trpm7 Regulation of in Vivo Cation Homeostasis and Kidney Function Involves Stanniocalcin 1 and fgf23

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The transient receptor potential melastatin 7 (trpm7) channel kinase is a primary regulator of magnesium homeostasis in vitro. Here we show that trpm7 is an important regulator of cation homeostasis as well as kidney function in vivo. Using zebrafish trpm7 mutants, we show that early larvae exhibit reduced levels of both total magnesium and total calcium. Accompanying these deficits, we show that trpm7 mutants express higher levels of stanniocalcin 1 (stc1), a potent regulator of calcium homeostasis. Using transgenic overexpression and morpholino oligonucleotide knockdown, we demonstrate that stc1 modulates both calcium and magnesium levels in trpm7 mutants and in the wild type and that levels of these cations are restored to normal in trpm7 mutants when stc1 activity is blocked. Consistent with defects in both calcium and phosphate homeostasis, we further show that trpm7 mutants develop kidney stones by early larval stages and exhibit increased levels of the anti-hyperphosphatemic factor, fibroblast growth factor 23 (fgf23). Finally, we demonstrate that elevated fgf23 expression contributes to kidney stone formation by morpholino knockdown of fgf23 in trpm7 mutants. Together, these analyses reveal roles for trpm7 in regulating cation homeostasis and kidney function in vivo and implicate both stc1 and fgf23 in these processes. (Endocrinology 151: 5700–5709, 2010)

The two closely related genes, TRPM7 and TRPM6, encode transient receptor potential (TRP) family proteins that function as divalent cation channels with C-terminal α-kinase domains that regulate channel activity (1–5). TRP melastatin 7 (Trpm7) and Trpm6 are preferentially permeable to a number of divalent cations (6–11), including magnesium and calcium, and studies of these channels have provided new insights into magnesium and calcium homeostasis (1, 9–15). Trpm7 and Trpm6 function as either homomeric channels or as heteromeric channels with one another (1, 10, 16–19). Whereas Trpm7 channels are expressed across a wide range of tissues (1, 19, 20), Trpm6 channels have a somewhat more limited distribution, being found primarily in organs that regulate physiological ion levels, such as the kidney and intestines (7, 20–22). Mammalian Trpm7 also functions in sensing extracellular calcium and magnesium in neurons (23–25) and in regulating cell adhesion (26, 27), whereas human mutations in TRPM6 are linked to hypomagnesemia with secondary hypocalcemia (14, 28).

Numerous studies have shown the importance of Trpm7 for cation homeostasis in vitro, yet the early lethality of Trpm7 mutations in mammals has precluded analyzing roles in whole-organism cation homeostasis (29). By contrast, zebrafish trpm7 mutants survive into embryonic and postembryonic stages, permitting analyses of developmental and physiological trpm7 functions in vivo (30). Zebrafish trpm7 mRNA is detectable in all adult tissues (Elizondo, M. R., and D. M. Parichy, unpublished data) and is expressed widely in embryos and larvae, with particularly high transcript abundance in the tubules of the pronephric and mesonephric kidneys, and in the corpuscles of Stannius (CS), a teleost-specific gland that regulates physiological ion homeostasis (30–32). As em-

Abbreviations: CaSR, Calcium-sensing receptor; CS, corpuscles of Stannius; dpf, days post fertilization; hpf, hours post-fertilization; EGFP, enhanced green fluorescent protein; FGF23, fibroblast growth factor 23; hpf, hours post fertilization; IRES, internal ribosome entry site; qPCR, quantitative RT-PCR; stc1, stanniocalcin 1; TRP, transient receptor potential; Trpm7, TRP melastatin 7.
bryos, trpm7 mutants have defects in the survival of melanized pigment cells, melanophores, and also develop a transient unresponsiveness to touch (33–35). As larvae, these mutants exhibit severe defects in growth and skeletogenesis while also developing kidney stones (30). The known functions of mammalian Trpm7 channels suggest the pleiotropic phenotypes of zebrafish trpm7 mutants may be related to altered cation homeostasis and kidney function.

Here we show that zebrafish trpm7 mutants exhibit multiple defects in physiological homeostasis. We find that trpm7 mutants have reduced levels of whole-embryo total calcium and total magnesium by 3 and 4 d post fertilization (dpf), respectively, and we demonstrate that the CS-specific gene, stanniocalcin 1 (stc1) is a downstream mediator of altered cation levels in trpm7 mutants. Additionally, we show that trpm7 mutants develop kidney stones by 5 dpf and express elevated levels of the anti-hyrophosphatemic factor fgf23, whereas morpholino knockdown of fgf23 reduces the incidence of kidney stones in the mutant background. Together, our findings provide important new information about trpm7 functions and lay the groundwork for further studies of its in vivo roles in cation and phosphate homeostasis as well as kidney function.

Materials and Methods

Strains and rearing conditions

Fish were reared at 28.5 C (except as noted below) with a 14-h light, 10-h dark cycle. Mutants were trpm7112461, trpm7112462, and trpm7120508, with most experiments using the latter allele.

Total calcium and magnesium assays

For each sample tested, 25 embryos or larvae were pooled, anesthetized with MS222, rinsed briefly in nanopure H2O, and collected in a 1.5-ml microcentrifuge tube. Fish were then dried at 65 C for 30–45 min, at which time 125 l of each sample was mixed with 125 l 1 M HCl was added to each tube and acid denatured overnight at 95 C with occasional tapping or brief centrifugation to collect solution at the bottom of the tube. Tubes were then centrifuged at maximum speed for 15 min, and supernatant was collected. To assess total calcium and magnesium content from the supernatant, we used QuantiChrom calcium and magnesium assays (BioAssay Systems, Hayward CA; DICA-500 and DIMG-250). Although trpm7 mutants exhibit growth retardation at later larval stages, sizes of embryos and early larvae are indistinguishable from wild type (30, 35).

For magnesium assays, the manufacturer’s protocol was used with half-reactions and 5 l of each sample tested for 2- to 5-dpf fish. Absorbances were read at 490 nm before and after addition of 10 l EDTA to obtain blank readings for individual wells. Statistical analyses were performed using JMP version 8.0.1 for Macintosh (SAS Institute, Cary, NC).

Total RNA isolation and cDNA synthesis

For RNA preparations, 10 embryos were pooled, anesthetized in MS222, and homogenized in 200 l TRizol (Invitrogen, Carlsbad, CA). RNA preps were performed as specified in the manufacturer’s protocol, resuspended in 13 l H2O, and quantitated using a NanoDrop 1000 spectrophotometer (ThermoScientific, Wilmington, DE). Superscript III and RNase Inhibitor (Invitrogen) were used in cDNA synthesis reactions primed with oligo dT primers, as per manufacturer’s protocol. cDNAs were diluted with 100 l TE before use.

Quantitative RT-PCR (qPCR)

For qPCR, 50-l reactions were performed in triplicate using 0.5 l AmpliTaq (Applied Biosystems, Foster City, CA), 5 l GeneAmp 10× PCR buffer, 1 l 12.5× SYBR Green (Sigma-Aldrich, St. Louis, MO), 1 l 10 mM dNTPs, 2 l 2.5 mM forward and reverse primer mix, and 1.5 l diluted cDNA. For cycling, a Chromo4 real-time instrument (MJ Research, Waltham, MA) was used with the following program: an initial denaturing step of 94 C for 3 min followed by 40 cycles of 94 C for 20 sec, 56 C for 20 sec, and 72 C for 30 sec and a final elongation step of 72 C for 5 min. For each primer set, no-template control reactions were performed. Before qPCR, multiple primer sets were tested for amplification efficiency, amplification without primer-dimer formation, and optimal annealing temperature using gradient PCR with annealing temperatures ranging from 54–62 C. Selected primers were determined to have optimal amplification efficiency at 56 C annealing without formation of primer-dimers. Primer sets (forward, reverse, 5’ to 3’) used for quantitative qPCR were: β-actin, GCATCACACCTTCTACAACCGAG, AGAGTCCATICAGTGACGATGCTG; stc1 (endogenous), GCAGGGCGGAGATTATTAGTGTCGAGAATCTTCACCAAGACATG; and stc1 (transgenic), TCACCTGTTCGCCAGAAGAC, CAAAGAAGCAGCAATGATTG. Primers used for molecular cloning (attB1F and attB1R sites underlined): stc1, (GGGGACAGTGTGTACAAAAAGCAGGCTACACATGCTCCTGAAAGCGGATTTC, GGGGAGACACTTGCGAAAACAGT; and

In situ hybridization

Gene expression analyses by in situ hybridization followed standard methods (38) using Sp6-synthesized digoxigenin riboprobes targeted to 753 bp of stc1 (NM_200539) and 758 bp of fgf23 (AY753222) cDNAs. Wild-type and mutant embryos were stained for identical times, and in situ hybridizations were replicated on three different occasions using embryos from multiple clutches (n > 300 total embryos examined). Typical expression patterns are shown, although mutants could sometimes show defects of greater or lesser severity.

Transgenic expression constructs

To construct an stc1-expressing transgenic line, we used the Tol2kit (39) and Multisite Gateway reagents (Invitrogen). We amplified a full-length stc1 cDNA with primers containing attB
flanking sequences, inserted the amplon into pDONR221 and verified integrity of the open reading frame by sequencing. We used the pDONR-stc1 clone as the middle entry vector, combined with pSE-hsp70 5′ entry vector, p3E-ires-EGFPpA 3′ entry vector, and the pDestTol2pA2 destination vector from the Tol2kit. The resulting vector comprised an expression cassette with a heat-shock protein hsp70 promoter controlling expression of the full-length stc1 followed by enhanced green fluorescent protein (EGFP) linked by an internal ribosome entry site (IRES). We co-injected this hsp70::stc1-ires-EGFP plasmid with Tol2 mRNA transcribed from the pCS26A-transposase plasmid (40). Injected embryos were heat shocked at 38.5°C for 30 min at 24 h post fertilization (hpf) and then screened for mosaic expression of EGFP at 4 h after heat shock. GFP-positive embryos were reared to adulthood and screened for germline integration through their progeny. Identified germline carriers were used to establish stable transgenic lines and the strongest GFP-expression line was used for experiments; we did not observe significant inter-embryo variation in the ubiquitous pattern of hsp70-induced expression as assayed by EGFP fluorescence. Similar results were observed in other transgenic lines.

**Heat-shock induction**

Two methods were used for heat-shock induction experiments. To screen transgenic progeny for GFP, a single heat shock was performed by placing embryos in 250-µl glass culture dishes (Carolina Biological, Burlington, NC) and then placing dishes in a shaking water bath set to 38.5°C and heat shocking embryos for 20 min. For repeated heat shocking over a period of several days, embryos were placed in clear plastic cups with mesh-covered holes to provide circulating water flow. Cups were then placed in a 10-gallon acrylic aquarium with a drain and 28.5°C flowing fish system water. Temperature was controlled using a ProcessTech heater and temperature controller (Aquatic Ecosystems, Apopka, FL). The heater controller was then plugged into an electrical timer set for a cycle for 30 min on, 5.5 h off.

**Antisense morpholino oligonucleotide injections**

Morpholinos to stc1 (stc1-MO: GAATTCGCTTTTACGAGCATGTC) and fgf23 (fgf23-sb1; GCCACAGTAGGCATTAGTACTTACTGTAT) were designed by GeneTools LLC (Philomath, OR). stc1-MO targets the translational start site, whereas fgf23-sb1 targets the exon1/intron1 splice donor site. Disrupted splicing of fgf23 pre-mRNA by fgf23-sb1 was verified by RT-PCR (41) (data not shown). Lyophilized morpholinos was resuspended in 300 µl 1 × Danieau buffer. Concentrations were determined by diluting 2 µl in 20 µl 0.1 M HCl, measuring the absorbance at 265 nm using a NanoDrop (ThermoScientific) spectrophotometer and calculating the concentration as recommended by the manufacturer. Morpholinos were then diluted to 0.2 mM in 1 × Danieau buffer, and 4.8–6.4 ng stc1-MO and 6.5 ng fgf23-sb1 were injected into wild-type embryos at the one- to two-cell stage.

**Detection of kidney stones**

We used a 0.5% (wt/vol) solution of alizarin red (Sigma-Aldrich) diluted in nanopure H2O and adjusted to pH 7.5 with sodium bicarbonate. For staining, we incubated embryos or larvae in petri dishes in a final concentration of 0.004% alizarin red diluted in 10% Hank’s solution (42). After overnight incubation, fish were briefly washed in 10% Hank’s before anesthetizing with MS222 and imaging under epifluorescence illumination using a Texas Red filter set. To assess kidney stone migration, individual fish were imaged immediately after a brief rinse and then allowed to recover in 10% Hank’s for 12 h, when they were imaged a second time.

**Results**

Reduced total calcium and magnesium in trpm7 mutants

In humans, mutations in TRPM6 lead to hypomagnesemia because of decreased Mg2+ reabsorption by the kidney and intestines, which in turn disrupts calcium homeostasis in the parathyroid gland, resulting in hypocalcemia (14, 28). Because Trpm6 channels are thought to act in heteromeric complexes with Trpm7 to regulate Mg2+ homeostasis (16, 19), defects arising from TRPM7 mutations might be expected to overlap with those exhibited by TRPM6 mutants. We therefore tested whether disruptions to cation homeostasis in zebrafish trpm7 mutants are similar to those arising from mammalian Trpm6 mutation. Because we could not extract sufficient serum from zebrafish embryos, we examined physiological cation levels as a proxy, by comparing the total calcium and magnesium contents of wild-type and mutant embryos from 2–5 dpf.

For total calcium, mutants did not differ significantly from wild type at 2 dpf but exhibited significantly reduced levels by 3 dpf and still more pronounced reductions at 4–5 dpf (Fig. 1A). For total magnesium, we found reduced levels in mutants at 4 and 5 dpf (Fig. 1B). Although not a direct measurement of serum cation levels, the reduced...
total cation levels are consistent with hypocalcemia and hypomagnesemia in mutants.

**Altered stanniocalcin-mediated regulation of divalent cation homeostasis in trpm7 mutants**

Terrestrial animals obtain calcium only from their diet and are typically challenged by hypocalcemic conditions. The primary regulators of calcium homeostasis, PTH and PTHrP, are, therefore, anti-hypocalcemic factors (43–45). In contrast, fish are surrounded by an abundant external supply of calcium and can be challenged with preventing hypercalcemia, which they accomplish by controlling ion influx through the gills, kidneys, and intestine. The teleost-specific CS regulates the rates of ion influx at these sites by secreting anti-hypercalcemic stanniocalcins (46–49) (reviewed in Ref. 50). Zebrafish stanniocalcin 1 (stc1) is expressed exclusively by the CS of embryos and early larvae (Fig. 2, A and B), although it is expressed more broadly in adults (31, 51). Because trpm7 is expressed particularly strongly in the CS as well (30, 31), we asked whether trpm7-dependent changes in calcium levels might be associated with changes in stc1 regulation.

To test this, we assayed stc1 expression in trpm7 mutant embryos by qPCR. We found significant increases in stc1 transcript abundance in trpm7 mutants compared with wild type beginning at 2 dpf and extending at least through 5 dpf (Fig. 2C). The increased stc1 expression in mutants preceded the detectable deficiency in total calcium (Fig. 1A), suggesting that up-regulated stc1 and its anti-hypercalcemic activity may be directly responsible for reduced total calcium in trpm7 mutants.

**stc1 overexpression reduces total calcium as well as total magnesium**

trpm7 mutants exhibited up-regulated stc1 expression followed by decreased total calcium and total magnesium (Figs. 1 and 2C). Although decreased calcium is explicable by the anti-hypercalcemic activity of stc1, we hypothesized that stc1 also might negatively regulate magnesium levels. To test this idea in a wild-type background, we generated a heat-shock-inducible transgenic line to over-express stc1, Tg(hsp70::stc1-IRES-EGFP). After 38.5°C heat shock for 30 min, we confirmed transgene-specific expression of stc1 (stc1T) by qPCR (Fig. 3).

To mimic the increased stc1 expression of trpm7 mutants in a wild-type genetic background, we heat shocked transgenic and nontransgenic sibling embryos for 30 min at 6-h intervals between 40 and 120 hpf. After heat shock, we sorted embryos for the presence or absence of the transgene by GFP fluorescence, and we performed ion assays as described above. We found that heat-shocked Tg(hsp70::stc1-IRES-EGFP) embryos exhibited reduced total calcium and total magnesium compared with heat-shocked, nontransgenic siblings (Fig. 4). This confirmed the anti-hypercalcemic activity of stc1 and supported the hypothesis that stc1 influences magnesium homeostasis, either directly or indirectly.

**Inhibition of stc1 in trpm7 mutants restores total calcium and total magnesium levels**

Inhibition of zebrafish stc1 activity increased calcium levels (51). Additionally, our results from overexpressing stc1 in wild-type embryos suggested that, in trpm7 mutants, decreased total calcium and total magnesium may result from stc1 up-regulation. We therefore tested whether normal total calcium and total magnesium levels could be restored in a trpm7 mutant background simply by inhibiting stc1 translation by morpholino oligonucleotide injection.

Embryos injected with a morpholino targeted to stc1 (stc1-MO) were morphologically indistinguishable from
Calcium and magnesium levels represent least squares means compared with heat-shocked nontransgenic [wild type (wt)] siblings. A and B, Reduced total calcium (A) and total magnesium (B) levels in heat-shocked transgenic \((Tg)\) larvae compared with wild-type (wt) siblings. Calcium and magnesium levels represent least squares means ± se after controlling for variation among batches \((n = 16\) mutant and wild-type samples at 5 dpf). By overall ANOVA, calcium \(F_{1,14} = 499\ (P < 0.0001)\) and magnesium \(F_{1,14} = 313\ (P < 0.0001)\). Samples with different letters in each panel are significantly different by Tukey-Kramer honestly significant difference test \((\alpha = 0.05)\).

Wild type (as observed also in Ref. 51). Moreover, \(trpm7\) mutants injected with \(stc1\)-MO exhibited total calcium and total magnesium restored to levels comparable to those of uninjected wild-type siblings (Fig. 5). As expected, wild-type siblings injected with \(stc1\)-MO showed an increase in total calcium relative to uninjected siblings (51) yet failed to exhibit altered levels of total magnesium. These results show that \(stc1\) influences magnesium levels in the \(trpm7\) mutant background and that wild-type \(trpm7\) masks this effect. Together, these data suggest that total calcium levels are misregulated in \(trpm7\) mutants due to the overexpression of \(stc1\) and that total magnesium levels can be restored in an \(stc1\)-dependent, but \(trpm7\)-independent, manner.

An early larval defect in kidney function

In addition to effects on calcium and magnesium homeostasis demonstrated above, we showed previously that zebrafish \(trpm7\) mutants develop mineralized deposits in the kidneys by late larval stages (30). Because \(trpm7\) is expressed in the earlier pronephros as well (30, 31, 52), we asked whether \(trpm7\) mutants exhibit defects in kidney function as embryos or early larvae. To test this possibility, we examined early larvae for signs of kidney stone formation using the vital dye alizarin red (53, 54). We detected kidney stones in \(trpm7\) mutants at 5 dpf (Fig. 6A) but not at 2–4 dpf (data not shown). Kidney stones were present in 57–94% of homozygous mutants per clutch but only 0–1.4% of wild-type siblings \((trpm7^+/+ : trpm7^+/+: \) mutants vs. wild-type: \(\chi^2 = 619; P < 0.0001)\). Although the incidence of kidney stone formation differed significantly between families \((\chi^2 = 27.1; P = 0.0001)\), it did not differ among mutant alleles that were lethal either at early larval stages \((trpm7^{124c2}, trpm7^{124c1})\) or viable \((trpm7^{50b58})\) (\(\chi^2 = 0.9; P = 0.6)\). By repeated imaging of individual larvae, we further showed that kidney stones transit through the pronephros, demonstrating their presence in the pronephric lumen rather than in the epithelium itself (Fig. 6B).

Kidney stones in humans most often comprise deposits of calcium oxalate or calcium phosphate (55, 56), and staining of \(trpm7\) mutant kidney stones with alizarin red and calcein (30, 57) suggests a similar composition in zebrafish. Given the effect \(stc1\) on calcium homeostasis, we asked whether elevated \(stc1\) might be responsible for kidney stone formation in \(trpm7\) mutants. Contrary to this expectation, however, heat-shocked, wild-type \(Tg(hsp70::stc1-IRES-EGFP)\) embryos failed to develop kidney stones, and morpholino knockdown of \(stc1\) in \(trpm7\) mutants failed to reduce kidney stone incidence \((\chi^2 = 0.01; P = 0.9)\).

We next considered the anti-hyperphosphatemic factor \(fgf23\) (58) as a candidate \(trpm7\)-dependent effector of kidney stone formation. Later larval skeletal defects in \(trpm7\) mutants are consistent with defects in both calcium and
phosphate homeostasis (30), and elevated fgf23 levels are associated with calcium-containing kidney stones in human patients with renal phosphate wasting and hypophosphatemia (59). We found that at 4–5 dpf, fgf23 is expressed principally in the CS and that trpm7 mutants exhibited a dramatic increase in transcript abundance compared with the wild type (Fig. 7A). To see whether increased fgf23 expression might contribute to kidney stone formation, we knocked down fgf23 using a splice-blocking morpholino, fgf23-sb1. The trpm7 mutants injected with fgf23-sb1 morpholino are not morphologically distinguishable from uninjected siblings. C, fgf23 knockdown in trpm7 mutants reduces the incidence of kidney stones compared with uninjected controls (χ² = 11.0; P < 0.001), whereas stc1 knockdown has no effect. Values are least squares means ± se, normalized to the incidence of kidney stones in uninjected trpm7 mutant siblings.

Discussion

Our study links mutation of trpm7 to stc1-dependent dysregulation of calcium and magnesium homeostasis and to fgf23-dependent early larval kidney stone formation. We demonstrated that trpm7 mutants exhibit reduced total calcium and reduced total magnesium at 3 and 4 dpf, respectively. By transgenic overexpression and morpholino oligonucleotide knockdown, we found that stc1 can modulate both calcium and magnesium levels in zebrafish; up-regulated stc1 in a wild-type background decreases total calcium and total magnesium, whereas inhibition of stc1 translation increases these cations to wild-type levels in trpm7 mutants. We also showed that kidney stones, previously detected in trpm7 mutants at later larval stages (30), are evident by 5 dpf in the pronephros. Finally, we demonstrated that trpm7 mutants overexpress fgf23, with the latter contributing to kidney stone formation.
whereas knockdown of $fgf23$ reduces the incidence of kidney stones in the mutant background.

This study demonstrates an association between $trpm7$ and physiological cation homeostasis in vivo. The reduced total magnesium and total calcium evident in $trpm7$ mutants is similar to the hypomagnesemia and hypocalcemia resulting from $Trpm6$ defects in mammals: decreased magnesium absorption likely via both intestine and kidney leads to parathyroid failure with attendant defects in calcium homeostasis leading to hypocalcemia (14, 28, 60–63). In zebrafish $trpm7$ mutants, however, reduced total calcium is evident at 3 dpf, whereas reduced total magnesium is not detectable until 4 dpf. This reversal in onset raises the possibility that reduced total magnesium is secondary to reduced total calcium in $trpm7$ mutants. An explanation for this difference may reside in overall differences in mammalian and teleost physiology and the relationships of these organisms with their environments as well as differences in specific molecular mediators that remain to be elucidated.

Our study revealed that $trpm7$ mutants exhibited higher levels of transcript for the anti-hypercalcermic factor $stc1$ and lower levels of calcium. Given that stanniocalcins are normally induced by high levels of serum calcium (50, 64), our data highlight the dysregulation of normal homeostatic mechanisms in the $trpm7$ mutant background. These observations further raise the possibility of a direct or indirect genetic interaction by which $trpm7$ regulates $stc1$. One possibility is that the kinase domain of $trpm7$ normally modulates the activity of Ca-sensing receptor (CaSR) within the CS. Consistent with this idea, a pharmacological activator of CaSR stimulates $stc1$ expression in salmon (65), and CaSR is expressed within the CS of flounder (49). Nevertheless, our data do not exclude the possibility that $trpm7$ effects on $stc1$ expression may be less direct and perhaps mediated through somatic tissues other than the CS. Indeed, it is also formally possible that increased $stc1$ expression in $trpm7$ mutants arises secondarily to decreased calcium and magnesium levels in this genetic background. Such an effect would run counter to the typical induction of $stc1$ by high calcium levels and would suggest a regulatory mechanism not previously described. The generation of transgenic lines to perturb $trpm7$ activity in a tissue-specific manner, and further studies to identify and characterize stanniocalcin 1 receptors and interactors will likely provide important insights into these questions.

An intriguing result from our study was the restoration of total magnesium levels in $trpm7$ mutants after morpholino knockdown of $stc1$. In contrast to calcium, stanniocalcin expression is not modulated by magnesium, and stanniocalcins are not known to directly influence magnesium homeostasis (64). Nevertheless, our results are concordant with a previous study, which showed that activation of CaSR led to increased levels of $stc1$ and decreases not only in serum calcium but in serum magnesium as well (49). The $trpm7$-independent correction of total magnesium levels we found implies a still-unknown mechanism for magnesium uptake. A potential mediator of such magnesium uptake in zebrafish would be claudin-16. In mammals, claudin-16 function in the loop of Henle is critical for passive, paracellular divalent cation reabsorption (66) and is distinct from the active, transcellular transport of magnesium by Trpm6/Trpm7 complexes. In zebrafish, we envision that claudin-16 may be regulated by $stc1$ and influences the paracellular transport of both magnesium and calcium in $trpm7$ mutants. The paracellular mechanism for claudin-16 transport is distinct from the transcellular mechanism of trpm6/trpm7 channels. Consequently, $stc1$-regulated claudin-16 activity could function as a $trpm7$-independent compensatory mechanism. Although a zebrafish ortholog of claudin-16 has not been identified, the presence of such genes in other fishes (67, 68) suggests one may yet be found.

Our study also provides insights into the development and physiological bases of kidney stone formation in $trpm7$ mutants. Consistent with a defect in phosphate homeostasis, we detected strongly increased expression of $fgf23$ in the CS, and we showed that knockdown of $fgf23$ reduces the incidence of kidney stone development. In humans, activating mutations in $FGF23$ cause autosomal dominant hypophosphatemic rickets, and tumor-produced fibroblast growth factor 23 ($FGF23$) results in osteomalacia (69–73), whereas mouse knockouts of $Fgf23$ develop hyperphosphatemia (74, 75). Alterations in $FGF23$ signaling are also associated with several other pathologies including chronic kidney disease (58) and the presence of calcium-containing kidney stones in patients with hypophosphatemia and urinary phosphate wasting (59). In mammals, $Fgf23$ is produced primarily by bone and affects phosphate regulation by signaling through $FGF$ receptor 1c and Klotho in the kidney (58, 76–78). Our findings suggest that CS-derived $fgf23$ is an early regulator of phosphate homeostasis in zebrafish, although low absolute levels of phosphate precluded the direct detection of differences in phosphate levels between wild-type and $trpm7$ mutants. We speculate that kidney stone formation in the pronephric tubules may result from a localized decrease in the reabsorption of calcium, owing to reduced $trpm7$ channel activity, leading to increased precipitation of calcium phosphate. That $fgf23$ knockdown did not eliminate kidney stone formation completely may reflect limited morpholino efficacy at 5 dpf (79) or a dependence on other factors. One candidate for such an ef-
fect is stc1. Although we did not observe a reduction in kidney stone incidence after injecting stc1 morpholino, either singly or in combination with fgf23 morpholino, and stc1 overexpression did not induce kidney stone formation in wild-type embryos, these outcomes may reflect limited perdurance of morpholinos as well as homeostatic regulation leading to resistance in the wild type that is absent or diminished in trpm7 mutants. Indeed, stc1 has been associated with phosphate homeostasis previously (49, 80). The isolation of stc1 and fgf23 mutants by targeted resequencing of mutagenized genomes or other approaches (81) would greatly facilitate the testing of homoeostatic roles for these factors at stages not amenable to approaches (81) would greatly facilitate the testing of homoeostatic roles for these factors at stages not amenable to these factors may contribute to later-stage trpm7 mutant defects in growth and bone development as well (30). Studies of mammalian stanniocalcins and fgf23 have linked each to skeletal and growth defects (50, 72, 73, 82–86) similar to those found in trpm7 mutants at later larval stages. Moreover, a mouse mutant for the trpm7-related gene transient receptor potential vanilloid 5 (Trpv5) exhibits urinary calcium and phosphate wasting as well as skeletal defects (87, 88), and mice doubly mutant for Trpv5 and CaSR have growth retardation and develop kidney stones (89), much like zebrafish trpm7 mutants. It will be interesting to identify the other parallels between molecular regulators of these phenotypes in teleost and mammalian models as well as in human disease.

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