LRRC52 (leucine-rich-repeat-containing protein 52), a testis-specific auxiliary subunit of the alkali-activated Slo3 channel

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Edited by Richard W. Aldrich, University of Texas, Austin, TX, and approved October 20, 2011 (received for review July 8, 2011)

KSper, a pH-dependent K+ current in mouse spermatozoa that is critical for fertility, is activated by alkalization in the range of pH 6.4–7.2 at membrane potentials between −50 and 0 mV. Although the KSper pore-forming subunit is encoded by the Slo3 gene, heterologously expressed Slo3 channels are largely closed at potentials negative to 0 mV at physiological pH. Here we identify a Slo3-associated protein, LRRC52 (leucine-rich-repeat-containing 52), that shifts Slo3 gating into a range of voltages and pH values similar to that producing KSper current activation. Messenger for LRRC52, a homolog of the Slo1-modifying LRRC26 protein, is enriched in testis relative to other homologous LRRC subunits and is developmentally regulated in concert with that for Slo3. LRRC52 protein is detected only in testis. It is markedly diminished from Slo3−/− testis and completely absent from Slo3−/− sperm, indicating that LRRC52 expression is critically dependent on the presence of Slo3. We also examined the ability of other LRRC subunits homologous to LRRC26 and LRRC52 to modify Slo3 currents. Although both LRRC26 and LRRC52 are able to modify Slo3 function, LRRC52 is the stronger modifier of Slo3 function. Effects of other related subunits were weaker or absent. We propose that LRRC52 is a testis-enriched Slo3 auxiliary subunit that helps define the specific alkalization dependence of KSper activation. Together, LRRC52 and LRRC26 define a new family of auxiliary subunits capable of critically modifying the gating behavior of Slo family channels.

The pore-forming subunit of the sperm-specific alkalization-activated K+ current, termed KSper (1), is encoded by the Slo3 gene (2). Deletion of the Slo3 gene abolishes pH-sensitive K+ current in testicular (3) and epididymal sperm (4) and results in infertile male mice (3, 4). However, the properties of Slo3 channels studied in heterologous systems (5, 6) differ from those of native KSper currents (1, 4). Whereas native KSper current is appreciably activated at pH 7.0 at potentials negative to 0 mV (1), Slo3 current studied in Xenopus oocytes exhibits little activation at pH 7.0 even up to +100 mV (4). This suggests that some unidentified regulatory components of Slo3 channels may contribute to native KSper current.

Recently, a new regulatory subunit of Slo1 BK-type channels, LRRC26, has been shown to markedly shift gating of Slo1, even in the absence of Ca2+, in prostate tumor cells (7). LRRC26 is one of a large number of LRRC (leucine-rich-repeat-containing) proteins, of which there are multiple superfamilies (8). LRRC26 belongs to an extracellular leucine-rich-repeat-only (Elron) cluster, which includes five other LRRC proteins: LRRC38, LRRC52, LRRC55, LRTM1 and LRTM2. Elron cluster members are all predicted to contain a single transmembrane segment with an N-terminal signal peptide resulting in extracellular localization of the LRR domain and a short cytoplasmic C-terminal tail containing a short stretch of acidic residues. Despite shared organization of leucine-rich-repeats among Elron family LRRC proteins, additional amino acid homology is modest. However, their common structural organization suggests they may share structurally similar interaction partners. Here we have evaluated the possibility that a testis-specific, Elron-family LRRC protein may interact with the Slo1 homolog, Slo3.

Our results establish that, of the Elron family members, LRRC52 protein is a candidate for a testis-specific, Slo3 interacting partner. The lrcc52 message and its encoded protein are enriched in testis. The developmental time-course of lrcc52 expression mirrors that of Slo3. Currents arising from coexpression of Slo3 and LRRC52 in Xenopus oocytes exhibit pH- and voltage-dependence more similar to native KSper currents than for Slo3 alone. Furthermore, deletion of Slo3 strikingly decreases LRRC52 protein abundance in mouse testis and abolishes the presence of LRRC52 in sperm. We propose that LRRC52 is a testis-specific, Slo3 auxiliary subunit that is essential for the activation of KSper current at physiological membrane potentials and pH. The results support the possibility that at least some Elron proteins may constitute a family of unique interacting partners for Slo family channels.

Results

**lrcc52 is the Most Abundantly Expressed Elron Cluster Gene in Mouse Testis.** The Slo3 gene is selectively expressed in mouse testis (2, 4). To assess whether an Elron family LRRC protein (8) might be a Slo3 channel auxiliary subunit, we determined the expression in mouse testis of the six Elron cluster members along with two testis-expressed non-Elron lrc messages, lrc6 and lrc28 (Fig. 1A). Of the Elron genes, only lrcc52 had absolute expression levels comparable to Slo3, while mRNA abundance of other Elron members was at least 10-fold lower than for Slo3 and lrcc52. Consistent with the biogps database (http://biogps.gnf.org), messages for lrc6 and lrc28 were also abundant in testis, but both define proteins without homology with Elron subunits (8). We also compared the time course of lrcc52 and Slo3 gene expression in testis from postpartum day 3 to day 150. Developmental expression of kcnmb4, which encodes the BK β4 auxiliary subunit, was also defined, since β4 was proposed as a potential interacting partner of Slo3 in testis (9). Slo1 was included as a control. The results revealed that lrcc52 and Slo3 genes share very similar expression time courses, being very low before P25, exhibiting pronounced enhancement between P25 and P30, and then persisting at a constant high level from P30 through P150 (Fig. 1B). In contrast, Slo1 message levels remained low through all 150 d postpartum while kcnmb4 gene expression increased modestly as early as P20.

We next tested the ability of Slo3 and HA-tagged LRRC52 subunits to coassemble, using coimmunoprecipitation (Co-IP)
Fig. 1. LRRC52 is a candidate auxiliary subunit of Slo3. (A) Relative abundance of message for all Elron-family LRRC subunits (LRTM1, LRTM2, LRRC26, LRRC38, LRRC52, and LRCC55) along with two other testis-associated LRRC proteins (LRRC6 and LRRC28) was determined using RT-qPCR. All estimates were normalized to the level of β-actin message. All determinations were from three separate RNA preparations, each run in triplicate. (B) Message for Slo3, lrcc52, slo1, and kcnmb4 were determined from mouse testis for different days postpartum. Each point is the mean of estimates from three different preparations, each run in triplicate. (C) Slo3 and HA-tagged LRRC52 coassemble in Xenopus oocytes. Batches of oocytes were injected with cRNA for either Slo3 alone, LRRC52-HA alone, or both together. The left five lanes show Western blotting of total oocyte proteins and staining with anti-HA antibody. HA-tagged LRRC52 protein is observed at ~47-55 kDa when glycosylated (PNGase F+) and at ~36 kDa following deglycosylation (PNGase F−). In the four right lanes, oocyte proteins were first immunoprecipitated with anti-Slo3. Anti-Slo3 only pulls down LRRC52-HA in the lane corresponding to coexpression of Slo3 and LRRC52. (D) Oocytes were separately incubated with or without biotin. Lanes 1–3 show anti-HA-stained labeled proteins from total oocyte proteins for oocytes injected with Slo3 alone, Slo3+LRRC52-HA, and LRRC52-HA alone. Lanes 4–6 show biotinylated proteins pulled down by streptavidin for sets of oocytes injected with Slo3 alone (lane 4), Slo3+LRRC52-HA (lane 5), and LRRC52-HA alone (lane 6), after staining with anti-HA. The right three lanes show blotting with anti-HA for proteins pulled down by streptavidin from nonbiotin labeled oocytes. (E) A polyclonal LRRC52 Ab identifies the LRRC52-HA protein in oocytes. To confirm validity of a polyclonal LRRC52 Ab, Slo3 was expressed with and without LRRC52-HA in oocytes. Oocytes expressing Slo3 alone exhibit minimal nonspecific bands either with or without glycanase treatment (lanes 1 and 2). For oocytes expressing both Slo3 and LRRC52-HA, the anti-LRRC52 Ab identified bands identical to those revealed by anti-HA Ab under both glycosylated (lane 3) and deglycosylated (lane 4) conditions. (F) LRRC52 protein is present in mouse testis. Anti-LRRC52 Ab labels multiple bands in total testis membrane proteins (lanes 1 and 2). However, the Ab identifies a 45-kDa band in glycosylated proteins (lane 1), which is absent following glycanase treatment (lane 2), with an increased density at ~33 kDa. When testis membrane proteins are first immunoprecipitated with anti-LRRC52 antibody, the anti-LRRC52 Ab pulls down a prominent glycosylated band at 45 kDa (lane 3) and a strong band at ~33 kDa (with a minor 24-kDa band) following deglycosylation (lane 4). IP with IP beads alone lacking the LRRC52 Ab identified a nonspecific band at 26 kDa (lane 5) also seen in all other lanes.
close to the predicted molecular weight of LRRC52 (with signal peptide cleaved). Following enrichment of candidate LRRC52 protein by sequential immunoprecipitation and Western blot with anti-LRRC52 antibody, the 45-kDa protein became the only prominent band in the IP products (Fig. 1F). As expected, with PNGase F treatment, the band shifted to ~33 kDa with a weak secondary band at 24 kDa. The glycosylated 45-kDa protein in native testis proteins has four properties consistent with LRRC52: it is a membrane protein, it is a glycosylated protein, following deglycosylation it runs with a molecular weight appropriate for LRRC52, and it is recognized by an Ab that recognizes heterologously expressed LRRC52. It should also be noted that the glycosylated molecular weight of the testis LRRC52 corresponds closely to that of the 47-kDa smaller form of LRRC52 expressed in oocytes and which selectively associates with Slo3. We conclude that both lrrc52 message and LRRC52 protein are expressed in mouse testis and LRRC52 is a candidate functional partner of Slo3 channel in testis. Although coassembly between Slo3 and LRRC52 was readily detected in oocytes (Fig. 1C), we were unable to obtain successful Co-IP of Slo3 and LRRC52 in testis proteins, despite the use of multiple solubilization conditions.

**LRRC52 Shifts Slo3 Gating to More Negative Potentials at a Given pH.**

We next tested the ability of LRRC52 to modify Slo3 gating behavior using heterologous expression of LRRC52 with Slo3 in *Xenopus* oocytes. Currents arising from Slo3 channels with or without LRRC52 were examined over voltages from −120 to +240 mV at pH values from 6.0 to 9.0 (Fig. 2A and Fig. S1). Conductance-voltage (GV) relationships were determined for Slo3 and LRRC52 currents and were normalized to the maximum values recorded at pH 9.0 and +240 mV (Fig. 2B). Similarly, GV curves for Slo3 alone were generated at pH up to 9.0. Compared with GV curves from Slo3 alone, the normalized G–V relationship from oocytes with Slo3+LRRC52 exhibited a substantial leftward shift and Slo3+LRRC52 currents were activated at pH 7.0 at membrane potentials negative to 0 mV (Fig. 2B and Fig. S1). At +240 mV, pH 8.5 produces near maximal activation of Slo3 conductance (5), although single Slo3 channels open to relatively modest Po (∼0.3) at these conditions (6). Examination of single Slo3+LRRC52 channels at +240 mV at pH 8.0 (where additional increases in pH have no effect) revealed similar Po estimates near 0.3 (Fig. S2), which was also confirmed in macroscopic current patches using analysis of current variance. This similarity between Slo3 and Slo3+LRRC52 in single channel Po at +240 and a at pH producing near maximal activation requires that the enhancement of relative conductance at lower pH and voltages observed for Slo3+LRRC52 results from a shift in channel gating properties and not an increase in channel number or maximal Po. Because of similarity in single channel Po at conditions approaching maximal activation, this allowed renormalization of the GV curves in terms of absolute conductance values (Fig. S3). Evaluation of such GV curves in terms of the Horrigan-Aldrich model used to describe allosteric gating of both Slo1 (10) and Slo3 (5) suggests that the association of LRRC52 with Slo3 produces strong effects on the constant describing the channel closing equilibrium (Fig. S3), with smaller effects on other allosteric constants (see SI Materials and Methods for details).

**KSPer currents in native sperm** are typically studied with cytosolic solutions containing 15 mM Na+ and 3 mM Mg2+ (1). These identical solutions result in voltage-dependent inhibition of Slo3 channels (4) thereby increasing relative Slo3 current at more negative potentials, but still insufficient to explain the apparent KSPer activation at negative pH and voltage. Here, when Slo3 and Slo3+LRRC52 currents were compared at pH 8.0 with cytosolic salines containing Na+ and Mg2+ (1), the Slo3+LRRC2 conductance at pH 8.0 exhibited a dependence on voltage that approached that of native clofilium-isolated KSPer current in mouse sperm (Fig. 2C). However, although Slo3+LRRC52 better approximates native KSPer current than Slo3 alone, the combination of Slo3+LRRC52 does not appear to fully account for the activation at low pH and negative voltages that is observed for native KSPer current.

**Deletions of the Slo3 Gene Abolishes LRRC52 Protein in Cauda Epididymal Sperm.** LRRC52, Slo3, and KCNMB4 protein levels were compared in WT, Slo3−/− and kcnmb4−/− mice using Western blotting, both in total testis membrane proteins and in immunoprecipitated proteins. Compared with WT testis, LRRC52 protein levels were markedly reduced in Slo3−/− testis but normal in kcnmb4−/− testis (Fig. 3A). Although the anti-LRRC52 Ab recognizes multiple nonspecific bands in testis membrane protein samples, IP with anti-LRRC52 resolved the specific LRRC52 protein band allowing comparison of relative protein abundance in the three mouse strains (Fig. 3B). The density of the LRRC52 band in each mouse strain (WT, Slo3−/−; kcnmb4−/−) was quantified and normalized to the WT value. For three independent experiments, the ratios of the LRRC52 levels were, for Slo3−/−: WT, 0.17 ± 0.05 and, for kcnmb4−/−: WT, 1.09 ± 0.05. In contrast, the ratio of KCNMB4 protein was, for Slo3−/−:WT, 1.19 ± 0.33.

Slo3, LRRC52, KCNMB4 protein levels in cauda epididymal sperm were also compared in WT and Slo3−/− animals. The polyclonal Slo3 Ab identified an ~120 kDa in WT caudal epididymal sperm proteins, but not from Slo3−/− (Fig. 3C). Using the anti-LRRC52 antibody, WT sperm proteins contained a 45-kDa protein band which shifted to bands of 33 kDa and a weak 24 kDa following deglycosylation (Fig. 3C). In similarly prepared proteins from Slo3−/− sperm, there was no detectable band either
in untreated or PNGase F-treated samples. No KCNMB4 protein could be detected in sperm proteins under conditions which readily identified the other two proteins, and which had revealed KCNMB4 protein in testis membrane proteins. RT-qPCR confirmed that lrrc52 message was identical in WT and Slo3−/− testis (Fig. 3D), suggesting that transcriptional regulation is not responsible for loss of LRRC52 protein in Slo3−/− sperm. Gene expression in all negative controls (−RT) was undetectable. The decreased LRRC52 protein in Slo3−/− testis suggests that Slo3 protein may be required to stabilize LRRC52 in native tissue and that loss of LRRC52 occurs when it is not properly assembled with Slo3. The apparently complete absence of LRRC52 in Slo3 null sperm indicates that the presence of LRRC52 in mature sperm depends entirely on the presence of Slo3.

**LRRC52 Is Selectively Enriched in Testis.** We next asked whether LRRC52 exhibits a tissue specificity appropriate to a selective role as a Slo3-associated protein. mRNA abundance of lrrc52 was determined for 18 mouse tissues (Fig. 4A). The lrrc52 message in testis was at least 10-fold higher than for any other tested tissue. Possible weak expression was also observed in kidney, ventricle, spinal cord and skeletal muscle. This result is consistent with EST profiles in the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?ugisid=Mm.159799) which reveals weak lrrc52 expression primarily in mouse testis and kidney. We also compared the relative abundance of lrrc26 and lrrc55 message among the same set of tissues (Fig. S4) and found that both exhibited a much broader distribution than observed for lrrc52.

Using the anti-LRRC52 Ab, the relative abundance of LRRC52 protein was compared in Western blots among different tissues, including kidney, heart, skeletal muscle and spinal cord. Cerebral cortex was included as a negative control. At comparable levels of protein loading, no LRRC52 protein was identified in any of the tested tissues except testis (Fig. 4B), indicating that LRRC52 protein may be restrictively expressed in testis, similar to Slo3 protein. Bands that appeared in Western blots in cortex and kidney could be excluded as candidate LRRC52 bands, since they did not shift to the size expected for deglycosylated LRRC52 following treatment with PNGase F.

**Other Efon Cluster Members also Interact with Slo3 in Oocytes but Are Less Effective at Shifting Slo3 Channel Gating.** We also tested the ability of other LRRC subunits to influence Slo3 activation. Although no other LRRC subunit produced the pronounced leftward shift in Slo3 activation observed with LRRC52, LRRC26

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**Fig. 3.** LRRC52 is absent in Slo3 null sperm. (A) Protein levels of LRRC52, Slo3 and KCNMB4 were compared in WT, Slo3−/− and kcnmb−/− mouse testis. Fifty micrograms of testis membrane proteins from each mouse strain was loaded in each well to examine target proteins by Western blotting. On the bottom, anti-α-tubulin was used to document comparable loading amounts from each sample. In the first row, proteins were blotted with anti-LRRC52 antibody revealing reduced LRRC52 protein in Slo3−/− testis. In the second row, anti-Slo3 Ab labeled multiple bands, but a band of ~120 kDa was absent in the Slo3−/− testis. In the third row, anti-KCNMB4 Ab labeled a 28-kDa band in WT and Slo3−/− testis proteins, but not in proteins from kcnmb−/− mice. (B) Total testis membrane protein samples as in A were first subjected to a round of immunoprecipitation. In the first row, IP with anti-LRRC52 Ab enriched the 45-kDa LRRC52 protein in WT and kcnmb−/− testis. This band is reduced to less than 20% in the Slo3−/− proteins. In the second row, IP with anti-Slo3 Ab resulted in enrichment of the Slo3 band in WT and kcnmb−/− testis proteins, but a complete absence of signal from Slo3−/− proteins. In the third row, IP with anti-KCNMB4 Ab enriched the KCNMB4 band in WT and Slo3−/− testis proteins, with no band in the kcnmb−/− proteins. For separation of IP products, anti-LRRC52 IP product (prepared from 0.3 mg testis membrane proteins of each mouse strain), anti-Slo3 IP product (prepared from 0.25-mg testis membrane proteins) and anti-KCNMB4 IP product (prepared from 1-mg testis membrane proteins) were respectively loaded in the lanes of SDS/PAGE gel. Protein abundance for target protein bands was analyzed with the use of ImageJ software and values were averaged from three independent experiments. (C) Slo3, LRRC52, and p4 were compared in proteins isolated from caudal epididymal sperm from WT and Slo3−/− mice. One microgram of sperm protein, prepared from about 2 × 10⁷ cauda epididymal sperm, was loaded in each well for Western blotting. The experiment was repeated thrice from separate animals. (Top) Samples were blotted with anti-Slo3 polyclonal Ab, revealing a band in WT samples at about 120 kDa. (Middle) Samples show robust presence of LRRC52 in WT, but not Slo3−/− samples. (Bottom) Samples show the complete absence of detectable p4 protein. (D) mRNA abundances of lrrc52, slo3 and kcnmb4 were compared in WT and Slo3−/− testis by RT-qPCR. Message levels were normalized to the level of β-actin message. All determinations were from three separate animals (filled circles), each run in triplicate.

**Fig. 4.** LRRC52 protein is enriched in testis. (A) lrrc52 message levels were examined in a variety of mouse tissues by RT-qPCR and normalized to the level of β-actin message. All determinations were from 3 separate RNA preparations each run in triplicate. (B) Following initial IP with anti-LRRC52 Ab, membrane proteins from several mouse tissues were blotted with anti-LRRC52 Ab for both glycosylated (−) and deglycosylated (+) conditions. In each lane, the IP products loaded in the gel were produced from 0.5-mg initial membrane proteins from each tissue.
and LRRC38 produced modest alterations in the resulting currents (Fig. S5A). Following normalization of NV curves at pH 8.0, GV curves both for Slo3+LRRC26 and Slo3+LRRC38 exhibited less inhibition at pH 7.6 and pH 7.0 than for Slo3 alone. However, in contrast to LRRC52, LRRC26 and LRRC38 failed to enhance activation of Slo3 currents at membrane potentials negative to 0 mV. The other tested Elron subunits, LRRC55, LRTM1, and LRTM2 did not alter Slo3 gating (Fig. S5B). Similarly, the non-Elron subunits, LRRC6 and LRRC28, had no effect (Fig. S6).

The absence of effects of some LRRC subunits on Slo3 currents might arise either from lack of subunit expression or failure to assemble with Slo3. The ability of HA-tagged LRRCs to express and coassemble with Slo3 was therefore examined. For each LRRC subunit, we determined: (i) HA-tagged LRRC subunit expression in total oocyte protein (Fig. S5C), (ii) the ability of anti-Slo3 Ab to pull down HA-tagged LRRC subunits from total oocyte proteins (Fig. S5D), and (iii) Slo3 subunit expression in the same batch of oocyte proteins (Fig. S5E). Because all eight tested LRRC proteins except LRRC28 are predicted to be glycoproteins (Table S1), the observed molecular weights (Fig. S5C) were somewhat higher than those predicted directly from their amino acid sequences. LRTM1, LRTM2, LRRC26, LRRC52, LRRC55, and LRRC6 were all abundantly expressed in oocytes (Fig. S5C), but only LRTM2, LRRC26 and LRRC52 showed strongly positive Co-IP with Slo3 (Fig. S5D). LRRC38 and LRRC55 exhibited only weak coassembly with Slo3. Co-IP between Slo3 protein and LRTM1, LRRC6 or LRRC28 appeared to be negative. Thus, five of the Elron family members, LRTM2, LRRC26, LRRC38, LRRC52 and LRRC55 were competent to interact with Slo3 protein. LRTM1, if it can coassemble with Slo3, appeared to do so much more weakly than for all of the other Elron members. For the non-Elron proteins, LRRC6 and LRRC28, coassembly with Slo3 was not observed, although expression of LRRC28 in oocytes was rather low. The Co-IP data are consistent with the functional results that LRRC52, LRRC26 and LRRC38 interact with Slo3 channels in oocytes.

**Discussion**

Previous work has demonstrated that Slo3 is an ion channel pore-forming subunit responsible for alkalization-activated KSper current in corpus epididymal sperm (4). However, a critical difference between sperm KSper current and heterologously expressed Slo3 current is that, unlike KSper, Slo3 channel was not appreciably activated at potentials negative to 0 mV and at pH less than 7.2 (5). This suggested that additional regulatory mechanisms are required for Slo3 activation. Indeed, previous work has demonstrated that Slo3 is an ion channel pore-forming subunit responsible for alkalization-activated KSper channel properties (11, 12). Native CatSper channels also appear to arise from multiple distinct subunits, presumably up to four pore-forming CatSper subunits, CatSper1-4 (12), while also requiring auxiliary β-, γ-, and δ-subunits (13–15). For native CatSper channels, KO of any of the four CatSper1-4 subunits results in male infertility and, similarly, KO of CatSper δ also produces infertile male mice. Our observation that LRRC52 subunits are totally absent in Slo3−/− sperm is similar to the effects of KO of various CatSper subunits on expression of their interacting partners. For example, in CatSper1−/− mice, CatSper δ (14) and CatSper β (13) protein is absent from sperm. Such results are taken to support the idea that these proteins are intimate partners of CatSper1-4 in the formation of the functional CatSper channel. Similarly, the absence of LRRC52 protein in sperm from Slo3−/− mice supports the idea that LRRC52 is intimately involved with Slo3 subunits and KSper channels.

What might be the expected consequences of LRRC52 deletion? We imagine two possibilities, both of which would result in infertile male mice. First, lrc52 KO may result in sperm with an alkalization-activated current similar to the Slo3 current recorded in oocytes. Based on the weak activation of Slo3 under conditions normally present in sperm, we would therefore expect that lrc52−/− male would be infertile. Alternatively, in native sperm, it may be the case that, in the absence of LRRC52 protein, Slo3 itself may not express, thereby also resulting in reproductive abnormalities. We predict that LRRC52 is an essential component of normal mouse male reproductive function and that KO of the lrc52 gene should essentially mimic all of the deficits in sperm function associated with Slo3 KO. Might there remain other unidentified participants in native KSper channels? The properties of Slo3+LRRC52 currents may still not fully account for activation of KSper at the lowest pH we have examined. As such, we cannot exclude that other components of KSper remain to be identified.

Another important aspect of this work is that LRRC52 is now the second Elron subfamily member found to interact with Slo family pore-forming subunits. In the first case, LRRC26 was shown to produce a strong negative shift in Slo1 gating in prostate cancer cells and LRRC26 may participate in Slo1 channels in other tissues (7). To our knowledge nothing is known about potential functions of the other Elron members, LRTM1, LRTM2, LRRC38, and LRRC55. Our results establish that at least some of these subunits are competent to associate with Slo3 and perhaps Slo1. It is natural to wonder whether some Elron subunits might play a role in the regulation of other ion channels, perhaps the less-studied Na+ regulated Slo2.1 and Slo2.2 channels (16, 17) that share some general structural similarity with Slo1 and Slo3 (18).

Taken together, this work suggests that LRRC52 is a Slo3 channel auxiliary subunit in mouse sperm critical for definition of the alkalization-dependence of native KSper current. Furthermore, the results identify Elron LRRC proteins, or at least a subset of them, as a family of unique auxiliary subunits of Slo family pore-forming K+ channels.

**Materials and Methods**

**Animal Husbandry and Procedures.** All animal husbandry and experimental procedures were approved by and performed in accordance with guidelines of the Washington University School of Medicine Animal Care and Use Committee.

**Preparation of Constructs.** LRRC Elron family subunits, along with LRRC6 and LRCC28 (Table S2), were subcloned into oocyte expression vector pXMX.
using PCR on EST clones obtained from Open Biosystems (mLRRC26, B1108309; mLRRC38, BC129963; mLRRC52, CB953430; hLRRC55, BC150572; mLRRTM2, B029902; hLRRTM1, BC428777; hLRRC6, BC046277; mLRRC21, BU946312). The HA- and His-tagged constructs were generated by over-lapping PCR to insert HA- and His-tags (GGYPVDVPYAGHGHHHHHHGGG) into the c-termini of LRRC proteins (mLRRC26 at position 331 between EDA and GSP; mLRRC38 at position 425 between CAP and NKD; mLRRC52 at position 293 between SRF and ANQ; hLRRC55 at position 333 between RWS and KAS; mLRRTM1 at position 345 between EKM and GSK; hLRRTM2 at position 354 between LMG and DPE; mLRRC6 at position 497 between STI and VQE; mLRRC28 at position 359 between TQC and LRT). cDNAs were in vitro synthesized after being linearized with MuIi1.

Electrophysiology. Recordings of macroscopic and single channel currents used inside-out patches from constructs expressed in Xenopus oocytes with additional details provided in SI Materials and Methods.

RNA Extraction and Quantitative RT-PCR. Total RNA from mouse tissue was isolated using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer’s recommendations and then treated with the DNA-free Kit (AM1906; Applied Biosystems) to remove genome DNA. cDNA was synthesized using the Retroscript Kit (AM1710; Applied Biosystems). Roche random hexamer was applied in reverse transcription. For the negative control groups, all components of the reverse transcription reaction mixture were replaced with water. The reaction mixtures, Real-Time PCR was performed with specific primers (Table S2) and Power SYBR Green PCR Master Mix (Applied Biosystems) under reaction conditions identical to that described previously (9). The slopes of primer efficiency equation for primer pairs used in this study were between −3.1 and −3.6, giving reaction efficiencies between 90% and 110%, which are typically acceptable for quantitative PCR assay. Message levels were normalized to the abundance of β-actin message in data analysis. In the Slo3−/− mice, exon 27 was deleted. Therefore, primers were designed to detect residual Slo3 message likely to be expressed in the Slo3−/− mice (4).

Protein Preparation. Protein preparation methods from mouse tissues, Xenopus oocytes and sperm follow standard procedures and are described in the SI Materials and Methods.

Immunoprecipitation and Western Blotting. The rabbit anti-LRRC52 antibody (ProSci) was raised to an epitope near the LRRC52 C terminus corresponding to residues NALRTSSGDDDETGSRFANQ. For mouse tissue, 12 mg of protein preparation was applied for IP with monoclonal anti-Slo3 antibody (3 μg/mL, ProSci), rabbit anti-LRRC52 antibody (3.75 μg/mL; ProSci), rabbit anti-KCNMB4 antibody (4 μg/mL; Alomone Lab) and mouse anti-α-tubulin antibody (1 μg/mL; Invitrogen). Secondary antibody was Trueblot anti-mouse IgG-HRP (1:1,000) and Trueblot anti-rabbit IgG-HRP (1:1,000) from eBioscience. The density of protein bands was quantified by the use of ImageJ.

Protein Deglycosylation. N-Glycanase PNGase F (Prozyme) was used to remove the N-linked glycosylated sugars. Thirty-five microliters protein or IP beads suspension was sequentially mixed with 10 μL 5X Reaction Buffer (Prozyme), 2.5 μL Denaturation Solution (Prozyme), 2.5 μL Detergent Solution (Prozyme) and 1 μL PNGase F (2 units/mL) and then incubated at 37 °C for 1 h. For PNGase F (−) control, every component but the glycanase was included in the reaction.

Cell Surface Biotinylation. EZ Link Sulfo NHS-SS-biotin (Pierce) was used to determine the expression of HA-tagged LRRC52 on the plasma membrane of oocytes. 50 oocytes were injected with appropriate amounts of dRNA of Slo3, Ircr52-HA or Slo3+Ircr52-HA and incubated for 6 d in a 16 °C incubator. Before biotin labeling, oocytes were equally divided into two Petri dishes and washed once for 10 min with 4 °C ND96 oocyte culture solution. 25 oocytes in biotin(+) dish were incubated in 5 mL NHS-SS-biotin solution (0.5 mg/mL in ND96 solution) at room temperature for 30 min and then at 4 °C for 1.5 h to label the cell surface proteins. As negative controls, 25 oocytes in biotin(−) dish were incubated in 5 mL ND96 solution without biotin. After labeling, the oocytes were washed twice with 5 mL quenching buffer (50 mM glycine in PBS) to scavenge the unreacted biotin, followed by 10-min rinse with 5 mL ND96 solution four times. Total proteins were prepared from the biotin(+) and biotin(−) oocytes, mixed with 35 μL Streptavidin agarose (Prozyme) and then incubated for overnight in 4 °C cold room. Agarose beads were then collected by a brief spin and washed thrice with 1 mL lysis buffer (containing 1% Triton X-100). Biotin-labeled cell surface proteins were eluted from the streptavidin agarose with 70 μL SDS loading buffer.

ACKNOWLEDGMENTS. We thank Robert Brenner (University of Texas Health Science Center at San Antonio) for providing the kcmrb+− mice and Dr. Jiusheng Yan for his suggestions and interest during this project. Work was supported in part by National Institutes of Health (NIH) Grant GM081748 (to C.L.). The monoclonal antibody N2216 was developed by or obtained from the University of California Davis/NIH NeuroMab Facility, supported by NIH Grant U24NS050606, and maintained by the Department of Neurobiology, Physiology, and Behavior, College of Biological Sciences, University of California Davis.

Supporting Information

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SI Materials and Methods

Electrophysiology. For functional studies of channels expressed in *Xenopus* oocytes, leucine-rich repeat-containing (LRRC) RNA was typically injected at a 1:1 ratio by weight with Slo3. Gigaohm seals were formed in frog Ringer (115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl$_2$•2H$_2$O, 10 mM Hepes, pH 7.4). The pipette/extracellular solution was 140 mM K-methanesulfonate, 20 mM KOH, 10 mM Hepes, 2 mM MgCl$_2$, pH 7.0. Preparation of solutions of different pH was as described (1), with 140 mM methanesulfonate, 20 mM KOH, 10 mM Hepes, 5 mM EGTA, and pH adjusted to nominal values. In some cases, 3 mM MgCl$_2$ and 15 mM NaCl was added to approximate the cytosolic calcium concentrations used in sperm recordings (2). Solutions were applied directly to patches via large bore pipette tip containing six independent solutions lines. Independent control of each line allowed switching between different test solutions with solution exchange occurring within less than 1 s. Current waveforms were analyzed either with Clampfit software or developed within this laboratory. All error bars correspond to SEM. All experiments were at room temperature (~22–25 °C). Chemicals were obtained from Sigma. Fitting of the H-A model to GV curves was done with custom software allowing simultaneous fitting of all conductance estimates over all pH and voltages.

Estimation of Allosteric Constants. GV curves were fit to the Horrigan-Aldrich formulation (3) with constants defined to reflect the idea that protonation of Slo3 negatively couples to other allosteric equilibria (1, 4). The general equation is:

$$Po(V, [H^+]) = \frac{1}{1 + \frac{[H^+]}{K_H} + [J + K + JKE]}^{L}$$

Here, $L = \frac{H(0)exp(z_1V/kT)}{1 + \frac{[H^+]}{K_H} + [J + K + JKE]}^{L}$ and represents the voltage-sensor equilibrium. $K = [H^+]^{K_H}$ where $K_H$ is the $p$K for protonatable sites. $D$ reflects the coupling of voltage-sensor equilibrium ($J$) to $L$. $C$ represents the inverse coupling between protonation and $L$. $E$ reflects coupling of protonation to voltage-sensing, while $z_1$ and $z_L$ reflect the charge associated with voltage-sensor movement and the C-O conformational change, respectively.

Macroscopic GV curves were normalized to absolute conductance values based on estimates of single channel Po for Slo3 (4) and Slo3+LRRC52 (Fig. S2) at near maximal activation conditions. Single channel Po at pH 8.5 and +240 mV for Slo3 was not obviously different from that for pH 8.0 and +240 mV for Slo3+LRRC52. Slo3+LRRC52 macroscopic currents exhibit clear saturation in activation over pH 8.0–9.0, while Slo3 alone exhibits only modest increases from pH 8.5–9.0. Previous description of allosteric gating of Slo3 depend on some assumptions, which were also used here. First, for Slo3, there is no measurable conductance at the lowest pH, thus precluding direct estimates of the constant C. As such, C was assumed to be identical to that estimated for Slo1. Second, no direct estimate of constant E was made so the value defined for Slo1 was also used. Thus, in evaluating Slo3+LRRC52, both C and E were constrained to these previous values. We also assumed that $z_L$ and $z_E$ were identical for Slo3 and Slo3+LRRC52. These voltage-dependencies are expected to involve movement during gating of charged residues within the electric-field of the membrane, which we assume to be similar for both channel complexes. Although these assumptions will require additional tests in the future, they provide a basis for an initial comparison of the effect of LRRC52 on Slo3 gating.

Preparation of Membrane Proteins from Mouse Tissues. One gram of tissue from 8- to 12-wk-old male mice was homogenized on ice with Teflon-glass pestle in 10 mL 0.32 M sucrose in PBS solution, including 100 μL 1 M PMSF in acetone and 100 μL Protease Inhibitor Mixture (Sigma). Specially, kidney, heart and skeletal muscle were frozen in liquid nitrogen, smashed into powder by liquid nitrogen pulverizer before being homogenized in Teflon-glass Pestle. The tissue suspension was centrifuged at 300 × g for 10 min at 4 °C and the supernatant was then transferred to an ultra-speed centrifuge tube. Membrane fraction was isolated by centrifuging the tube at 150,000 × g for 60 min and the obtained membrane pellet was resuspended in 10 mL 2% Triton X-100 lysis buffer (50 mM Na phosphate, 150 mM NaCl, 10 mM KCl, 1% Triton X-100, pH 7.2) with 100 μL 1 M PMSF in acetone and 100 μL Protease Inhibitor Mixture. Suspension was rocked in 4 °C cold room for 1 h and then centrifuged at 150,000 × g for 30 min to spin down insoluble materials and the supernatant was saved at ~80 °C.

Oocyte Total Protein Preparation. Appropriate amounts of cRNA of Slo3 or Slo3+lrcc were injected into *Xenopus laevis* oocytes. Oocytes were cultured in medium ND96 for 6 d before harvesting. To prepare the total protein, 50 oocytes were homogenized in 1 mL lysis buffer (50 mM Na phosphate, 150 mM NaCl, 10 mM KCl, 1% Triton X-100, pH 7.2) with 10 μL Protease Inhibitor Mixture by seven passages through an 18-gauge needle and another seven passages through a 27-gauge needle. Samples were centrifuged at 300 × g for 10 min at 4 °C to remove the yolk. Next, 0.12 mL 10% Triton X-100 was added to the collected supernatant, followed by rocking at 4 °C for 30 min to solubilize membrane proteins. After centrifugation at 15,000 × g for 10 min, only the supernatant was collected and saved at ~80 °C for future use.

Sperm Protein Preparation. Cauda epididymal sperm were released from two cauda epididymis of one mouse into 1.5 mL HTF medium (Irvine Scientific) at 37 °C 5% CO$_2$ incubator for 20 min. The sperm suspension was centrifuged at 3,000 × g for 3 min and the sperm pellet was resuspended in 0.3 mL lysis buffer (50 mM Na phosphate, 150 mM NaCl, 10 mM KCl, pH 7.2) with 6 μL Protease Inhibitor Mixture. Five microliters of sperm suspension were used for co-precipitation of Slo3 and LRRC52 from testis proteins. A variety of detergents was tested in the lysis buffer to solubilize Slo3 channels as intact complexes; this included 1% Nonidet P-40, 1–2% Triton X-100, 1% dodecyl maltoside and 1% Triton X-100+0.5% Na deoxycholate+0.1% SDS. Conceivably, interactions between Slo3 and LRRC52 may be highly sensitive to the conditions of solubilization.

Coimmunoprecipitation and Western Blotting in Native Testis. Numerous attempts were made to demonstrate coimmunoprecipitation (Co-IP) of Slo3 and LRRC52 from testis proteins. A variety of detergents was tested in the lysis buffer to solubilize Slo3 channels as intact complexes; this included 1% Nonidet P-40, 1–2% Triton X-100, 1% dodecyl maltoside and 1% Triton X-100+0.5% Na deoxycholate+0.1% SDS. Conceivably, interactions between Slo3 and LRRC52 may be highly sensitive to the conditions of solubilization.


Fig. S1. Comparison of current activation for Slo3 alone and Slo3+LRRC52 at potentials near 0 at pH 6.0, 7.0 and 8.0. (A) The currents arising from Slo3+LRRC52 channels in an inside-out patch are shown for activation steps of −80, −40, 0, +40, and +80 at pH 8.0, 7.0, and 6.0. Red traces correspond to activation at −40 mV and blue to activation at +40 mV. For Slo3+LRRC52, no activation was seen at pH 6.0, but at pH 7.0, steps to both −40 and +40 mV produce time-dependent current activation. (B) Traces show Slo3 currents under the same activation conditions as in A. At pH 7.0, there was essentially no detectable activation of Slo3 current at either −40 or +40 mV.
Fig. S2. Single channels arising from Slo3+LRRC52 coexpression open to low open probabilities at pH 8.0 and +240 mV. (A) Single Slo3+LRRC52 channels were activated by depolarizing voltage steps from +120 mV to +280 mV. Typically, 50 sweeps were obtained at each voltage. Dotted lines correspond to a single channel conductance of 103.2 pS. Total amplitude histograms were generated from sweeps at individual voltages and displayed here for +240 mV (B) and +160 mV (C). The vertical axis plots square root of the total number of counts. Red lines correspond to the fit of a two Gaussian function to the distribution: the baseline and the largest amplitude component (component 2). Separate fits with three components were also used, as done previously for Slo3 alone (4). The complexity of open amplitude current levels was similar to properties of Slo3 alone (4). The fractional time spent in components 1 and 2 was determined arithmetically by subtraction of the total baseline counts from the total distribution and then subtraction of the component 1 counts. At +240 mV, the total time spent in nonbaseline current levels was in the range of 0.25–0.35 for a set of three patches, but overall time spent in nonbaseline current levels varied only modestly over voltages from +160 through +280 mV. This is generally consistent with the less than twofold change in conductance seen in macroscopic recordings over the same voltage range. (D) Single channel amplitude of the largest conductance level was directly measured from sets of 10 separate openings at the indicated voltages for a set of patches with one to three channels. Error bars corresponding to SD are hidden by the symbols. The line corresponds to a fitted conductance of 103.2 pS. In separate experiments, mean-variance (iσ²) analysis of multichannel patches provided an estimate of the Slo3+LRRC52 single channel conductance of 66.0 ± 5.2 pS, which reflected the averaged contributions of both component 1 and component 2 in the single channel traces. This finding was also consistent with properties of Slo3 alone (4).
Fig. S3. Comparison of pH dependence of Slo3 and Slo3+LRRC52 conductance. (A) Activation of normalized conductance at different voltages for Slo3+LRRC currents is plotted as a function of cytosolic pH. The lines correspond to fit of a Hill equation, with the concentration of half activation occurring at pH of 7.43–7.55 with \( n = 1.6–1.7 \) at different voltages. Compared with Slo3 (4), Slo3+LRRC52 resulted in an \(-0.25\) pH unit shift (to more acidic pH) in the pH at which half-maximal conductance is activated. At any activation voltage, Slo3+LRRC52 currents require lower pH to produce inhibition. (B–E) Normalized GV curves were converted to absolute Po based on estimates of single channel Po. For Slo3 alone, single channel Po at +240 mV and pH 8.5 was estimated to be 0.28 based on previous single channel estimates (4). For Slo3+LRRC52, from examples as in Fig. S2, single channel Po was estimated to be 0.25–0.35 at pH 8.0 over voltages from +200 to +280 mV. Because the difference in maximal conductance is small between pH 8.0 and pH 9.0 for Slo3+LRRC52, we normalized Slo3+LRRC52 GV curves to a value of 0.32 at +240 mV and pH 9.0. (B) Slo3 GV curves were fit to the HA model. Values for \( z_L = 0.04 \) e, \( z_J = 0.34 \) e, \( E = 0.7 \), \( C = 0.127 \), as described in SI Materials and Methods. Note that \( E \) and \( C \) are reciprocals of constants used to describe Slo1 gating, as protonation is negatively coupled to voltage-sensing \( J \) or channel activation \( L \). The best fit to this set of curves (red lines) yielded values comparable to those previously obtained (1): \( L(0) = 0.00205 \), \( D = 3.92 \), \( J = 0.33 \), \( K = 33.8 \). K was not well defined, as at the lowest pH activation of conductance was not well-defined. (C) The fit in B is shown on a log(Po) scale to highlight the adequacy of the fit at negative voltages and lower Po. In all case, Po values used for the fit reflected conductance estimates there were \( >-2.5\% \) of the maximal evoked conductance. Background leak conductance estimated from currents at negative potentials with pH 6.0 were subtracted in all cases. (D) GV curves for Slo3+LRRC52 currents were fit with the HA model with the same constraints used to fit Slo3 currents in B and C. The optimal fit resulted from \( L(0) = 0.0251 \), \( D = 1.98 \), \( J = 0.66 \), and \( K = 91.4 \), with \( L(0) \) exhibiting the largest changes relative to the constants used to fit Slo3 alone. (E) The fit in C is shown on a log(Po) scale to highlight the adequacy of the fit at negative voltages and lower Po. Estimates of relative conductance at low Po were based on patches such as that in Fig. S2. Net current levels at pH 7.0 and negative potentials were clearly different from those at pH 6.0, allowing an estimate of fractional activation relative to that at stronger activation conditions. Based on the assumptions of identical \( z_L \), \( z_J \), \( C \), and \( E \), this analysis suggests that a change in allosteric constant \( L \) best accounts for differences between Slo3 GV curves and GV curves for Slo3+LRRC52.
Fig. S4. *lrrc26* and *lrrc55* messages exhibit a broad distribution among various tissues. RT-qPCR for *lrrc26* (A) and *lrrc55* (B) was performed on the indicated tissues. Each point is the mean of three independent tissue samples, each repeated in triplicate.
Fig. S5. Other Elron (extracellular leucine-rich-repeat-only) family subunits are less effective than LRRC52 at influencing Slo3 activation. (A) LRRC26 and LRRC38 produce modest alterations in the pH-dependent inhibition of Slo3 gating. GV curves were generated for Slo3+LRRC26 (Left) and Slo3+LRRC38 (Right) recorded in inside-out patches and compared with Slo3 alone. (B) The Elron subunits, LRTM1, LRTM2, LRRC55, when coexpressed with Slo3, had little effect on pH- and voltage-dependence of activation. (C) Slo3 was coexpressed with HA-tagged LRRC proteins. In all cases, total oocyte proteins were separated on Western blots and stained with anti-HA Ab. For each subunit combination, the left lane shows Slo3+HA-tagged–LRRC subunit coexpressed in the same oocytes and the right lane shows separation of proteins mixed from batches of oocytes separately injected either with Slo3 alone or HA-tagged–LRRC subunit alone. In general, coexpression with Slo3 did not influence the total detected LRRC protein. LRTM1, LRTM2, LRRC26, LRRC52, LRRC55, and LRRC6 were all robustly expressed in oocytes, but LRRC38 and LRRC28 were either weakly or not expressed. (D) Total oocyte proteins were immunoprecipitated with monoclonal anti-Slo3 Ab, separated on Western blot, and stained with anti-HA Ab. LRTM2, LRRC26, LRRC38, and LRRC52 were pulled down with anti-Slo3 Ab. LRRC55 may weakly coassociate with Slo3, but LRTM1 showed no evidence of coassembly. The non-Elron subunits, LRRC6 and LRRC28, were not associated with Slo3. (E) Immunoprecipitated proteins were also blotted with the anti-Slo3 polyclonal Ab to provide a measure of total loaded protein in each sample. Sample sizes for LRRC26 lanes were reduced because of its robust expression.

Fig. S6. Non-Elron LRRC subunits, LRRC6 and LRRC28, have no obvious effects on Slo3 gating. (A) The G-V relationships for Slo3 alone (black) and Slo3+LRRC6 (red) are shown for pH 7.0 (circles), pH 7.6 (diamonds), and pH 8.0 (squares). (B) GV relationships are shown for Slo3 alone and Slo3+LRRC28.
Table S1. Predicted molecular weight and glycosylation potential of LRRC proteins

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<th>Protein</th>
<th>Length of amino acid sequence</th>
<th>MW predicted by amino acid sequence (kDa)</th>
<th>Potential N-linked glycosylation sites (by OPPF protein analysis linker)</th>
<th>Potential O-linked glycosylation sites (by OPPF protein analysis linker)</th>
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<tr>
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<tr>
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Table S2. Primers used in real-time PCR

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<th>Gene</th>
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<th>Primer efficiency equation</th>
<th>Primer efficiency (%)</th>
<th>Length of amplicon (bp)</th>
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<tr>
<td>Lrtm1</td>
<td>Forward: 5'-TGGTACTTTCTAAGGACAACCTT-3' Reverse: 5'-CAGGGTTTCAGGCCAGCTGCAGATG-3'</td>
<td>$y = -3.59 \times +31.64$</td>
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<td>127</td>
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<td>Lrtm2</td>
<td>Forward: 5'-TGCAACCTCTGGAATTTCAACAC-3' Reverse: 5'-TCTCATGGGGACCCGACGCATGT-3'</td>
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<td>127</td>
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<td>Forward: 5'-TGTCGCCTGCCCTTTGACT-3' Reverse: 5'-CGCATCGGAGAAAAGCTGCAGTA-3'</td>
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<td>127</td>
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<tr>
<td>Lrc38</td>
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<td>127</td>
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<tr>
<td>Lrc52</td>
<td>Forward: 5'-GTGGTGCTCCAGACTGTGTACCTA-3' Reverse: 5'-CCGTACAGTGCGCTTGGGCAT-3'</td>
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<tr>
<td>Lrc55</td>
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