Loss of NFAT5 results in renal atrophy and lack of toxicity-responsive gene expression


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The transcription factor NFAT5/TonEBP, a member of the NFAT/Rel family of transcription factors, has been implicated in diverse cellular responses, including the response to osmotic stress, integrin-dependent cell migration, T cell activation, and the Ras pathway in Drosophila. To clarify the in vivo role of NFAT5, we generated NFAT5-null mice. Homozygous mutants were genetically underrepresented after embryonic day 14.5. Surviving mice manifested a progressive and profound atrophy of the kidney medulla with impaired activation of several osmotoprotective genes, including those encoding aldose reductase, Na+/Cl−-coupled betaine/γ-aminobutyric acid transporter, and the Na+/myo-inositol cotransporter. The aldose reductase gene is controlled by a toxicity-responsive enhancer, which was refractory to hypertonic stress in fibroblasts lacking NFAT5, establishing this enhancer as a direct transcriptional target of NFAT5. Our findings demonstrate a central role for NFAT5 as a toxicity-responsive transcription factor required for kidney homeostasis and function.

Because water can diffuse freely across most membranes, animal cells must preserve a balanced osmolarity to prevent dehydration and maintain viability. All nucleated cells possess a programmed response to hypertonic stress in which acute compensatory changes in cell volume are followed by a coordinated transcriptional response that results in the intracellular accumulation of small, cell-compatible osmolytes, such as sorbitol, myo-inositol, betaine, and taurine, which increase intracellular osmolality, restore cell volume, and provide a buffer against osmotic stress (1–3). The genes that are transcriptionally up-regulated during the osmoprotective response encode enzymes and membrane proteins involved in synthesis and transport of organic osmolytes. Among them, aldose reductase (AR) is responsible for sorbitol synthesis, whereas the sodium-dependent myo-inositol cotransporter (SMIT), the betaine/γ-aminobutyric acid transporter (BG1T1), and the sodium and chloride-dependent taurine transporter (TattT) are membrane-localized cotransporters that rely on coupled influx of Na+ and/or Cl− to mediate entry of osmolytes into osmotically stressed cells (1, 3).

The cells in the kidney are specialized for water and ion retention and serve as the primary regulators of electrolyte concentration in extracellular fluid (4). The rodent kidney medulla is regularly exposed to extreme hypertonic stress (up to 4,000 mOsm compared with 300 mOsm of serum (4), a hyperosmolality constant under antidiuretic (hydropenic) conditions. An increase in extracellular osmolality causes water to diffuse out of the medullary cells, so they must counter this stress by synthesizing and accumulating osmolytes. Diseases that impair kidney functions lead to pathological imbalances in the tonicity of body fluids, which disturb other organ systems.

NFAT5 (TonEBP and OREBP) is a member of the NFAT/Rel family of transcription factors (5–6), which contains a DNA-binding domain related to both the NFAT and NFκB families (5–9). NFAT5 is highly expressed in the kidney medulla and in other tissues, and several lines of evidence suggest its involvement in the osmoprotective response (see ref. 10 for a review). NFAT5 binds toxicity-response elements that are present in the control regions of osmotically regulated genes, and it is hyperphosphorylated and translocates to the nucleus in response to hypertonic stimulation of cells in culture (9–11). NFAT5 also undergoes nuclear translocation in the kidney during antidiuresis, which is most apparent within the outer and inner regions of the medulla (12).

In addition to its potential involvement in the osmotic stress response, NFAT5 has been shown to be regulated by other stimuli and to participate in a diverse set of cellular responses. In response to T cell receptor stimulation, NFAT5 displays a dependence on the calcium/calmodulin-dependent phosphatase calcineurin (11, 13). On the other hand, the activation of NFAT5 by osmotic stress is independent of calcineurin (11). Intracellular signals transmitted by the prometastatic integrin α6β4 also lead to an increase in the levels and activity of NFAT5 that enhances the migratory capacity of carcinoma cells (14). Finally, genetic analyses in Drosophila suggest a role for dNFAT, the likely ortholog of mammalian NFAT5, in Ras-mediated cell growth (15).

To define the functions of NFAT5 in the mouse, we disrupted the mouse NFAT5 gene. The homozygous NFAT5 null allele resulted in midembryonic lethality with incomplete penetrance. Surviving mutant mice displayed progressive growth retardation and perinatal lethality associated with severe renal abnormalities and impaired activation of osmoprotective genes, including AR. Cells lacking NFAT5 do not express AR mRNA because of their inability to activate its toxicity-responsive enhancer. Our findings demonstrate a central role for NFAT5 as a toxicity-responsive transcription factor required for renal homeostasis and function.

Materials and Methods

Generation of a Targeted NFAT5 Allele. A 14-kb region surrounding the mouse NFAT5 gene was isolated from a genomic library. We constructed a mutant NFAT5-targeting vector by subcloning 2.3-kb and 2.9-kb sequences surrounding the sixth exon, which encodes the DNA-binding loop of NFAT5. Gene targeting was

Abbreviations: AQP, aquaporin; AR, aldose reductase; BGT1, Na+/Cl−-coupled betaine/γ-aminobutyric acid transporter; En, embryonic day n; SMIT, Na+/dependent myo-inositol transporter; TattT, Na+ and Cl−-dependent taurine transporter; Pn, postnatal day n.

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performed as described in Supporting Text, which is published as supporting information on the PNAS web site.

Histology and Immunostaining. Histology and terminal deoxynucleotidyltransferase-mediated dUTP end-labeling analysis were performed as described in Supporting Text. Formalin-fixed and paraffin-embedded kidney sections were incubated with rabbit primary antibodies to aquaporin 2 (AQP2, Mark Knepper, National Institutes of Health, see ref. 16) at 1:2,000 dilution or aquaporin 3 (AQP3, Santa Cruz Biotechnology) at 1:1,000 dilution at room temperature for 2 h, followed by further incubation with Alexa Fluor 488 anti-rabbit IgG (Vector Laboratories) at 1:400 dilution at room temperature for 40 min. Stained sections were photographed under epifluorescence illumination by using a Zeiss Axioplan microscope, and the images were analyzed with OPENLAB software (Improvision, Boston, MA).

In Situ Hybridization. In situ hybridization was performed as described (17). Details can be found in Supporting Text.

Mouse Embryo Fibroblast Isolation and Reporter Assays. Mouse embryo fibroblasts were isolated from NFAT5+/− and NFAT5−/− mice at embryonic day (E)13.5 as described (18). Mouse embryo fibroblasts were cultured in DMEM supplemented with 10% FBS/10 mM Hepes/2 mM L-glutamine and transfected by using Effectene (Qiagen, Valencia, CA) according to manufacturer’s instructions. Hypertonic treatments were performed 24 h after transfection, and cells were stimulated by addition of an NaCl solution to a final concentration of 100 mM. Sixteen hours after stimulation, cells were harvested and luciferase activity was assessed as described (5). Photinus luciferase values were normalized to an independent reporter (Renilla luciferase). All experiments were performed at least twice, and a representative experiment is shown in the figure.

Quantitative RT-PCR. RNA isolation and RT-PCR were performed as described in Supporting Text.

Results

Generation of NFAT5−/− Mice. We inactivated the mouse NFAT5 gene by replacing the Rel-DNA binding domain within the sixth exon with an inverted neomycin-resistance cassette (Fig. 1A). Homologous recombination introduced an additional BamHI restriction site into the NFAT5 locus, generating 7.3- and 9.0-kb fragments instead of the 15.3-kb fragment of the wild-type locus (Fig. 1B). Injection of targeted embryonic stem cells into blastocysts yielded chimeric mice that transmitted the mutant allele through the germ line. NFAT5-null mice showed embryonic and perinatal lethality with incomplete penetrance (Table 1). We did not observe variations in the NFAT5 mutant phenotype in mixed 129Sv/C57BL6 or inbred 129Sv backgrounds. No major deviation from the expected Mendelian ratios was observed at E14.5, but only 50% of the expected number of NFAT5−/− embryos was obtained at E17.5 (Table 1). A majority of the NFAT5-null mice that were born died around postnatal day (P)10, with only 3.4% of the expected number living past P21 (Table 1). Despite their underrepresentation, NFAT5−/− mice showed no obvious abnormalities at birth, but the small proportion of these mice that
survived to adulthood failed to thrive and their weight was about half that of wild-type littermates (Fig. 2).

Western blots of T cell lysates of homozygous mutant mice showed no NFAT5 protein (Fig. 1C). Expression of NFAT1 protein was unaffected in the same extracts, providing an internal control.

Kidney Abnormalities in NFAT5−/− Mice. Histological examination of null mice at 3 weeks of age revealed kidney hypoplasia and an altered medullary morphology (Fig. 3A). The normal kidney consists of distinct regions: the cortex, the outer and inner stripes of the outer medulla, the inner medulla, and the papilla. The outer and inner stripes of the medulla are clearly demarcated in normal kidneys (Fig. 3Aa), but they were not well defined in the kidneys of NFAT5−/− mice, which had a higher cellular density than normal (Fig. 3Ab). Serial sections through the kidneys of NFAT5−/− mice showed a disruption of the normal architecture of the outer medulla, the lack of a complete papilla, and enlargement of the renal pelvis indicative of progressive atrophy of the medulla (Fig. 3Ab). In the inner stripe of the outer medulla within the normal kidney, a clear demarcation exists between the vascular bundles and the surrounding loops of Henle and collecting ducts, which was lost in the mutant (Fig. 3Ac and Ad). Tubules in the medulla of the mutant were also curved rather than straight, and epithelial cells lacked the typical cuboidal character seen in wild-type kidneys (Fig. 3Ad–h). Instead, these cells were tightly packed with an increased nuclear-to-cytoplasmic ratio (Fig. 3Ah), suggesting they are unable to maintain normal cell volume in the presence of hypertonic stress. The renal cortex in immature NFAT5−/− mice appeared relatively normal (Fig. 3Ai and j). Gross histological analysis of adult kidney sections showed that in contrast to wild-type kidneys (Fig. 3B a and b), kidneys from mature NFAT5−/− mice (P70–P120) were grossly malformed, having an irregular surface and severe segmental atrophy (Fig. 3Bc and e). Large irregular areas of the medulla and cortex were distorted by tubular and interstitial inflammation (Fig. 3Bd and f), resulting in dramatic atrophy and loss of nephrons. In adjacent areas of the medulla, micro-vascular dilatation of the tubules was evident, a change representing a compensatory response to the functional loss suffered by the remainder of the kidney (Fig. 3Bc–f).

Increased Apoptosis in the Inner Medulla of NFAT5−/− Kidneys. The progressive renal atrophy observed in NFAT5−/− mice was associated with the presence of apoptotic bodies in the renal medulla (not shown). Terminal deoxynucleotidyltransferase-mediated dUTP end-labeling analysis revealed a significant number of apoptotic cells only in NFAT5−/− mice (Fig. 3C), indicating that the absence of NFAT5 affected survival of epithelial cells in the kidney medulla. The cell loss observed in the medulla and papilla of NFAT5−/− kidneys likely stems from an inability of medullary cells to compensate for hypertonic stress, resulting in atrophy of the affected zones of the kidney. NFAT5-null mice displayed a slight increase in mitotic cells within the kidney (not shown), most likely as a secondary compensatory response. Despite this increase in cell division, cell loss was the dominating outcome.

Loss of NFAT5 Alters a Gene Expression Program that Regulates Osmotic Homeostasis in the Kidney Medulla. Because NFAT5−/− mice exhibit medullary atrophy, we performed immunostaining with antibodies to the proteins and water transporters specifically expressed in renal tubules in the medulla (Fig. 4A). Expression of Tamm-Horsfall, which is expressed in the thick ascending limbs of loops of Henle in the outer medulla, was only slightly reduced in mutant mice (not shown). Similarly, expression of AQP3, which is primarily expressed in cortical and outer medullary collecting ducts, was only moderately down-regulated (Fig. 4At–d). In contrast, expression of AQP2, which is expressed in both inner and outer medullary collecting ducts, was markedly inhibited. Neither AQP2 nor AQP3 was expressed in the inner medulla, and no differences occurred in the expression of cortical markers, such as Fx1A (not shown). Higher-magnification images showed that AQP3 was expressed normally in the basolateral membrane of both wild-type and mutant principal cells. However, AQP2 was primarily localized to the apical membrane in the mutant but was primarily located in the cytoplasm in wild-type kidney (Fig. 4Ae–h). The apical localization of AQP2 in the mutant suggests the translocation of AQP2 from the cytoplasm to the apical membrane under conditions of high osmolarity that result from atrophy of the medulla.

To test the involvement of NFAT5 in the osmoprotective transcriptional response, we analyzed expression of the Ar, BGT1, SMIT, and taurine transporter (TaTuT) genes by in situ hybridization on kidney sections from NFAT5−/− and wild-type mice. The mice were water-deprived for 24 h before killing in conditions that physiologically increase hypertonic stress and result in enhanced up-regulation of tonicity-responsive genes. We observed marked reduction of AR, BGT1, and SMIT mRNAs in kidneys of NFAT5−/− mice in comparison with those of wild-type mice (Fig. 4B). Wild-type mice showed abundant

Table 1. Embryonic and perinatal lethality of NFAT5 mutant mice

<table>
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<tr>
<th>Day</th>
<th>+/+</th>
<th>+/−</th>
<th>−/−</th>
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<tbody>
<tr>
<td>E14.5</td>
<td>11 (11)</td>
<td>28 (22)</td>
<td>8 (11)</td>
</tr>
<tr>
<td>E17.5</td>
<td>10 (10)</td>
<td>12 (20)</td>
<td>5 (10)</td>
</tr>
<tr>
<td>P18–21</td>
<td>205 (205)</td>
<td>305 (410)</td>
<td>7 (205)</td>
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Numbers are based on predicted Mendelian inheritance. At least three litters were analyzed for each time point.
expression of AR transcripts in the inner medulla (Fig. 4Ba) and BGT1 transcripts in the inner stripe of the medulla between the vascular bundles and papilla (Fig. 4Bc), but NFAT5+/− mice completely lacked AR and BGT1 expression (Fig. 4 Bb and d). SMIT expression is normally localized to both stripes of the outer medulla and tubules within the cortex (Fig. 4Be) but was expressed only in the cortical tubules in NFAT5−/− mice, which clearly lacked SMIT expression in the outer medulla (Fig. 4Bf). In contrast, expression of the TauT gene, which was localized to the outer stripe of the outer medulla that contained the proximal straight tubules in wild-type mice (Fig. 4Bg), was unaltered in the kidneys of NFAT5−/− mice (Fig. 4Bh); we note, however, that mutant mice showed TauT expression in glomeruli of the kidney cortex (Fig. 4Bh), an expression pattern not observed in dehydrated wild-type mice (Fig. 4Bg). The decreased expression of osmoregulatory genes was confined to the medulla because the interstitial fluid in the cortex is isosmotic with plasma, whereas the medulla is hypertonic.

Altered Transcriptional Induction of Osmoprotective Genes in NFAT5−/− Cells. To test whether NFAT5 is responsible for regulating expression of osmoregulatory genes, we isolated fibroblasts from NFAT5−/− and wild-type mice at E13.5, subjected them to hypertonic conditions by exposing them to media containing an additional 100 mM NaCl for 16 h, and evaluated expression of the AR, BGT1, TauT, and SMIT genes. No significant induction of the latter three genes was observed in control fibroblasts (not shown), possibly reflecting their kidney-specific expression. However, wild-type fibroblasts subjected to hypertonic stimulation showed a 3-fold increase in the expression of AR mRNA relative to fibroblasts maintained under isotonic conditions; this increase depended on the presence of NFAT5, as shown by the complete lack of AR mRNA expression in NFAT5−/− fibroblasts (Fig. 5A).

Transcription of the AR gene is regulated by a 132-bp enhancer that contains three consensus NFAT5-binding elements (ref. 19 and Fig. 5B). To test whether NFAT5 regulates AR transcription through this enhancer element, we transfected NFAT5−/− and control fibroblasts with a luciferase reporter construct by the AR enhancer and tested reporter expression in cells subjected to osmotic stress. Although control fibroblasts showed strong induction of luciferase activity, NFAT5−/− cells did not (Fig. 5B), suggesting that the AR gene is a direct target of the transcriptional activity of NFAT5.

Discussion

The results of this study reveal a key role of NFAT5 in maintenance of kidney morphology and the transcriptional response to hypertonic stress. Mice lacking NFAT5 display progressive disruption of kidney morphology and function after birth. The severe kidney dysfunction in mutant mice seems to arise from an inability of cells of the kidney medulla to activate the expression of osmoregulatory genes, which depend on NFAT5. This tran-
scriptional defect is likely to result in apoptotic cell death of medullary cells and consequent renal failure.

The kidney phenotype of NFAT5−/− mice is most pronounced in the medulla, the region under greatest hypertonic stress (4). Consistent with the expected dependence of this tissue on NFAT5-regulated expression of osmoprotective genes, NFAT5 is expressed at high levels in the kidney medulla, and its expression follows the tonicity gradient along the corticomedullary axis, with highest NFAT5 levels concentrated in the inner medulla and the inner stripe of the outer medulla (12). Atrophy of the kidney medulla in the mutant mouse is most prevalent in the loops of Henle and collecting ducts, which must endure the highest extracellular tonicity. Previous reports have shown that medullary cells that cannot compensate for high osmolarity undergo apoptosis (20). Our findings are consistent with a model in which cells respond to an increase in extracellular osmolality by acutely shrinking, which activates membrane transport proteins that allow the influx of NaCl that restores normal cell volume (the so-called volume regulatory increase). Under conditions of chronic hypertonicity, cells must replace the inorganic ions with compatible organic osmolytes. In the absence of NFAT5, the latter response is lost and cells undergo apoptosis.

Medullary cells normally compensate for extracellular hypertonic stress by activating the expression of genes that encode membrane transporters and enzymes that synthesize compatible osmolytes (1, 3). Genes involved in these processes, such as AR,
NFAT5 was observed in older NFAT5 knockout mice to compromise the ability of the medullary cells to maintain cell volume and protect themselves from hypertonicity, leading to a reduced capacity of the kidney epithelium to endure high osmotic stress and explaining the severe renal abnormalities observed in older NFAT5−/− mice. Because of the relatively small number of viable mutant mice, the precise cause of death could not be determined. The decrease in surviving mutant mice at or about P10 correlates with the progressive development of urinary concentrating ability of rodents, suggesting renal failure as the most likely cause of postnatal lethality.

A substantial fraction of NFAT5−/− mice died by midgestation. We do not currently know the basis for this embryonic lethality, but kidney dysfunction seems unlikely to be responsible, because maintenance of the extracellular milieu of the fetus depends on the placenta, not the fetal kidney. Mice with bilateral renal agenesis, such as caused by Pax-2 deficiency (21), are born at expected Mendelian ratios and succumb only postnatally. The kidney phenotype of NFAT5−/− mice is reminiscent of other genetic models of kidney failure, such as AQPO2 knockin mice, which display papillary atrophy, enlarged renal pelvis, and dilated collecting ducts (22). Inhibition of myo-inositol transport also leads to medullary injury and acute renal failure (23). Mice lacking AR also display hypercalcemia and hypercalciuria and have a reduced ability to concentrate their urine (24). The phenotype of NFAT5−/− mice suggests possible disorders that might be observed in humans lacking functional NFAT5. In this regard, it is notable that the NFAT5 gene is located in a region of human chromosome 16(q22) that is associated with several kidney-related diseases.

In addition to the kidney, NFAT5 is highly expressed in heart, brain, and cells of the immune system, suggesting other possible abnormalities in NFAT5−/− mice. The emergence of NFAT5 in evolution predates the development of a functioning kidney. Thus, although the phenotype of NFAT5−/− mice provides a dramatic demonstration of the role of this transcription factor in the osmoprotective response, it is likely that NFAT5 has additional functions that may have been preserved and expanded in vertebrates.

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