDNA synthesis. Primer pairs used in the mRNA studies are listed in Supplemental Table 2.

**Microscopy and Immunostaining**

**Human Specimens**

After fixation with 10% buffered formalin, renal biopsy specimens were routinely processed and embedded in paraffin. Sections cut at 3 μm were stained with hematoxylin and eosin. Renal tissues were stained with an ANKS6 antibody recognizing amino acid residues between 726 and 855 of human NPHP1, INVS/NPHP2, NPHP3, NPHP4, IQCB1/NPHP5, CEP290/NPHP6, GLIS2/NPHP7, RPGRIP1L/NPHP8, NEK8/NPHP9, SDCCAG8/NPHP10, TMEM67/NPHP11, TTC21B/NPHP12, WDR19/NPHP13, ZNF423/NPHP14, CEP164/NPHP15, FAN1/MTMR15, MRE11A, SLC41A1, ANKS6, XPNPEP3/NPHPL1; 414 coding exons in total) were targeted by a custom SeqCap EZ choice sequence capture library (NimbleGen) and were subsequently sequenced on an Illumina MiSeq platform (2 × 150 PE) according to the manufacturer’s protocol. The patients described in the present article were analyzed with an average coverage of >120-fold. Bioinformatic analysis was performed using the SeqPilot SeqNext module (v3.5.2; JSI Medical Systems) and in-house bioinformatic pipelines established at the Center for Human Genetics at Bioscientia. All mutations were confirmed by Sanger sequencing. The homozygous NPHP1 deletion found in patient 4Cis3009 was confirmed by multiplex ligation probe amplification.
ANKS6 (HPA008355; Sigma-Aldrich) and an antibody to β-catenin (M3539; Dako). Paraffin-embedded tissue sections (5 μm) were used for immunoperoxidase staining on an autostainer (Bond Max; Leica) using a detection system (Bond Max; Leica) with diaminobenzidine.

Because the sample for electron microscopy (EM) revealed no glomeruli, one of the paraffin blocks was processed for EM. The renal tissue was dewaxed by heating at 50°C and then was immersed in xylene at room temperature. The sample was rehydrated in descending concentrations of ethanol followed by a wash in phosphate buffer.

A standard EM tissue process schedule was followed as fixation of 4% glutaraldehyde and postfixed 1% osmium tetroxide. After dehydration and infiltration, the schedule was followed at room temperature, and the specimen was embedded in epoxy resin. Sections (70-nm thin) were stained with uranyl acetate-lead citrate, examined with a Jeol Jem 1400 electron microscope, and photographed with a Gatan Orius digital camera.

**Animal Studies**

Rabbit polyclonal total β-catenin (D10A8; Cell Signaling Technology) and mouse monoclonal active β-catenin (dephosphorylated on Ser37 or Thr41, clone 8E7; EMD Millipore) antibodies were utilized. Anks6 antibody was from Aviva (OAA810271).

Rat kidneys were collected after perfusion with PBS and snap-frozen in liquid nitrogen, left in a solution of 30% sucrose in PBS at 4°C overnight, embedded in optimum cutting temperature compound, and stored at −80°C. Tissue sections of 5 μm were rehydrated in PBS, permeabilized in 0.1% Triton X-100, and rinsed in PBS. Sections were then incubated in a solution of 0.1% sodium borohydride (NaBH₄) to quench autofluorescence, rinsed with PBS, and incubated in blocking solution (10% goat serum, 0.1% BSA in PBS) for 1 hour at room temperature. The tissues were incubated overnight at 4°C in primary antibody, washed with PBS, and incubated in fluorescent-labeled secondary antibody and mounted with ProLong (Invitrogen). Images were acquired using a Zeiss Axioplan microscope.

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