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Received for publication, March 14, 2018, and in revised form, November 7, 2018 Published, Papers in Press, November 9, 2018, DOI 10.1074/jbc.RA118.002896

The renal medulla is the only tissue that is constantly under hypertonic conditions to concentrate urine through water reabsorption in mammals (1). The driving forces for water reabsorption are elevated concentrations of sodium chloride and urea, which create exceptionally hypertonic conditions (600 and 2,000 mosmol/kg H2O under conditions of diuresis and antidiuresis in rodents, respectively) (2). Renal cells, particularly renal medullary cells, partly adapt to the hypertonic conditions by accumulating intracellular organic osmolytes that reduce intracellular ionic strength. Nuclear factor of activated T cells 5 (NFAT5)3/tonicity-responsive enhancer binding protein plays a key role in the adaptation to hypertonic conditions by binding to the consensus DNA-binding element RNRNNTTTCCA (where N is any nucleotide, and R is any purine) (3, 4). NFAT5 stimulates the transcription of aldose reductase (AR/ACKR1B1) (5, 6), sodium- and chloride-dependent betaine transporter (BGT1/SLC6A13) (4, 6), sodium myo-inositol co-transporter (SMIT/SLC5A3) (3, 6), and heat shock protein 70 (HSP70/HSPA1B) (7, 8), which mediate the intracellular accumulation of sorbitol, betaine, and myo-inositol and function as an intracellular molecular chaperone, respectively. In addition to these genes, many downstream genes of NFAT5 are known (9–15). However, a ubiquitin ligase that is directly activated by NFAT5 has not yet been reported.

Ubiquitin ligases are involved in a wide variety of cellular events through ubiquitination (16, 17). RING finger protein 183 (RNF183) is a member of the RNF family, which functions as a ubiquitin ligase (18). Previous studies have demonstrated that RNF183 mRNA expression in the colon of patients with inflam-
matory bowel disease (IBD) and colorectal cancers was 5- and 2-fold higher than that in control subjects; in these patients, RNF183 promotes intestinal inflammation (19) and proliferation and metastasis of cancers (20), respectively. On the contrary, we previously demonstrated that RNF183 was specifically expressed in human and mouse kidney, and that mouse Rnf183 mRNA expression in the kidney was 324-fold higher than in the colon (21). To date, however, the reason why Rnf183 mRNA is selectively expressed in the kidney remains unclear.

In this study, we demonstrated that RNF183 is dominantly expressed in the renal medulla and that NFAT5 regulates Rnf183 transcription in mouse inner-medullary collecting duct (mIMCD-3) cells.

**Results**

**RNF183 is predominantly expressed in the renal medulla**

RNF183 has been described as a ubiquitin ligase, which is expressed in normal colonic epithelial cells and colorectal cancer cells (19, 20). In addition, we previously reported that Rnf183 mRNA expression in the kidney was 324-fold higher than in the colon (21); however, RNF183 protein expression in the kidney remains unclear. To detect RNF183 protein expression, we generated an affinity-purified antibody using recombinant deletion mutant RNF183 (amino acids 61–158) lacking a RING finger domain at its N terminus and a transmembrane domain at its C terminus (Fig. 1A). The antibody specifically recognized GFP-RNF183 at 50 kDa, which was transiently transfected into human embryonic kidney 293 (HEK293) cells (Fig. 1B). Immunofluorescence staining using HeLa cells transiently transfected with GFP-RNF183 showed that punctate signals of GFP-RNF183 were recognized by the anti-RNF183 antibody (Fig. 1C). GFP signals were localized in the late endosome/lysosome (Lamp1) and early endosome (EEA1), with additional weak signal in the recycling endosome (transferrin receptor) and Golgi complex (GM130) but not in the endoplasmic reticulum (calnexin) or mitochondria (COX IV) (Fig. 1D and Fig. S1). To evaluate endogenous RNF183 protein expression, we performed RT-PCR and Western blot analysis using tissue extracts in 4-week-old mice. Western blotting revealed that endogenous RNF183 protein was expressed markedly in the kidney, particularly in the renal medulla, and in the thymus (Fig. 1, F and H), consistent with Rnf183 mRNA expression (Fig. 1, E and G). Megalin and AKR1B1 were used as positive controls for the renal cortex and medulla, respectively.

**NFAT5 regulates Rnf183 transcription**

In this study, we demonstrated that RNF183 is dominantly expressed in the renal medulla and that NFAT5 regulates Rnf183 transcription in mouse inner-medullary collecting duct (mIMCD-3) cells.
RNF183 expression is up-regulated in response to hypertonic stress

Renal medullary cells are constantly exposed to extreme hypertonic stress to concentrate the urine (1). Therefore, using quantitative real-time RT-PCR (qRT-PCR), we examined whether RNF183 expression and the other transmembrane RNF family members, those containing RING finger (C3H2C3 or C3HC4) and transmembrane domains, are up-regulated in response to hypertonic stress. Mouse IMCD-3 cells were incubated with isotonic medium or NaCl- or sucrose-supplemented medium. The extracellular tonicity was increased by adding 75 mM NaCl or 150 mM sucrose. Akr1b1 (5, 6) and Hapa1b (7, 8) were used as positive controls for tonicity dependence.

We found that Rnf183 mRNA expression was markedly up-regulated in a tonicity-dependent manner in both hyper-tonic NaCl- and sucrose-treated cells compared with that in isotonic control cells (Fig. 2A), which was consistent with the Akr1b1 and Hapa1b up-regulation patterns (Fig. 2A). Rnf128 and Rnf19B were modestly up-regulated in a tonicity-dependent manner. Further, Rnf24, Rnf13, Rnf167, Rnf43, Syvn1, Amfr, Rnf145, Rnf5, Rnf185, and Rnf170 were slightly up-regulated in cells treated with only 75 mM NaCl; the other RNF family members were not up-regulated (Fig. 2A). Western blot analysis showed that RNF183 and AKR1B1 protein expression was up-regulated in a tonicity-dependent manner (Fig. 2B–E). However, Rnf183 mRNA was not up-regulated under hypoxic conditions (oxygen concentration, 1 and 0.3%) (Fig. S2), which is another characteristic of the renal medulla. These results suggest that hypertonic conditions play a more important role in regulating RNF183 expression.

**Figure 2. RNF183 is up-regulated in response to hypertonic stress.** A, the hypertonic response of Rnf183 (top) and the other transmembrane RNF family members (bottom, 17 of the C3HC4 type) mRNA levels in mIMCD-3 cells. Akr1b1 and Hspa1b were used as positive controls (top). Cells were treated with isotonic or the indicated NaCl- or sucrose-supplemented medium for 12 h and analyzed by qRT-PCR (n = 5). B and D, the tonicity-dependent induction of RNF183 and AKR1B1 protein in mIMCD-3 cells. Cells were cultured under hypertonic conditions by adding the indicated NaCl (B) or sucrose (D) supplementation for 12 h and analyzed by Western blotting. C and E, quantitative analysis of RNF183 (left) and AKR1B1 (right) protein expression in NaCl (B) and sucrose (D) (n = 5). F, the up-regulation patterns of RNF183 and AKR1B1 proteins in four renal cell lines in response to hypertonic stress. NRK-52E, NRK-49F, mIMCD-3, and HEK293 cells were treated with isotonic or 75 mM NaCl–supplemented medium for 12 h and analyzed by Western blotting. HEK293 cells transfected with mouse RNF183 were used as a positive control. Data were analyzed by one-way ANOVA, followed by post hoc tests using t tests with Bonferroni correction. Values represent mean ± S.D. (error bars), *p < 0.05; **, p < 0.01; ***p < 0.001 (versus isotonic control).

NFAT5 regulates Rnf183 transcription

Renal medullary cells are constantly exposed to extreme hypertonic stress to concentrate the urine (1). Therefore, using quantitative real-time RT-PCR (qRT-PCR), we examined whether RNF183 expression and the other transmembrane RNF family members, those containing RING finger (C3H2C3 or C3HC4) and transmembrane domains, are up-regulated in response to hypertonic stress. Mouse IMCD-3 cells were incubated with isotonic medium or NaCl- or sucrose-supplemented medium. The extracellular tonicity was increased by ~150 mosmol/kg H2O (final osmolality: 450 mosmol/kg H2O) by adding 75 mM NaCl or 150 mM sucrose. Akr1b1 (5, 6) and Hapa1b (7, 8) were used as positive controls for tonicity dependence. We found that Rnf183 mRNA expression was markedly up-regulated in a tonicity-dependent manner in both hypertonic NaCl- and sucrose-treated cells compared with that in isotonic control cells (Fig. 2A), which was consistent with the Akr1b1 and Hapa1b up-regulation patterns (Fig. 2A). Rnf128 and Rnf19B were modestly up-regulated in a tonicity-dependent manner. Further, Rnf24, Rnf13, Rnf167, Rnf43, Syvn1, Amfr, Rnf145, Rnf5, Rnf185, and Rnf170 were slightly up-regulated in cells treated with only 75 mM NaCl; the other RNF family members were not up-regulated (Fig. 2A). Western blot analysis showed that RNF183 and AKR1B1 protein expression was up-regulated in a tonicity-dependent manner (Fig. 2B–E). However, Rnf183 mRNA was not up-regulated under hypoxic conditions (oxygen concentration, 1 and 0.3%) (Fig. S2), which is another characteristic of the renal medulla. These results suggest that hypertonic conditions play a more important role in regulating RNF183 expression.
NFAT5 regulates Rnf183 transcription

RNF183 expression is up-regulated concurrently with NFAT5 activation

The NFAT5 transcription factor is the master regulator for hypertonic stress in mammals (22). In response to hypertonic stress, NFAT5 activation is achieved by a combination of NFAT5 induction and localization into the nucleus (4, 23, 24). Thus, we evaluated the effects of hypertonicity on NFAT5 activation in mIMCD-3 cells. We performed immunofluorescence staining of NFAT5 and plotted the fluorescence intensity along a line drawn through the nucleus (Fig. 3A). Hypertonic stimulation for 3 h reduced NFAT5 cytoplasmic expression and slightly enhanced nuclear expression, and stimulation for 12 h drastically enhanced nuclear expression (Fig. 3A). Western blot analysis demonstrated that the total abundance of NFAT5 protein significantly increased after 3 h of hypertonic stimulation and peaked at 9 h (Fig. 3B and C). qRT-PCR analysis revealed that the expression of Rnf183 and Ak1b1 downstream genes, Akr1b1 (5, 6) and Hspa1b (7, 8), concurrently increased after 3 h of hypertonic stimulation (Fig. 3D). The RNF183 and AKR1B1 protein levels significantly increased after 6 and 9 h of hypertonic stimulation (Fig. 3E and F). In contrast to the downstream genes of NFAT5, the Tnfa mRNA level, which is that of NF-κB, peaked after 3 h of hypertonic stimulation and decreased after 6 h (Fig. S3). These results demonstrated that RNF183 expression was up-regulated under hypertonic conditions and was accompanied by NFAT5 activation and the up-regulation of Rnf183 expression in renal cells.
NFAT5 regulates Rnf183 transcription

NFAT5 knockdown and p38/MAPK inhibitor SB203580 attenuate hypertonicity-induced RNF183 expression

To determine the effect of NFAT5 inhibition on RNF183 expression, mIMCD-3 cells were transfected with siRNA oligonucleotides targeting NFAT5 (NFAT5 siRNA 1) or negative control siRNA (NC siRNA). After 12 h of hypertonic stimulation, the NFAT5 protein levels were significantly reduced in control siRNA (NC siRNA). After 12 h of hypertonic stimulation using siRNA. Mouse IMCD-3 cells transfected with NFAT5 siRNA 1 or NC siRNA were treated with isotonic or 75 mM NaCl– or 150 mM sucrose–supplemented medium for 12 h and analyzed by Western blotting.

Figure 4. NFAT5 knockdown and p38/MAPK inhibitor SB203580 attenuate hypertonicity-induced RNF183 expression.

A, inhibition of NFAT5 expression using siRNA. Mouse IMCD-3 cells transfected with NFAT5 siRNA 1 or NC siRNA were treated with isotonic or 75 mM NaCl– or 150 mM sucrose–supplemented medium for 12 h and analyzed by Western blotting. B, quantification of data from A (n = 4). C, effect of NFAT5 knockdown on RNF183 protein expression induced by hypertonicity. D, quantitative analysis of RNF183 in C (n = 4). E, effect of NFAT5 knockdown on Rnf183 (left) and Akr1b1 (right) mRNA (n = 5). F and G, effect of p38/MAPK inhibitor SB203580 on mRNA expression of Rnf183, Akr1b1, and Sgk1 (F) and protein expression of RNF183 (G). Cells were treated with 75 mM NaCl for 12 h in the presence of the indicated concentrations of SB203580 (1 h of preincubation) and analyzed by qRT-PCR (F) (n = 6) or Western blotting (G). Data were analyzed by one-way ANOVA, followed by post hoc tests using Bonferroni correction. Values represent mean ± S.D. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001 (versus NC).

Identification of the Rnf183 enhancer region that responds to hypertonic stress

To identify the putative enhancer region in the Rnf183 promoter, we searched for an evolutionarily conserved region (ECR) within 5,000 bp upstream of the human RNF183 gene using the ECR browser (27). The 319-bp region (−4,367 to −4,049 bp region; numbers indicate nucleotide positions relative to the transcription start site) upstream of the human RNF183 gene was identified as conserved in mammals (blue; Fig. 5A). Although its actual position from the transcription start site varies among the species (Fig. 5B), it shows high sequence identity with the corresponding region in the following mammals: Mus musculus, 71%; Rattus norvegicus, 73%; Canis familiaris, 81%; Bos taurus, 80%; Macaca mulatta, 94%; and Pan troglodytes, 98%. We next searched for potential binding sites for various transcription factors within the mammalian conserved region of RNF183 genes (−3,466 to −3,136, mouse (Table S1); −1,978 to −1,664, rat (top right); −4,367 to −4,049, human (bottom right)) using the JASPAR database (28). This analysis identified one binding site for NFAT proteins conserved among mouse, rat, and human (Table 1 and Table S1). The sequences (GTACTTTTCCA (−3,342 to −3,332; numbers indicate the nucleotide position relative to the transcription start site of mouse Rnf183), GTACTTTTCCA (−1,870 to −1,860; numbers indicate the nucleotide position
stress on luciferase activities. Mouse IMCD-3 cells transfected with 3.5 kbp-Luc or 2.0 kbp-Luc constructs were treated with isotonic or 75 mM NaCl–

Figure 5. Identification of the \textit{Rnf183} enhancer region that responds to hypertonic stress. A, screen capture from the ECR Browser website (http://ecrbrowser.dcode.org/) (57) (please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site) of the human \textit{Rnf183} gene with ECR in the genomes of mouse, rat, canis, cattle, rhesus, and chimpanzee. The \textit{peaks in red} show comparative pairwise ECR between human and the indicated mammals. The 319-bp region (−4,367 to −4,049 region; numbers indicate nucleotide positions relative to the transcription start site) is conserved in mammals (\textit{blue}). \textit{B}, location and sequence of the conserved NFAT5 DNA–binding site (\textit{yellow background with underline}; mouse, −3,342 to −3,332 bp; rat, −1,870 to −1,860 bp; human, −4,256 to −4,246 bp). Conserved bases around the binding site are indicated as \textit{blue background}. NFAT5 consensus sequence (RNRNNTTTCCA) is indicated \textit{below} the site. \textit{C}, scheme of the mouse \textit{Rnf183} promoter region and reporter constructs. Two NFAT5 DNA–binding sites are indicated in 3.5 kbp upstream of the mouse \textit{Rnf183} start site. 3.5 kbp-Luc and 2.0 kbp-Luc constructs consist of −3.5 kbp and 2.0 kbp upstream of the mouse \textit{Rnf183} transcription start site, respectively. \textit{D}, effect of hypertonic stress on luciferase activities. Mouse IMCD-3 cells transfected with 3.5 kbp-Luc or 2.0 kbp-Luc constructs were treated with isotonic or 75 \textit{mM NaCl}–supplemented medium for 24 h. The pGL4-Luc without the promoter was used as a control. Firefly luciferase activities were normalized to Renilla luciferase signals. Data were analyzed by one-way ANOVA, followed by post hoc tests using Bonferroni correction. Values represent mean ± S.D. (error bars). **, \(p < 0.01\); N.S., \(p > 0.05\).

Table 1
Conserved putative transcription factor binding sites in the \textit{Rnf183} enhancer region

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<th>Relative score</th>
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<th>End</th>
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NFAT5 regulates Rnf183 transcription

Rnf183 enhancer region depends on NFAT5

To determine whether the putative Rnf183 enhancer region depends on NFAT5, we examined the effects of NFAT5 knockdown, p38/MAPK inhibition, and the dominant negative (DN) form of NFAT5 on luciferase activities. NFAT5 knockdown markedly reduced the reporter activity of pGL4–3.5 kbp-Luc (Fig. 6A and Fig. S4D), whereas FLAG-NFAT5 overexpression fully rescued the down-regulated reporter activity (Fig. 6B and Fig. S5). p38/MAPK inhibitor SB203580 significantly inhibited the hypertonicity-induced Rnf183 luciferase activities (Fig. 6C), which is consistent with mRNA and protein expression of RNF183 (Fig. 4, F and G). Moreover, Rnf183 luciferase activities were inhibited by overexpression of DN-NFAT5, whereas they were up-regulated by that of the full-length NFAT5 (Fig. 6D). These results suggest that the putative Rnf183 enhancer region depends on NFAT5.

Rnf183 is a direct target of NFAT5

We next performed luciferase reporter assays using pGL4–3.5 kbp-Luc of WT and mutant having a mutation in the conserved NFAT5 DNA–binding site (Fig. 7A). In mLMD-3 cells transfected with the mutant, reporter activities were markedly reduced even in hypertonic conditions (Fig. 7B). Consistent with this, NFAT5 overexpression markedly induced reporter activity of WT, which was inhibited by the mutation (Fig. 7C). To confirm whether NFAT5 directly binds to the conserved binding site in the Rnf183 promoter, we performed ChIP assays using mLMD-3 cells under hypertonic conditions (Fig. 7D) and detected a high level of NFAT5 binding to the conserved binding site in the endogenous Rnf183 promoter (Fig. 7, E and F). These results indicated that NFAT5 directly acts on the conserved NFAT5 DNA–binding site within the Rnf183 promoter and facilitates its transcription in mLMD-3 cells.

NFAT5 expression provides protection against hypertonicity-induced apoptosis

Stress induced by an elevated NaCl concentration mediates apoptosis in mLMD-3 cells when osmolality is acutely raised to >600 mosmol/kg (29–31). Moreover, several NFAT5 downstream genes show a protective effect against hypertonicity-induced apoptosis (9, 32, 33). Thus, to determine whether RNF183 expression affects the osmotic tolerance of mLMD-3 cells to high NaCl levels, mLMD-3 cells were transfected with siRNA oligonucleotides targeting RNF183 (RNF183 siRNA) or NC siRNA. RNF183 siRNA effectively reduced the Rnf183 mRNA levels compared with NC siRNA (Fig. 8A). After 48 h of transfection, cells were treated with hypertonic medium for an additional 24 h (n = 4). C. effect of p38/MAPK inhibitor SB203580 on Rnf183 luciferase activities. Cells transfected with 3.5 kbp-Luc were treated with 75 mM NaCl for 24 h in the presence of the indicated concentrations of SB203580 (1 h of preincubation) (n = 3). D. effect of overexpression with FLAG-DN-NFAT5 and FLAG-NFAT5 on luciferase activities. Cells cotransfected with 3.5 kbp-Luc and either empty, pFLAG-DN-NFAT5, or pFLAG-NFAT5 vectors were analyzed after 48 h of transfection (n = 4). Data were analyzed by one-way ANOVA, followed by post hoc tests using t tests with Bonferroni correction. Values represent mean ± S.D. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 6. Rnf183 enhancer region depends on NFAT5. A, effect of NFAT5 knockdown on hypertonicity-induced luciferase activities. Cells cotransfected with 3.5 kbp-Luc and siRNA (NFAT5 1 or NC) were treated with isotonic or 75 mM NaCl– or sucrose–supplemented medium for 24 h (n = 3). B, effect of rescue with FLAG-NFAT5 on luciferase activities. Cells pretransfected with siRNA (NFAT5 3 or NC) were cotransfected with 3.5 kbp-Luc and either empty or pFLAG-NFAT5 vectors after 12 h of knockdown. After 24 h of cotransfection, cells were treated with isotonic or 75 mM NaCl–supplemented medium for an additional 24 h (n = 4). C, effect of p38/MAPK inhibitor SB203580 on Rnf183 luciferase activities. Cells transfected with 3.5 kbp-Luc were treated with 75 mM NaCl for 24 h in the presence of the indicated concentrations of SB203580 (1 h of preincubation) (n = 3). D, effect of overexpression with FLAG-DN-NFAT5 and FLAG-NFAT5 on luciferase activities. Cells cotransfected with 3.5 kbp-Luc and either empty, pFLAG-DN-NFAT5, or pFLAG-NFAT5 vectors were analyzed after 48 h of transfection (n = 4). Data were analyzed by one-way ANOVA, followed by post hoc tests using t tests with Bonferroni correction. Values represent mean ± S.D. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 7. Rnf183 promoter is direct target of NFAT5. A, luciferase reporter assays. Cells transfected with 3.5 kbp-Luc were treated with different concentrations of NaCl for 24 h, and luciferase activities were measured. B, effect of NFAT5 knockdown on hypertonicity-induced luciferase activities. Cells transfected with 3.5 kbp-Luc and siRNA (NFAT5 1 or NC) were treated with isotonic or 75 mM NaCl–supplemented medium for 30 min. C, effect of NFAT5 knockdown on hypertonicity-induced caspase-3 activity and apoptosis. Cells transfected with 3.5 kbp-Luc and siRNA (NFAT5 1 or NC) were treated with isotonic or 75 mM NaCl–supplemented medium for 30 min. D, effect of NFAT5 knockdown on hypertonicity-induced caspase-3 activity and apoptosis. Cells transfected with 3.5 kbp-Luc and siRNA (NFAT5 1 or NC) were treated with isotonic or 75 mM NaCl–supplemented medium for 30 min. E, effect of NFAT5 knockdown on hypertonicity-induced caspase-3 activity and apoptosis. Cells transfected with 3.5 kbp-Luc and siRNA (NFAT5 1 or NC) were treated with isotonic or 75 mM NaCl–supplemented medium for 30 min. F, effect of NFAT5 knockdown on hypertonicity-induced caspase-3 activity and apoptosis. Cells transfected with 3.5 kbp-Luc and siRNA (NFAT5 1 or NC) were treated with isotonic or 75 mM NaCl–supplemented medium for 30 min.
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Figure 7. Rnf183 is a direct target of NFAT5. A, location and site-directed mutagenesis of the conserved NFAT5 DNA–binding site. The sequences from the pGL4–3.5 kbp–Luc of the WT and mutant are shown. Mutagenized bases are indicated by lowercase letters. B, effect of conserved site mutation on luciferase activities. Cells transfected with WT or mutant were cultured in isometric or 75 mM NaCl–supplemented medium for 24 h. The pGL4-Luc was used as a control. Firefly luciferase activities were normalized to Renilla luciferase signals (n = 4). C, effect of NFAT5 overexpression on luciferase activities. Cells transfected with WT, mutant, empty, or pFLAG-NFAT5 were cultured in an isotonic medium for 36 h (n = 5). D, schematic representation of the Rnf183 promoter and the annealing sites of the primer set used in the ChIP assays. E, ChIP assay analysis of the NFAT5 binding to the Rnf183 promoter in mIMCD-3 cells. ChIP assays were performed with the indicated antibodies. Immunoprecipitated (IP) DNA and input DNA were subjected to RT-PCR. IgG was used as a control. F, quantitative analysis of RT-PCR in E. Results were normalized to input DNA (n = 3). Data were analyzed by one-way ANOVA, followed by post hoc tests using t tests with Bonferroni correction. Values represent mean ± S.D. (error bars), *, p < 0.05; **, p < 0.01; ***, p < 0.001 (versus isotonic control).

Demonstrate that RNF183 protects inner-medullary cells from hypertonicity-induced apoptosis.

Rescue of RNF183 knockdown attenuates hypertonicity-induced apoptosis

To eliminate the possibility of siRNA off-target effects, mIMCD-3 cells transfected with RNF183 siRNA or NC siRNA were rescued with siRNA-resistant 3×FLAG-RNF183, which was expressed in mIMCD-3 cells under RNF183 knockdown (Fig. 9A). After 4 h of exposure to 200 mM NaCl–supplemented medium, cleaved caspase-3 protein levels significantly decreased in mIMCD-3 cells rescued with 3×FLAG-RNF183 (Fig. 9B). Crystal violet assays showed that the rescue of RNF183 knockdown significantly improved cell viability under hypertonic conditions (Fig. 9C). These results confirm that RNF183 protects inner-medullary cells from hypertonicity-induced apoptosis.

Discussion

We identified the conserved NFAT5 DNA–binding site in the Rnf183 enhancer region and found that this site induced RNF183 reporter activity under hypertonic conditions, whereas mutation at the site resulted in almost complete inhibition of Rnf183 reporter activity. In addition, ChIP assays revealed a high level of NFAT5 binding to the site. Thus, these results demonstrated a mechanism of RNF183 up-regulation under hypertonic conditions by which NFAT5 directly binds to the conserved NFAT5 DNA–binding site in the Rnf183 enhancer region and leads to its transcriptional activation. This is the first study to demonstrate that ubiquitin ligase can be directly regulated by NFAT5. However, Yu et al. (19) demonstrated that microRNA-7 (miR-7) directly bound to the 3' UTR of RNF183 mRNA, leading to RNF183 degradation and translational inhibition, and that down-regulation of miR-7 induced RNF183 elevation in inflamed colon tissues of IBD patients and colitic mice. Of the two regulators of RNF183 expression, we considered NFAT5 to be more important than miR-7 in a normal kidney because the tissue distribution pattern of RNF183 is different from that of miR-7 (34). The expression of miR-7 is restricted to specific tissues of the brain, colon, and thymus, whereas it is extremely low in the kidney, heart, liver, lung, spleen, and stomach (34), which is not consistent with our results on RNF183 expression (Fig. 1, E and F). Whereas NFAT5 is broadly expressed as an essential factor during exposure to the hypertonic environment (4), systemic or immature thymocyte-specific NFAT5 knockout mice display profound defects in the renal medulla and reduced thymocyte compartment in the thymus, respectively (6, 35, 36). This is because NFAT5 is activated by hypertonicity-dependent and -independent pathways in the renal medulla (6, 37, 38) and thymus (36), respectively. Consistent with these findings, RNF183 was highly expressed in the renal medulla and was also expressed in the thymus (Fig. 1, E–H). Therefore, we hypothesized a mechanism by which high RNF183 expression in the normal kidney and thymus is predominantly regulated by the NFAT5 activation, but not miR-7.

We observed an ~2–3-fold increase in the induction of Rnf183 reporter activity following hypertonic stimulation. One possible explanation for this mild induction of the Rnf183 promoter is that only a short sequence of the Rnf183 promoter was investigated. Consistent with our results, Hasler et al. (39) and Chen et al. (12) have demonstrated that the activity of the AQP2 and SGK1 promoters (each containing one NFAT5-binding element) increased ~1.5- and 5-fold, respectively, following hypertonic stimulation. However, at least five NFAT5-binding
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Figure 8. RNF183 expression provides protection against hypertonicity-induced apoptosis. A, inhibition of Rnf183 expression using siRNA. Mouse IMCD-3 cells transfected with RNF183 siRNA or NC siRNA were analyzed using qRT-PCR (n = 3). B and C, effect of RNF183 knockdown on hypertonicity-induced apoptosis. Cells transfected with the indicated siRNAs were treated with isotonic or 200 mM NaCl–supplemented medium for 4 h and analyzed using Western blotting (B) or immunofluorescence staining (C) using anti-cleaved caspase-3–specific antibody. The accompanying bar graph summarizes the quantification of relative amounts of cleaved caspase-3 (n = 3). C, the cleaved caspase-3 fluorescence–positive cells (red) are marked with white arrows. Bars, 100 μm. The number of cleaved caspase-3–positive cells was counted in five different fields of view and divided by the number of DAPI-stained cells (blue) to yield the ratio of cleaved caspase-3–positive cells; the results are summarized in the accompanying bar graph (n = 3). D, effect of RNF183 knockdown on cell viability under hypertonic conditions. Cells transfected with the indicated siRNAs were treated with isotonic or 200 mM NaCl–supplemented medium for 12 h and analyzed using a crystal violet assay (n = 3). Data were analyzed using a t test (A and D) or one-way ANOVA, followed by post hoc tests using t tests with Bonferroni correction (B and C). Values represent mean ± S.D. (error bars). *, p < 0.05; **, p < 0.01; ***, p < 0.001 (versus NC).

elements spread over 50-kb pairs, which participate in NFAT5-mediated transcriptional stimulation, were revealed in the SMIT promoter (3). Similarly, three NFAT5-binding sequences that were identified in the AKR1B1 promoter may collectively participate in the hyperosmotic response (5). Therefore, other NFAT5-binding elements may be present in regions of the Rnf183 promoter that are further upstream or downstream. Moreover, we demonstrated that Rnf183 reporter activity and Rnf183 and Akr1b1 mRNA expression were concurrently reduced by NFAT5 knockdown under isotonic conditions. Several previous studies have shown that, to some extent, NFAT5 knockdown or dominant negative NFAT5 expression inhibited mRNA expression or promoter activity of its downstream genes under isotonic conditions (7, 9, 39, 40). In contrast, two studies have shown that NFAT5 inhibition did not affect the basal expression of its downstream genes under isotonic conditions (12, 41). This difference in NFAT5 inhibition may result from variation in the basal NFAT5 protein levels because basal AKR1B1 protein expression levels were high in mIMCD-3 cells compared with those in other renal cell lines (Fig. 2F).

We further examined whether Rnf183 was up-regulated by hypoxia in mIMCD-3 cells because the renal medulla is characteristically hypoxic (42). Although vascular endothelial growth factor A (Vegfa) mRNA, which is downstream of the hypoxic master regulator hypoxia-inducible factor 1 (HIF-1) (43), was significantly up-regulated under hypoxia (oxygen concentration, 1 and 0.3%), Rnf183 was not up-regulated (Fig. S2). Moreover, the HIF-1 DNA–binding site (ACGTG) was not observed in the Rnf183 enhancer region (Table S1). These results suggest that the high RNF183 expression in the renal medulla does not result from hypoxia. Our results demonstrated that Rnf183 mRNA was markedly up-regulated compared with the other transmembrane RNf family members under hypertonic conditions and that the siRNA-induced knockdown of RNF183 exacerbates apoptosis and reduces cell viability in mIMCD-3 cells at ~700 mosmol/kg. Although the effects of RNF183 on adaptation to hypertonicity appear to be mild, we consider RNF183 to be as important as other NFAT5-regulated genes. Lee et al. (44) demonstrated up to a 1.5-fold increase in the hypertonicity-induced LDH release in MEF cells transfected with siRNA targeting SMIT, BGT1, Akr1b1, Hspa1b, etc. Shim et al. (33) have also demonstrated an ~20–30% decrease in cell viability following 100 mM NaCl supplementation in Hspa1b-knockout MEF
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**Figure 9. Rescue of RNF183 knockdown attenuates hypertonicity-induced apoptosis.** A, resistance of 3×FLAG-RNF183 to RNF183 siRNA. Mouse IMCD-3 cells were transfected with RNF183 siRNA or NC siRNA. After 12 h, the cells were electroporated with empty or 3×FLAG-RNF183-pcDNA vectors and were analyzed by Western blotting 48 h after electroporation. B and C, effect of rescue of RNF183 knockdown on hypertonicity-induced apoptosis (B) and cell viability (C). Cells transfected with RNF183 siRNA or NC siRNA were electroporated with empty or 3×FLAG-RNF183-pcDNA vectors. After 48 h of electroporation, cells were treated with 200 mM NaCl-supplemented medium for 4 (B) and 12 h (C) and then analyzed using Western blotting (B) and a crystal violet assay (C) (n = 3), respectively. The accompanying bar graph summarizes the quantification of the relative amounts of cleaved caspase-3 (n = 3). Data were analyzed by one-way ANOVA, followed by post hoc tests using t tests with Bonferroni correction. Values represent mean ± S.D. (error bars). *p < 0.05.

cells. Their effects on adaptation to hypertonicity appear to be similar to that of RNF183 in the present study. Therefore, RNF183 expression is induced by hypertonic stress and plays an important role in the kidney’s osmoprotective function under hypertonic conditions. However, we could not clarify the substrate of RNF183 under hypertonic conditions. To date, three previous studies have demonstrated the different functions of RNF183 in cancers and the colon as follows. (i) In tumors, RNF183 interacts with fetal and adult testis–expressed 1 component of FATE1 complex, leading to increased tumor cell viability (45). (ii) In the colon, RNF183 degrades IκBα downstream gene, thereby inducing NF-κB activation, which might contribute the pathogenesis of IBD (19). (iii) In the colorectal cancers, RNF183 induces the activation of NF-κB and expression of its downstream gene, IL8, resulting in increased proliferation and metastasis of colorectal cancer cells (20). However, FATE1 expression is restricted to cancer or testis and is absent in the other tissues, such as the kidney (45). Moreover, NF-κB activation by hypertonicity is transient and starts decreasing after 3 h of hypertonic stimulation in cortical collecting duct cells due to the restoration of IκBα protein levels (46, 47). Consistent with this, the mRNA level of Tnfα, an NF-κB downstream gene, peaked after 3 h of hypertonic stimulation and decreased after 6 h at the time of RNF183 protein level elevation (Fig. S3). Hence, these results suggest that RNF183 ubiquitylates other substrates under hypertonic conditions as there is a contradict-

**Experimental procedures**

**Plasmids**

A full-length mouse RNF183 construct with a stop codon was transferred from pENTR/D-TOPO (catalog no. 45-0218, Invitrogen) into the Vivid Colors pcDNA6.2/N-EmGFP-DEST vector (catalog no. 35-1017, Invitrogen) containing a N-terminal emerald GFP and into pcDNA6.2-DEST (catalog no. 35-1264, Invitrogen) using Gateway LR Clonase II enzyme mix (catalog no. 12538-120, Invitrogen). The 3×FLAG-RNF183 construct synthesized using GeneArt Strings DNA fragments (Invitrogen) was cloned into the pENTR/D-TOPO (Invitrogen). The entry clone product was recombined into the pcDNA6.2-DEST vector using LR Clonase II enzyme mix. The deletion mutant mouse RNF183 (amino acids 61–158) with a stop codon was amplified by PCR using the following primer sets and was subcloned into pENTR/D-TOPO: 5′-CACCATG-CAGGCCACCGT-3′ (forward) and 5′-TCAGAAAGTGAG-GTTGCCGACCAC-3′ (reverse). Subsequently, using Gateway LR Clonase II enzyme mix, the sequence was transferred into the pDest-565 vector (catalog no. 11520, Addgene), containing a GSH S-transferase (GST) at the N terminus of insert sequences and into the pDest-566 vector (catalog no. 11517, Addgene) containing a maltose-binding protein (MBP) at the
N terminus of the insert sequences. pFLAG-NFAT5, the expression vector of NFAT5, was gifted to us by Dr. B. C. Ko (23) and Dr. Takashi Ito (9). The pFLAG-DN-NFAT5 was a generous gift from Dr. Takashi Ito (9).

Rnf183 promoter fragments were amplified from C57BL/6 murine genomic DNA by PCR using the following primer sets: 3,525 bp (3,465 to +60 region; numbers indicate the nucleotide position relative to the transcription start site), 5′-GGTACCCAGCGAGGCCATCCC-3′ (forward) and 5′-TTTCTGGTAATGTCACCTCGTCTTC-3′ (reverse); 2,012 bp (1,952 to +60 region), 5′-CTGGTTAATGTCACCTCGTCTTC-3′ (forward) and 5′-TTTCTGGTACCAGGCCATCCC-3′ (reverse). Subsequently, the purified products were subcloned into the pGL4.10[luc2] vector (catalog no. 9PIE665, Promega) between KpnI and XhoI sites, respectively. The resulting vectors were named pGL4-3.5 kbp-Luc and pGL4–2.0 kbp-Luc, respectively. Mutagenesis of oligonucleotides (sequence represents antisense strand, with mutagenized bases represented in lowercase type): mutant, ACCTTTCCCAGAAAGCCCATCCC-3′; wild type, GCTTAGGCCAGGTACTTTTCCAGCTGGCCTGC; 3,358 GTACT-/H11002 of the conserved NFAT5 DNA–binding site (assay ID: Mm.PT.58.43776563.g), mouse Rnf183 (assay ID: Mm.PT.56a.31556976), mouse Rnf128 (assay ID: Mm.PT.58.8505793), mouse Rnf24 (assay ID: Mm.PT.58.9219868), mouse Rnf150 (assay ID: Mm.PT.58.31570020.g), mouse Vegfa (assay ID: Mm.PT.58.9635653), and mouse Vegfc (assay ID: Mm.PT.58.32670381). For knockdown experiments, mIMCD-3 cells were transfected with three types of NFAT5-targeting Silencer siRNA (catalog no. 4390771 (ID: s21069 and s203948) and catalog no. 4399665 (ID: s550857), Invitrogen) or an RNF183-targeting Silencer siRNA (catalog no. 4390771 (ID: s94062), Invitrogen) or a negative control Silencer siRNA (catalog no. 4390843, Invitrogen) as a control using ScreenFect siRNA (catalog no. 295-75003, Wako Pure Chemical Industries, Osaka, Japan) in 6-well plates (12.5 pmol/well). HeLa or HEK293 cells were transfected with each expression plasmid using ScreenFect A (catalog no. 297-73204, Wako Pure Chemical Industries). For inhibition of p38/MAPK, mIMCD-3 cells were preincubated with SB203580 at 0–25 μM (catalog no. 199-16551, Wako Pure Chemical Industries) for 1 h and treated with 75 mM NaCl–supplemented medium for the indicated time, according to the method reported by Chen et al. (12).

Analysis of mRNA levels in murine tissues

Total murine tissue RNA was extracted from C57BL/6 mice using ISOGEN (catalog no. 311-02501, Nippon Gene, Toyama, Japan) according to the manufacturer’s protocol. Reverse transcription was performed with ReverTra Ace (catalog no. TRT-101, Toyobo Co., Ltd., Osaka, Japan). PCR was performed using the following primer sets in a total volume of 20 μl containing 0.2 μM of each primer, 0.2 mM dNTPs, 1 unit of Paq5000 DNA polymerase, and 10× PCR buffer (catalog no. 600680, Agilent Technologies): mouse Rnf183, 5′-GACCAGCGCCAGGCGGTATA-3′ (forward) and 5′-CCCCAAGAAGAATGCTAGTCTTACAC-3′ (reverse); mouse Megalin, 5′-CTCCTGAGAATGGGTTTTTAT-3′ (forward) and 5′-GAGAGCATGGTGAA-AGGAGCC-3′ (reverse); mouse Akr1b1, 5′-AGAGCAGATGGTGAAATGGAGGCC-3′ (forward) and 5′-AACCTGCTGATCTGGTTTTAC-3′ (reverse). The PCR conditions were as follows: 94 °C for 2 min, 22–26 cycles at 98 °C for 10 s, 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 3 min. The PCR products were resolved by electrophoresis on a 4.8% acrylamide gel. The density of each band was quantified using the Adobe Photoshop Elements version 2.0 program (Adobe Systems Inc.).
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Mouse Znf3 (assay ID: Mm.PT.56a.5645486), mouse Syvn1 (assay ID: Mm.PT.58.14132695), mouse Amfr (assay ID: Mm.PT.58.9856954), mouse Rnf121 (assay ID: Mm.PT.58.6378114), mouse Rnf145 (assay ID: Mm.PT.58.10110397), mouse Rnf139 (assay ID: Mm.PT.58.45812642), mouse Rnf103 (assay ID: Mm.PT.58.9763784), mouse Rnf19A (assay ID: Mm.PT.58.28600163), mouse Rnf19B (assay ID: Mm.PT.58.28736280), mouse Rnf15 (assay ID: Mm.PT.58.14209781), mouse Rnf185 (assay ID: Mm.PT.56a.11642500), mouse Rnf170 (assay ID: Mm.PT.56a.13272002), mouse Rnf186 (assay ID: Mm.PT.56a.29338718.g), mouse Rnf152 (assay ID: Mm.PT.58.29277956.g), mouse Rnf182 (assay ID: Mm.PT.58.30902864.g), mouse Trim59 (assay ID: Mm.PT.58.30417218), mouse Trim13 (assay ID: Mm.PT.56a.9751937), mouse Bfar (assay ID: Mm.PT.56a.28494992), mouse Rnf180 (assay ID: Mm.PT.58.45835696), mouse Rnf1 (assay ID: Mm.PT.58.6836835), mouse Rnf26 (assay ID: Mm.PT.58.5764306.g), mouse Cgrnf1 (assay ID: Mm.PT.58.5664724), mouse Mull1 (assay ID: Mm.PT.58.11670267), and mouse Rnf217 (assay ID: Mm.PT.56a.33323466). Mouse β-actin TaqMan primer and probe set was as follows: 5'-GCCGTTGGCCATGCGCTT-3' (forward), 5'-CATGGATGCCAGGATTCC-3' (reverse), and 5'-AGGCCCTCTTCCAGCCTTCTTGG-3' (probe).

**Western blotting**

Tissue or cell lysates were prepared in 1× Laemmli sample buffer. Equivalent protein samples were loaded onto SDS-polyacrylamide gels. Gels were transferred to Immobilon-P polyvinylidene difluoride membranes (catalog no. IPVH00010, Millipore) and blocked in 5% nonfat dry milk at room temperature for 1 h. The membrane was incubated with primary antibody at 4°C overnight and with secondary antibody at room temperature for 1 h. The following primary antibodies were used: anti-β-actin (catalog no. sc47778, Santa Cruz Biotechnology, Inc.), anti-aldolase reductase (catalog no. sc271007, Santa Cruz Biotechnology), anti-cleaved caspase-3 (catalog no. 9661, Cell Signaling Technology), anti-GFP (catalog no. 598, Medical Biological Laboratories, Nagoya, Japan), anti-megalin (catalog no. 101-M144, ReliaTech GmbH, Germany), anti-NFAT5 (catalog no. ab3446, Abcam, UK), and anti-RNF183. The secondary antibodies used were as follows: horseshad peroxidase–conjugated goat anti-mouse IgG antibody (catalog no. 1031-05 Southern Biotech) and horseshad peroxidase–conjugated goat anti-rabbit antibodies (catalog no. 4050-05 Southern Biotech). Images were obtained using a WSE-6100 LuminoGraph (ATTO Corp., Tokyo, Japan).

**Immunofluorescence**

Cells were fixed in 4% paraformaldehyde at room temperature for 10 min and then permeabilized in methanol at −20°C for 10 min. Cells were blocked with 5% anti–goat serum at room temperature for 30 min and incubated with primary antibody at 4°C overnight and then with Alexa Fluor–conjugated secondary antibodies at room temperature for 30 min. The following primary antibodies were used: anti-GFP (catalog no. 598, Medical Biological Laboratories), anti-NFAT5 (catalog no. ab3446, Abcam), anti-cleaved caspase-3 (catalog no. 9661, Cell Signaling Technology), anti-calnexin (catalog no. 2679, Cell Signaling Technology), anti-COX IV (catalog no. 4850, Cell Signaling Technology), anti-EA1 (catalog no. 3288, Cell Signaling Technology), anti-GM130 (catalog no. 12480, Cell Signaling Technology), anti-transferrin receptor (catalog no. 13-6800, Invitrogen), and anti-Lamp1 (catalog no. 9091, Cell Signaling Technology). The secondary antibodies used were as follows: goat anti-mouse Alexa Fluor 555 (catalog no. A-21422, Invitrogen), goat anti-rabbit Alexa Fluor 568 (catalog no. A-11036, Invitrogen), goat anti-mouse Alexa Fluor 488 (catalog no. A-11001, Invitrogen), and goat anti-rabbit Alexa Fluor 488 (catalog no. A-11070, Invitrogen). ProLong Diamond Antifade Mountant with DAPI (catalog no. P36962, Invitrogen) was used to mount slips on glass slides. Fluorescence images were acquired using an Olympus Fluoview FV1000 confocal microscope (Olympus, Tokyo, Japan). Apoptotic cells were counted in five different fields of view, and the ratio of the number of cleaved caspase 3–positive cells to that of DAPI-stained cells was calculated.

**Luciferase assay**

Mouse IMCD-3 cells were transiently transfected with 0.2 μg of pGL4.10[luc2] carrying both the firefly luciferase gene and the referenced constructs and 0.02 μg of pGL4.74[hRluc/TK] carrying the Renilla luciferase gene under the control of the herpes simplex virus thymidine kinase enhancer and promoter (catalog no. 9PIE692, Promega) in the presence of NFAT5 siRNA 1, NFAT5 siRNA 2 (catalog no. 4390771, Invitrogen), NFAT5 siRNA 3 (catalog no. 4399665, Invitrogen), NC siRNA (catalog no. 4390843, Invitrogen), or empty, pFLAG-DN-NFAT5, or pFLAG-NFAT5 vectors; the transfection was performed using ScreenFect A (catalog no. 297-73204, Wako Pure Chemical Industries) under conditions recommended by the manufacturer. For rescue of NFAT5 knockdown, cells pretransfected with siRNA (NFAT5 siRNA 3 or NC) using ScreenFect siRNA (catalog no. 295-75003, Wako Pure Chemical Industries) were cotransfected with 3.5 kbp-Luc and either empty or pFLAG-NFAT5 vectors after 12 h of knockdown. Twenty-four hours after transfection, cells were treated with 75 mM NaCl or 150 mM sucrose in DMEM/F-12 for 24 h. Cells were washed twice with PBS and subsequently lysed with Passive Lysis Buffer (catalog no. 194A, Promega). Luciferase activities were measured with the Dual-Luciferase Reporter Assay System (catalog no. 1960, Promega) and a GloMax Multi Detection System (Promega), according to the manufacturer's protocol. Relative activity was defined as the ratio between the activities of firefly luciferase and Renilla luciferase. The vector pGL4.10[luc2] without the promoter (pGL4-Luc) was used as a negative control.

**ChIP assay**

Two 10-cm plates of ~80% confluent mIMCD-3 cells were cultured for 24 h and treated with 75 mM NaCl or 150 mM sucrose in DMEM/F-12 for 24 h prior to cross-linking. Cells were treated with 1% (v/v) formaldehyde for 15 min at 37°C, followed by an additional 5 min in 150 mM glycine. Subsequently, cells were washed with cold PBS and lysed in nuclear lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS, and protease inhibitor mixture) for 10 min on ice. Nuclear lysates were sonicated to a final average fragment length of 1,000 bp using a Bioruptor II (BM Equipment, Tokyo, Japan).
Japan). Sonicated chromatin was cleared by centrifugation at 13,000 rpm for 10 min and then diluted 10-fold with dilution buffer (16.7 mM Tris-HCl (pH 8.0), 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, 167 mM NaCl, and protease inhibitor mixture). The soluble chromatin was incubated with 2 μg of normal IgG rabbit (catalog no. 2729, Cell Signaling Technology) or anti-NFAT5 antibody (catalog no. ab3446, Abcam) on a rotating platform at 4 °C overnight, followed by incubation with 20 μl of Mab Chip Protein A + G magnetic beads (catalog no. 16-663, Millipore) for 1 h. Subsequently, the beads were washed with the following four buffers: low-salt buffer (20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 150 mM NaCl), high-salt buffer (same as low-salt buffer with 500 mM NaCl), LiCl buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate), and TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Precipitated complexes were eluted twice from the beads in fresh elution buffer (1% SDS, 0.1 M NaHCO₃) for 30 min each at room temperature and subsequently heated at 65 °C overnight to reverse formaldehyde-induced cross-linking. The DNA was purified by phenol–chloroform extraction and ethanol precipitation. The purified DNA was amplified by PCR in a total volume of 20 μl containing a 0.45 μM concentration of each primer, 0.4 mM dNTPs, 1 unit of KOD FX DNA polymerase, and 2× PCR buffer (catalog no. KFX-101, Toyobo). The following primer set was used, yielding a 303-bp product: 5′-GTTTTACCCTTTTG-CTTTGGTAAACA-3′ (forward) and 5′-CAGGCTAAGAAGTCCAGGCTAAAG-3′ (reverse). The PCR conditions were as follows: 94 °C for 5 min, 33 cycles at 94 °C for 15 s, 60 °C for 30 s, 72 °C for 1 min. The PCR products were resolved by electrophoresis on a 4.8% acrylamide gel. The density of each band was quantified using the Adobe Photoshop Elements version 2.0 program (Adobe Systems Inc.).

Cell viability assay

mIMCD-3 cells were transfected with RNF183 or NC siRNA in 6-well plates (12.5 pmol/well). After 24 h, the cells were reseeded in a 24-well plate and cultured in 5% CO₂ at 37 °C for an additional 24 h. Subsequently, cells were treated with isotonic or 200 mM NaCl–supplemented medium in 5% CO₂ at 37 °C for 12 h and washed with PBS and then stained with crystals violet. The wells were washed with water and air-dried, and the dye was eluted with water-containing 0.5% SDS. The absorbance was measured at 590 nm using a Bio-Rad SmartSpec 3000 spectrophotometer, according to the method reported by Omura et al. (56).

Electroporation

For RNF183 rescue experiments using electroporation, mIMCD-3 cells were transfected with RNF183 or NC siRNA in 10-cm dishes (62.5 pmol/dish). After 12 h, the cells were trypsinized and resuspended in DMEM/F-12. They were then pelleted at 1,000 rpm for 5 min and resuspended in Opti-MEM at a working concentration of ~1.0 × 10⁶ cells/ml. One hundred microliters of cell suspension was added to 10 μg of empty or 3×FLAG-RNF183–pcDNA vectors in electroporation cuvettes (catalog no. SE-202, BEX Co., Ltd., Tokyo, Japan) and then transfected by one direct-current 150-V electrical pulse and five direct-current 20-V electrical pulses at intervals of 50 ms using an electroporator (CUY 21 Vitro-EX, BEX Co.). After 48 h, cells were treated with 200 mM NaCl in DMEM/F-12 for the indicated time and analyzed using Western blotting or a crystal violet assay.

Statistical analysis

Results are expressed as the mean ± S.D. Statistical evaluation was performed using JMP statistical software (version Pro 12). Comparisons between two groups were analyzed using the two-tailed t test. For multiple-group comparisons, one-way analysis of variance (ANOVA), followed by two-tailed Student’s t test with Bonferroni correction were applied. Significant p values are as follows: *p < 0.05; **p < 0.01; and ***p < 0.001. Nonsignificant p values are shown as N.S. (p > 0.05).

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NFAT5 regulates Rnf183 transcription

Author contributions—M. K. and Y. M. conceptualized the study; Y. M. performed formal analysis; M. K. and K. I. acquired funding; Y. M. performed the investigation; S. K., T. O., A. S., R. A., and K. M. designed the methodology; K. I. oversaw project administration; T. M. supervised the study; Y. M., Y. W., and X. G. performed data validation; Y. M. performed visualization of cells and tissues; Y. M. and M. K. wrote the original draft; and K. I., T. O., and S. K. reviewed and edited the manuscript.

Acknowledgments—We appreciate the advice and expertise of Isao Naguro, Shigehiro Doi, Ayumu Nakashima, Toshiaki Doi, Shuma Hirashio, and Kensuke Sasaki. We sincerely appreciate Keiji Tanimoto for handling equipment for the hypoxia experiment. Sincere appreciation is extended to Ryoji Kojima for supplying mIMCD-3 cells. Gratitude is expressed to Dr. Takashi Ito and Dr. B. C. Ko for the generous donation of the expression vector pFLAG-NFAT5 and pFLAG-DN-NFAT5. We are grateful to Takeshi Ike and Yui Tanita for assistance. Part of this work was performed at the Analysis Center of Life Science, Natural Science Center for Basic Research and Development, Hiroshima University. We thank Enago for providing an English language review.
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doi: 10.1074/jbc.RA118.002896 originally published online November 9, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.002896

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