



**A Mutation in the TRPC6 Cation Channel Causes  
Familial Focal Segmental Glomerulosclerosis**

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# A Mutation in the TRPC6 Cation Channel Causes Familial Focal Segmental Glomerulosclerosis

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Focal and segmental glomerulosclerosis (FSGS) is a kidney disorder of unknown etiology, and up to 20% of patients on dialysis have been diagnosed with it. Here we show that a large family with hereditary FSGS carries a missense mutation in the *TRPC6* gene on chromosome 11q, encoding the ion-channel protein transient receptor potential cation channel 6 (TRPC6). The proline-to-glutamine substitution at position 112, which occurs in a highly conserved region of the protein, enhances TRPC6-mediated calcium signals in response to agonists such as angiotensin II and appears to alter the intracellular distribution of TRPC6 protein. Previous work has emphasized the importance of cytoskeletal and structural proteins in proteinuric kidney diseases. Our findings suggest an alternative mechanism for the pathogenesis of glomerular disease.

Focal and segmental glomerulosclerosis (FSGS) is an important cause of end-stage renal disease worldwide, and up to one-fifth

of dialysis patients have been diagnosed with it (1, 2). The prevalence of FSGS is increasing yearly, and the incidence is particularly

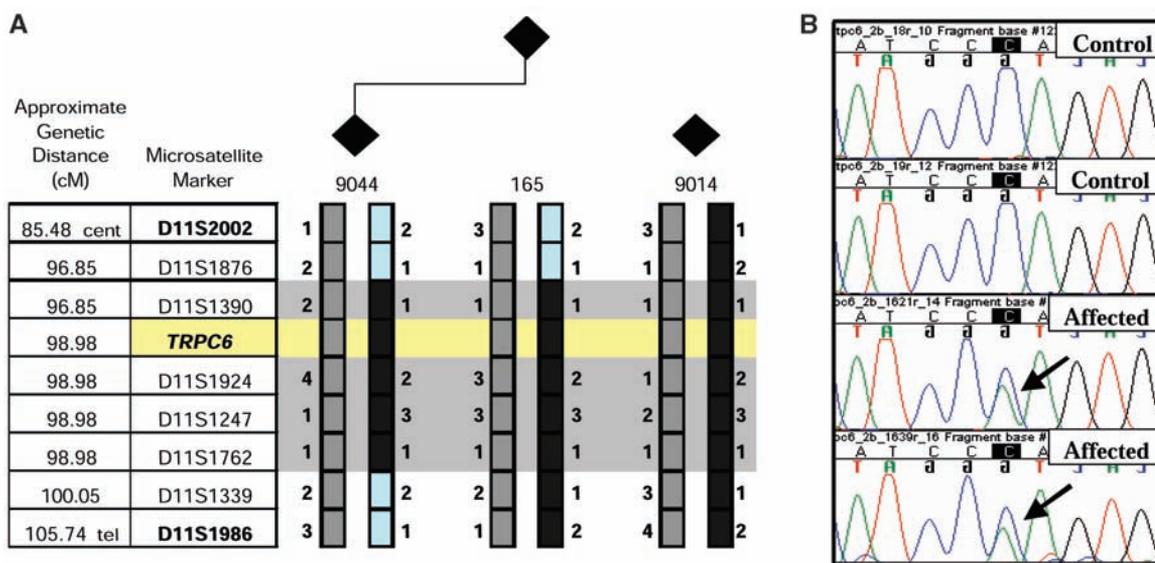
high in the black population (1, 3). FSGS is a pathological entity in which the glomerulus is primarily targeted. Typical manifestations of FSGS include proteinuria, hypertension, renal insufficiency, and eventual kidney failure. Our understanding of the pathogenesis of FSGS is incomplete, and there are no consistently effective treatments.

Analysis of disease-causing mutations in hereditary FSGS and congenital nephrotic syndromes has provided new insights into the pathogenesis of nephrotic syndrome. The previous identification of at least three genes causing familial FSGS and hereditary nephrotic syndromes underscores the substantial genetic heterogeneity in this disorder (4–6). These studies have highlighted the importance of abnormalities in the podocyte and the slit

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**Fig. 1. (A)** Minimal candidate region (MCR) of FSGS on human chromosome 11q. The area of interest is flanked by markers D11S1876 and D11S1339. The genetic distance from the centromere (cent) was obtained from <http://genome.cse.ucsc.edu>. Black squares indicate common alleles among affected individuals. Blue squares indicate recombination. Gray squares are alleles inherited from the non-affected parent. Individual 165 is the parent of individual 9044. Individual 9014 provides an example of the ancestral affected haplotype. All individuals represented are affected. The MCR is defined by individual 9044. Microsatellite markers in bold indicate original flanking markers. The horizontal gray box (including *TRPC6* in yellow) indicates the candidate gene region. tel, telomere. **(B)** Sequence chromatogram of exon 2 of *TRPC6*. The arrows highlight the C/A mutation.



diaphragm of the glomerulus in the development of the severe proteinuria that characterizes the nephrotic syndrome.

Previously, we identified and characterized a large New Zealand family of British origin who have autosomal dominant hereditary FSGS (fig. S1) (7). The character of the disease in this family is particularly aggressive. Affected individuals typically present with high-grade proteinuria in their third or fourth decade, and approximately 60% progress to end-stage renal disease (ESRD). The average time between initial presentation and the development of ESRD is 10 years. A genomic screen performed on this kindred localized the disease-causing mutation to chromosome 11q (8).

Haplotype analyses reduced the minimal candidate region to an approximate 2.1-centimorgan (cM) area defined by critical recombination events at D11S1390 and D11S1762 (Fig. 1A). This region contains several known genes as well as multiple novel and predicted genes, which were systematically screened for mutations by direct sequencing. After examination of 42 other candidate genes, *transient*

*receptor potential cation channel 6 (TRPC6)* (GenBank accession number NP\_004612) emerged as a candidate on the basis of reports of detection of *TRPC6* mRNA in the kidney (9, 10). We therefore sequenced each of the 13 exons of the *TRPC6* gene, along with their intron/exon boundaries. Primer sequences are provided (table S1). As shown in Fig. 1B, we discovered a missense mutation (C335A) in exon 2 from affected individuals, causing a proline-to-glutamine substitution at position 112 (P112Q) within the first ankyrin repeat of the *TRPC6* protein. This variant was present in all of the affected individuals (20 affected; 1 probably affected) in our kindred, and there were no nonpenetrant carriers. The change was not found in any of the public databases of single-nucleotide polymorphisms. Furthermore, we found no evidence of the substitution in 614 chromosomes screened from a group of Caucasian controls without known renal disease, 33 of whom were from New Zealand. The allele frequencies from all markers used for linkage in this kindred and from the New Zealand controls are similar to those from the other Caucasian controls. Pro<sup>112</sup> is high-

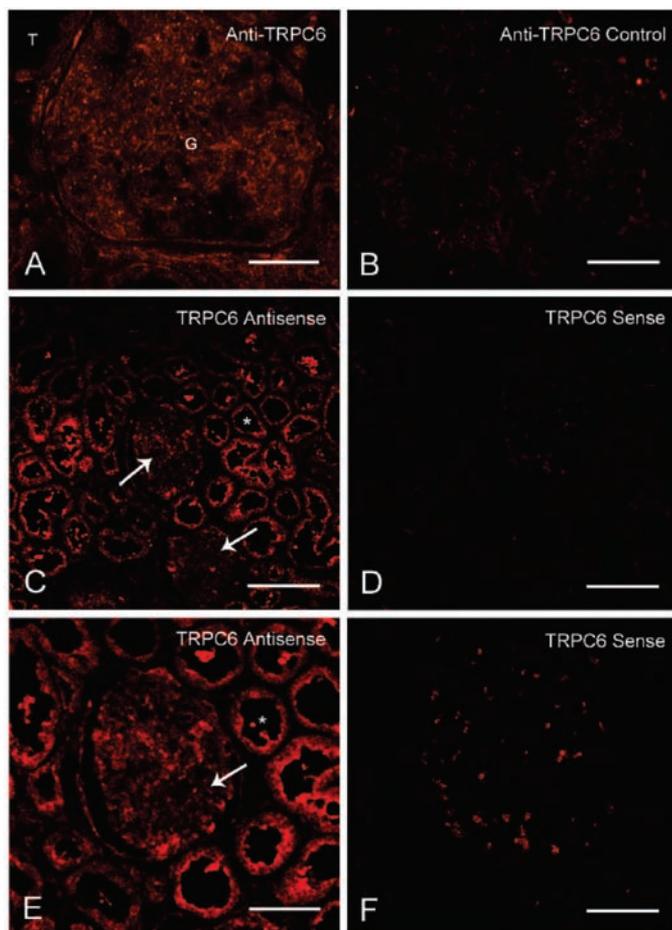
ly conserved in evolution and is present in TRPC protein homologs from multiple species (fig. S2).

Our previous finding that familial FSGS does not recur in affected patients after renal transplantation indicates a critical role for the kidney in disease pathogenesis (11). Although expression of *TRPC6* mRNA has been reported in multiple tissues, including the kidney, its distribution in the kidney is not clear (9, 10). Therefore, to define the spatial distribution of *TRPC6* protein expression in the human kidney, we performed immunohistochemistry of normal human renal cortex tissue with a rabbit antibody raised against a specific human *TRPC6* peptide (Fig. 2, A and B). Immunofluorescence staining revealed *TRPC6* expression throughout the kidney in glomeruli and tubules. This is consistent with a recent study detecting *TRPC6* mRNA in isolated glomeruli (12). The expression of *TRPC6* in glomeruli is particularly noteworthy because abnormal podocyte function appears to be a final common pathway in a variety of proteinuric kidney diseases (13). To verify these immunofluorescence findings, we carried out fluorescence in situ hybridization (FISH) in human kidney sections (Fig. 2, C to F). These studies confirmed diffuse expression of *TRPC6* mRNA in glomeruli and tubules in a pattern that is virtually identical to that seen with staining for antibody to *TRPC6*.

To determine the effect of the P112Q mutation on *TRPC6* function, we studied human embryonic kidney (HEK) 293 cells transfected with mutant (*TRPC6*<sup>P112Q</sup>) or wild-type (WT) *TRPC6* (14). The WT *TRPC6* was cloned from a human kidney cDNA library. On Western blots, the abundance and mobility of the P112Q mutant were comparable to those of WT *TRPC6* (fig. S3). Diacylglycerol (DAG) is a potent activator of *TRPC6* (15). We therefore measured the intracellular calcium concentration ( $[Ca^{2+}]_i$ ) using Fura fluorescence in HEK 293 cells expressing either the WT *TRPC6* or *TRPC6*<sup>P112Q</sup> after exposure to the DAG analog OAG (1-oleoyl-2-acetyl-sn-glycerol). OAG perfusion increased late  $Ca^{2+}$  transients in cells transfected with WT *TRPC6* as expected (Fig. 3A and fig. S4). Peak intracellular concentrations were significantly higher in cells expressing *TRPC6*<sup>P112Q</sup> as compared with WT controls ( $[Ca^{2+}]_i$  of *TRPC6*<sup>P112Q</sup> = 181 ± 25 nM versus  $[Ca^{2+}]_i$  of WT *TRPC6* = 106 ± 15 nM;  $P < 0.05$ ).

Angiotensin II, acting through its AT1 receptor, plays a critical role in the generation of proteinuria and in the progression of kidney injury in a number of kidney diseases, including FSGS (16). AT1 receptors, coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins), activate phospholipase C-β (PLC-β) isoforms that hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>). This triggers the production of inositol 1,4,5-

**Fig. 2.** Immunofluorescence staining and FISH of normal human renal cortical tissue for *TRPC6*. (A) Immunofluorescent staining of normal human renal cortical tissue with rabbit antibody against human *TRPC6*. In this representative photomicrograph, specific staining within a glomerulus (G) and the epithelium of surrounding tubules (T) is easily seen. (B) Negative control of an adjacent section also stained with the primary antibody to *TRPC6* in the presence of the immunizing peptide. There is minimal nonspecific staining. Scale bars in (A) and (B), 25 μm. [(C) and (E)] FISH of *TRPC6* mRNA in normal human renal cortex. (C) *TRPC6* antisense probe generated from nucleotides 2301 to 3621 from *TRPC6* mRNA (GenBank accession number AJ006276). (D) Hybridization with the corresponding *TRPC6* sense probe. Scale bar, 90 μm. (E and F) High-power photomicrographs of the same sense and antisense probes. Scale bar, 40 μm. Widespread expression of *TRPC6* mRNA was detected throughout the kidney in both glomeruli and tubular epithelia. Background staining in (D) and (F) reflects autofluorescence from red blood cells trapped at the time of kidney harvest. Arrows highlight glomeruli. Asterisks are centered in renal tubules.



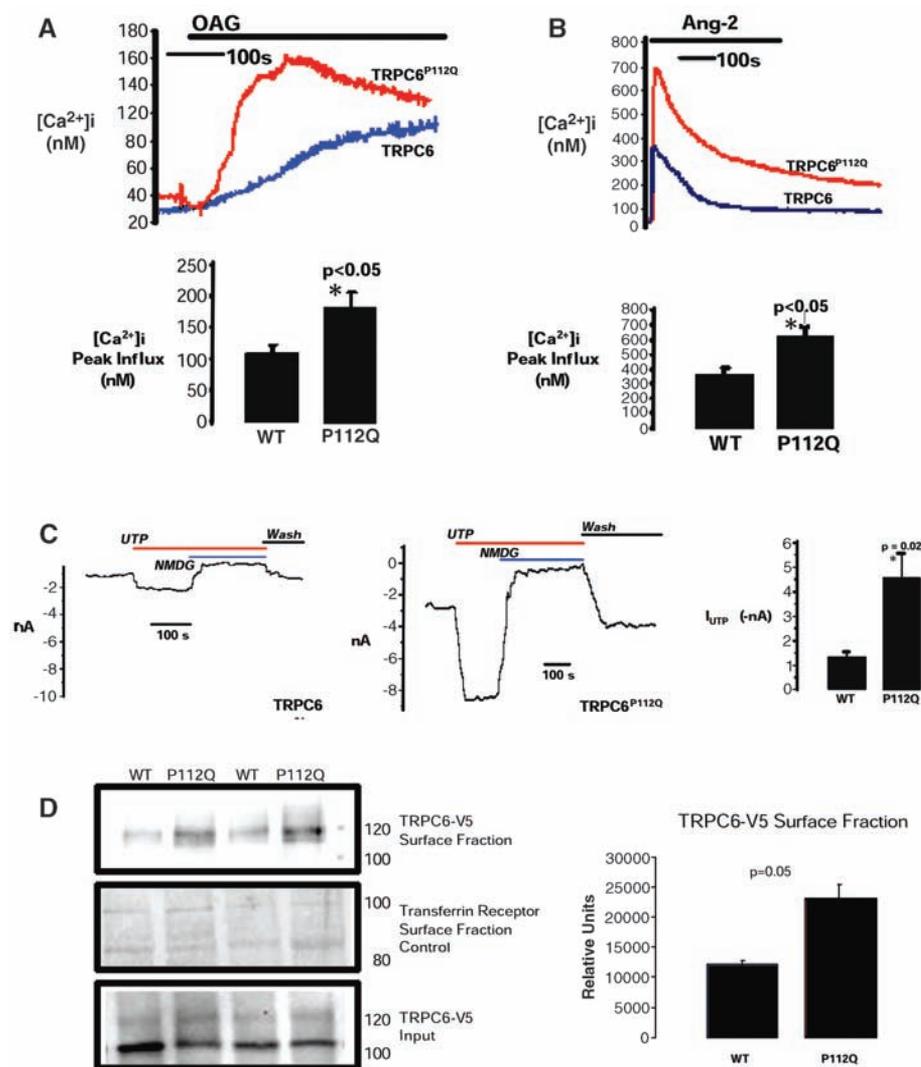
trisphosphate (InsP<sub>3</sub>), and DAG releases internal calcium stores and activates Ca<sup>2+</sup> entry (17). We examined whether the P112Q mutation would affect angiotensin II–dependent (that is, receptor-operated) calcium signaling. HEK 293 cells were cotransfected with the AT1 receptor (AT1–yellow fluorescent protein) and with either WT *TRPC6* or *TRPC6*<sup>P112Q</sup>. [Ca<sup>2+</sup>]<sub>i</sub> changes were measured after exposure to angiotensin II (Fig. 3B and fig. S5). As in the OAG experiments, the peak angiotensin II–stimulated [Ca<sup>2+</sup>]<sub>i</sub> was higher in cells expressing the mutant protein as compared with WT controls ([Ca<sup>2+</sup>]<sub>i</sub> *TRPC6*<sup>P112Q</sup> = 640 ± 66 nM versus [Ca<sup>2+</sup>]<sub>i</sub> WT *TRPC6* = 357 ± 46 nM; *P* < 0.05).

To examine the effects of the P112Q mutation on ion flux, we measured current using the whole-cell patch-clamp technique (Fig. 3C). After patch break, HEK 293 cells expressing WT *TRPC6* or *TRPC6*<sup>P112Q</sup> channels were held at –60 mV for the duration of the experiment. In normal Na<sup>+</sup> extracellular solution, we detected large inward currents in cells transfected with WT *TRPC6*. These currents were significantly increased in cells transfected with the mutant *TRPC6*<sup>P112Q</sup> protein (WT *TRPC6* = 1.03 ± 0.23 nA versus *TRPC6*<sup>P112Q</sup> = 4.21 ± 0.10 nA; *P* = 0.02). The addition of uridine triphosphate (UTP), carbachol, or angiotensin II augmented the inward currents in cells expressing WT *TRPC6* or *TRPC6*<sup>P112Q</sup>. However, the magnitude of the agonist-stimulated current was two to three times greater in cells transfected with mutant than with WT *TRPC6* protein. Control green fluorescent protein–transfected cells showed no appreciable UTP current in comparison to the *TRPC6* transfected cells. The enhanced UTP currents established at baseline and after stimulation with G<sub>q</sub>/11 agonist support the Ca<sup>2+</sup> measurements found in our Fura-2 experiments. Thus, the results of the [Ca<sup>2+</sup>]<sub>i</sub> measurements, along with whole-cell current recordings using both endogenous (P2Y<sub>2</sub>; UTP) and ectopically expressed (AT1R; Ang-2) G<sub>q</sub> receptors for *TRPC6* WT and mutant channels, indicate that the P112Q mutation in *TRPC6* causes a gain of function. Ca<sup>2+</sup> entry is enhanced and is particularly exaggerated in response to G-protein agonists such as angiotensin II.

We also evaluated the subcellular localization of the mutant *TRPC6* protein by surface biotinylation experiments (Fig. 3D). A greater fraction of the mutant protein was associated with the plasma membrane as compared with the WT protein (densitometry measurements were as follows: WT *TRPC6* = 1210.33 versus *TRPC6*<sup>P112Q</sup> = 23126.67 units; *P* = 0.05). In control experiments, no difference was found in the surface expression of the transferrin receptor (TfR) in cells transfected with either WT *TRPC6* or *TRPC6*<sup>P112Q</sup>. Our findings are in accordance with reports by others

(18, 19). This enhanced cell surface expression of *TRPC6*<sup>P112Q</sup> protein suggests a mechanism of exaggerated calcium signaling and flux.

Mutations in several other proteins have been identified in familial nephrotic syndrome and hereditary FSGS. Nephhrin (*NPHS1*), the



**Fig. 3.** The *TRPC6*<sup>P112Q</sup> mutant enhances the influx of calcium into cells via DAG-mediated and receptor-operated pathways. (A) [Ca<sup>2+</sup>]<sub>i</sub> was measured after OAG perfusion. *TRPC6*<sup>P112Q</sup>-transfected cells had significantly higher calcium concentrations than cells transfected with WT *TRPC6*. The peak influx [Ca<sup>2+</sup>]<sub>i</sub> is depicted in the bar graph below the tracing. (B) Angiotensin II (Ang-2)-induced [Ca<sup>2+</sup>]<sub>i</sub> was measured. The peak influx [Ca<sup>2+</sup>]<sub>i</sub> is depicted in the bar graph below the tracing. Again, *TRPC6*<sup>P112Q</sup>-transfected cells had significantly higher calcium concentrations than cells transfected with WT *TRPC6*. Each trace represents the mean value derived from 15 to 20 cells in a single experiment, and each experiment was replicated three times, with similar results. The error bars represent the standard deviation. (C) Whole-cell current recordings of HEK 293 cells expressing either WT *TRPC6* or *TRPC6*<sup>P112Q</sup> protein. Considerable inward currents in normal Na<sup>+</sup> extracellular solution were observed in WT *TRPC6* cells. However, inward currents were significantly larger in *TRPC6*<sup>P112Q</sup> cells. When cells were perfused with 100 μM UTP, even larger inward currents were obtained. Cells expressing the *TRPC6*<sup>P112Q</sup> mutation conducted two to three times more current than did the WT *TRPC6*-expressing cells, as depicted in the bar graph next to the current recordings. NMDG, *N*-methyl-*D*-glucamine; wash, washout of NMDG with physiological solution. (D) Surface expression experiments in HEK 293 cells transfected with *TRPC6* protein. Biotinylation was used to quantitate cell surface expression of *TRPC6* proteins. Cells expressing *TRPC6*-V5 or *TRPC6*<sup>P112Q</sup>-V5 were incubated with biotin-SS reagent, followed by pull-down with streptavidin agarose beads. Immunoblotting with an antibody to V5 made of surface and whole-cell lysates demonstrates increased surface expression of the *TRPC6*<sup>P112Q</sup> as compared to WT *TRPC6* protein. Immunoblotting with an antibody to TfR is located in the middle row and shows no difference in the surface expression of the constitutively active plasma membrane receptor (the 95-kD band). Each experiment was replicated four times, with similar results. Densitometry measurement in relative units are depicted in the bar graph next to the immunoblot (the results from all four replicants are quantitated). The error bars represent the standard error.

cause of Finnish nephropathy, is a protein of unknown function that localizes to the glomerular slit diaphragm and appears to form a zipperlike structure (20). Podocin (*NPHS2*) appears to anchor elements of the slit diaphragm to the cytoskeleton (21). Mutations in alpha-actinin 4 (*ACTN4*) may alter functions of the actin cytoskeleton in the podocyte (6, 22). CD2-associated protein (*CD2AP*) has been implicated in glomerular function on the basis of mouse studies (23). *CD2AP* also appears to have important interactions with nephrin and podocin at the slit diaphragm.

TRP channels have been implicated in diverse biological functions such as cell growth, ion homeostasis, mechanosensation, and PLC-dependent calcium entry into cells. Calcium as a second messenger affects many of these same cellular functions. We speculate that the exaggerated calcium signaling conferred by the *TRPC6*<sup>P112Q</sup> mutation disrupts glomerular cell function or causes apoptosis (24). We further speculate that the mutant protein may amplify injurious signals triggered by ligands such as angiotensin II that promote kidney injury and proteinuria. Clinical manifestations of renal disease do not appear until the third decade in individuals with the *TRPC6*<sup>P112Q</sup> mutation. This is in contrast to individuals with Finnish nephropathy and steroid-resistant nephrotic syndrome, who typically develop

proteinuria in utero or at birth (5). This delay may reflect the difference between these recessive disorders and the autosomal-dominant mechanism of inheritance in the family described here; in that family, the presence of one normal *TRPC6* allele may postpone the onset of kidney injury. Patients with autosomal-dominant FSGS due to mutations in the *ACTN4* gene also have a delayed onset of kidney disease.

Our studies identify *TRPC6* as a disease gene causing hereditary FSGS. Because ion channels tend to be amenable to pharmacological manipulation, our study raises the possibility that *TRPC6* may be a useful therapeutic target in treating chronic kidney disease.

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