Knockout of the LRRC26 subunit reveals a primary role of LRRC26-containing BK channels in secretory epithelial cells

Chengtao Yang*, Vivian Gonzalez-Perez*, Taro Mukai*, James E. Melvin†, Xiao-Ming Xia*, and Christopher J. Lingle*•

*Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO 63110; †Secretory Mechanisms and Dysfunction Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892-4320; and •Department of Oral Reconstruction and Rehabilitation, Kyushu Dental University, Kitakyushu, Fukuoka 803-8580, Japan

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Leucine-rich-repeat-containing protein 26 (LRRC26) is the regulatory γ1 subunit of Ca2+- and voltage-dependent BK-type K+ channels. BK channels that contain LRRC26 subunits are active near normal resting potentials even without Ca2+, suggesting they play unique physiological roles, likely limited to very specific cell types and cellular functions. By using Lrrc26 KO mice with a β-gal reporter, Lrrc26 promoter activity is found in secretory epithelial cells, especially acinar epithelial cells in lacrimal and salivary glands, and also goblet and Paneth cells in intestine and colon, although absent from neurons. We establish the presence of LRRC26 protein in eight secretory tissues or tissues with significant secretory epithelium and show that Lrrc26 protein coassembles with the pore-forming BK α-subunit in at least three tissues: lacrimal gland, parotid gland, and colon. In lacrimal, parotid, and submandibular gland acinar cells, Lrrc26 KO shifts BK gating to be like α-subunit-only BK channels. Finally, Lrrc26 KO mimics the effect of SLO1/BK KO in reducing K+ efflux in saliva. LRRC26-containing BK channels are competent to contribute to resting K+ efflux at normal cell membrane potentials with resting cytosolic Ca2+ concentrations and likely play a critical physiological role in supporting normal secretory function in all secretory epithelial cells.

Significance

Ca2+- and voltage-regulated K+ channels (termed BK channels) are expressed in a diverse variety of cells, playing distinct physiological roles often defined by cell-specific regulatory subunits. Here, genetic deletion of one particular regulatory subunit, LRRC26, reveals that LRRC26-containing BK channels are found, perhaps exclusively, in secretory epithelial cells, including salivary glands, airways, and gastrointestinal tract. Such cells mediate fluid, peptide, and mucus secretion, influencing digestion, airway function, gut resistance to infection, and lactation. The absence of LRRC26 in secretory epithelial cells renders BK channels inactive during normal physiological conditions and alters ion efflux from salivary gland. LRRC26-containing BK channels are critical for normal ionic flux in secretory epithelial cells, likely impacting on a variety of epithelial cell pathologies.


References

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on candidate tissues with high message levels, we confirmed the presence of LRRC26 protein in various tissues. In the three tissues with the most abundant protein: parotid gland, lacrimal gland, and colon, we demonstrate immunoprecipitation (co-IP) of LRRC26 and SLO1. Furthermore, using lacrimal, parotid cells, and submandibular gland acinar cells, we show that LRRC26 KO results in large positive shifts in the activation range of BK channels. Finally, we show that the absence of LRRC26 is sufficient to account for the effects of SLO1 KO in reducing potassium secretion in saliva from mouse salivary glands. These results suggest that LRRC26-containing BK channels are suited to a specific role in secretory epithelial cells, contributing to maintenance of normal fluid and electrolyte secretion.

Results

Survey of Potential Loci of Expression of Lrrc26 Message. To provide a general overview of tissues likely to express LRRC26 protein, we used quantitative RT-PCR to test for Lrrc26 message abundance relative to β-actin and compared this to expression levels of the Kenhuali (Slol) message for the BK pore-forming subunit. In SI Appendix, Fig. S1A, loci of expression are grouped by general similarity of function, e.g., neuronal tissues, glandular tissues, and so on. Results for different tissues were also plotted in rank order of detected Lrrc26 message (SI Appendix, Fig. S1B). Of 38 tested mouse tissues, lacrimal, parotid, and submandibular glands show the highest Lrrc26 message abundance. In contrast, hippocampus, cerebral cortex, liver, adrenal, and heart ventricle show the lowest levels of Lrrc26 message expression, more than 500-fold lower than observed in tissues most enriched in Lrrc26 message. It is worth noting that, in intestine, there is a trend for Lrrc26 message to increase distally (duodenum, 0.0035 ± 0.0007 < jejunum, 0.0041 ± 0.0007 < ileum, 0.0071 ± 0.0017 < colon, 0.0152 ± 0.0038, values normalized to β-actin). Also, Lrrc26 message in vagina is markedly increased during pregnancy.

LRRC26 Protein Can Be Readily Detected in Most Native Tissues with High Lrrc26 Message. To facilitate definition of the loci of expression of LRRC26 protein, we used LRRC26 KO mice obtained from the Knockout Mouse Program (KOMP) (University of California-Davis) in which a lacZ reporter gene replaces the full-length Lrrc26 coding sequence (SI Appendix, Fig. S2A), along with a custom polyclonal antibody made to an epitope on the cytosolic C terminus of mouse LRRC26 (Materials and Methods). We first confirmed successful disruption of the Lrrc26 gene via PCR of mouse tails (SI Appendix, Fig. S2B). Subsequently, because both our qRT-PCR results and earlier results suggest LRRC26 protein is present in prostate (12, 20), membrane proteins were prepared from isolated prostate glands of wt and Lrrc26−/− mice and the LRRC26 protein was concentrated by immunoprecipitation (IP) with the rabbit anti-LRRC26 antibody. The LRRC26-IP products were then blotted with the same antibody (SI Appendix, Fig. S2C). Mouse LRRC26 protein has 331 amino acids and, according to the sequence, is predicted to be about 36 kDa. It has a potential N-linked glycosylation site at N56 in the extracellular domain. After the removal of the N-terminal signal peptide, the mature LRRC26 is predicted to be about 33 kDa. In the Western blot (WB) of LRRC26-IP products from prostate, a single band (~40 kDa) is seen only in WT sample and totally absent in KO sample (SI Appendix, Fig. S2C). After PNGase F treatment to remove N-linked oligosaccharides, the resulting deglycosylated product is shifted to ~35 kDa, generally consistent with the expected molecular weight (MW) of mouse LRRC26. Together these results confirm that the bands detected by the rabbit anti-LRRC26 Ab correspond to the LRRC26 protein. Because the MW of PNGase F-treated LRRC26 (35 kDa) is still somewhat larger than the predicted MW of the mature LRRC26 protein (33 kDa), it is possible that LRRC26 in mouse prostate has additional modifications other than N-linked glycosylation. The absence of LRRC26 protein in the Lrrc26−/− prostate confirms the successful deletion of the Lrrc26 gene.

Using a similar sequential IP and WB strategy with the anti-LRRC26 Ab along with a comparison of tissues from wt and Lrrc26−/− mice, we next determined that LRRC26 protein could also be identified both in tissues with relatively high Lrrc26 message levels, including lacrimal gland, parotid, and submandibular gland (Fig. 1A), and also tissues with lower Lrrc26 message levels, including trachea, mammary gland, and lung (Fig. 1B) and also colon and glandular stomach (Fig. 1C). LRRC26 protein could not be detected from membrane protein samples from the cerebellum (Fig. 1C).

We noticed that LRRC26 protein exhibited different forms in different tissues. In lacrimal gland and submandibular gland, LRRC26 migrated as a single band in Western blots as observed in the prostate samples. However, multiple LRRC26 bands were clearly observed in parotid, colon, trachea, and lung with the MW ranging from 38 kDa to 48 kDa. The results suggest that different tissues may exhibit differential posttranslational processing of LRRC26 protein or perhaps alternative splicing of Lrrc26 message that has not as yet been reported. Although all bands were shifted to smaller sizes with PNGase F treatment, the number of bands and their relative densities appeared unchanged by deglycosylation. Similar to these observations, multiple bands of LRRC26 were reported from the human MDA-231 breast cancer cell line. However, to our knowledge, there is no current proposal that LRRC26 may contain modifications other than N-glycosylation. In this regard, the online O-linked glycosylation prediction server, NetOGlyc 4.0 (www.cbs.dtu.dk/services/NetOGlyc/), identifies nine Thr/Ser residues in the mouse LRRC26 protein sequence, is predicted to be about 36 kDa. It has a potential N-linked glycosylation site at N56 in the extracellular domain. After the removal of the N-terminal signal peptide, the mature LRRC26 is predicted to be about 33 kDa. In the Western blot (WB) of LRRC26-IP products from prostate, a single band (~40 kDa) is seen only in WT sample and totally absent in KO sample (SI Appendix, Fig. S2C). After PNGase F treatment to remove N-linked oligosaccharides, the resulting deglycosylated product is shifted to ~35 kDa, generally consistent with the expected molecular weight (MW) of mouse LRRC26. Together these results confirm that the bands detected by the rabbit anti-LRRC26 Ab correspond to the LRRC26 protein. Because the MW of PNGase F-treated LRRC26 (35 kDa) is still somewhat larger than the predicted MW of the mature LRRC26 protein (33 kDa), it is possible that LRRC26 in mouse prostate has additional modifications other than N-linked glycosylation. The absence of LRRC26 protein in the Lrrc26−/− prostate confirms the successful deletion of the Lrrc26 gene.

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LRRC26 May Be Exclusively Expressed in Secretory Epithelial Cells. In the Lrrc26 KO mice, the β-galactosidase reporter under control of the Lrrc26 promoter provides another tool to reveal potential loci of Lrrc26 message expression and offers the advantage that the cellular loci of β-gal activity can potentially be visualized. To evaluate loci of Lrrc26 promoter activity, 20- to 25-μm sections were prepared from various tissues and incubated with the BlueGal substrate. Incubation conditions that were required to reveal β-gal reaction product varied appreciably among tissues (Materials and Methods), possibly reflecting cell-specific differences in regulation of promoter activity or differences in success in tissue permeation with substrate. Despite variations in optimal reaction conditions among cells, direct side-by-side comparison of wt vs. KO tissues provides assurance regarding the specificity of any observed reaction product. In all tissues for which a positive β-gal reaction product is observed, the positive staining is seen only in likely epithelial cells.

Positive BlueGal staining is readily observed in acinar epithelial cells in lacrimal gland and salivary glands. Fig. 2 A–D shows sections from wt (Left) and Lrrc26 KO (Right) glandular tissues reacted for BlueGal. All sections are counterstained with eosin. Lower gain sections showing most of each gland are displayed in SI Appendix, Fig. S3 A–D. Even at the shortest tested BlueGal incubation times (1–2 h), BlueGal staining in the lacrimal gland exceeded other tissues (Fig. 24), generally consistent with an abundance of message (SI Appendix, Fig. S1) and protein (Fig. 1). Strong staining is observed throughout the acinar cells in the lacrimal gland KO section, but not the wt section. Although parotid had a similar level of Lrrc26 message (SI Appendix, Fig. S1) and protein (Fig. 1) compared with lacrimal gland, dark blue staining in Lrrc26 KO parotid sections required about 24 h of
incubation to develop, compared with 1–2 h for lacrimal gland, with Bluo-Gal staining in the parotid occurring in both acini cells and ducts (SI Appendix, Fig. S4 A and B). Unlike lacrimal and parotid glands, where serous acini are most dominant, the mouse submandibular gland contains exclusively seromucous cells interspersed with primarily granular duct cells. The submandibular KO sections exhibit strong positive Bluo-Gal staining (SI Appendix, Fig. S3C), which is most prominent in seromucous acini (Fig. 2C). Finally, in the sublingual gland, which contains primarily mucous cells, sections from the LRRC26 KO tissue exhibit strong Bluo-Gal staining (SI Appendix, Fig. S3D and Fig. 2D) comparable to that in the submandibular gland (SI Appendix, Fig. S3C). Staining in LRRC26 KO sections showing both sublingual and submandibular glands was more prominent after 24-h Bluo-Gal incubation time than after 2 h (SI Appendix, Fig. S4 C and D), but ductal cells in the wt submandibular gland also revealed some nonspecific darkening.

Positive Bluo-Gal staining was also observed in goblet cells and Paneth cells in the epithelium of small intestine (Fig. 2 E and G and SI Appendix, Fig. S3E) and colon (Fig. 2F and SI Appendix, Fig. S3F). The proportion of goblet cells among epithelial cell types increases caudally from duodenum (4%) to jejunum (6%) to ileum (12%) to distal colon (16%) (21). We found a qualitatively similar rostral-to-caudal increase of Lrcc26 message from duodenum to colon in our qRT-PCR data (Fig. 1), raising the possibility that β-gal positive cells are goblet cells. Because goblet cells produce mucins, they can be visualized with the periodic acid-Schiff (PAS) staining method (22, 23). For both the small intestine (Fig. 2G) and the colon (Fig. 2F), tissue sections were therefore prepared with both Bluo-Gal staining and the PAS counterstain. In wt sections, PAS stained the mucin granules with magenta in the apical part of goblet cells, whereas in LRRC26 KO sections additional dark blue Bluo-Gal staining was observed in the narrow base of the same cells stained with PAS. This confirms that Bluo-Gal–stained cells in small intestinal villi and in colon are goblet cells. Furthermore, in the crypts of the small intestine, positive Bluo-Gal staining signals appeared not only in PAS+ goblet cells, but also in PAS+ Paneth cells (24) at the base of crypts (Fig. 2G). In the colon, positive Bluo-Gal activity is most prominent in the upper half of the colonic crypts.

Positive Bluo-Gal staining specific to sections from LRRC26 KO mice was also obvious in both the vomeronasal organ (VNO) (SI Appendix, Fig. S5 A and B) and conjunctiva of the eye (SI Appendix, Fig. S5 C and D). In the VNO, the PAS stain identifies the vomeronasal gland, also termed Jacobson’s gland (25). In VNO sections from LRRC26 KO animals, the magenta signal of PAS staining colocalized with the dark blue signals from the β-gal reaction (SI Appendix, Fig. S5 A and B). Positive staining was also seen in the receptor-free epithelium of the VNO. Similarly, in the conjunctiva, Bluo-Gal staining (SI Appendix, Fig. S5C) appears to overlap with PAS staining (SI Appendix, Fig. S5D). In the conjunctiva, PAS staining identifies mucus-secreting goblet cells (26, 27).

Positive Bluo-Gal staining cells were also observed in epithelium of trachea (SI Appendix, Fig. S6A), bronchiolo of the lung (SI Appendix, Fig. S6B), oviduct (SI Appendix, Fig. S6C), prostate (SI Appendix, Fig. S6D), uterus (SI Appendix, Fig. S6E), cervix, vagina (SI Appendix, Fig. S6F), and mammary gland of lactating females (SI Appendix, Fig. S6G). The epithelial layer exhibiting Bluo-Gal staining in cervix and vagina was markedly thickened during pregnancy (for cervix, SI Appendix, Fig. S7 A vs. B; for vagina, SI Appendix, Fig. S7 C vs. D). In both cervix (SI Appendix, Fig. S6A) and vagina (SI Appendix, Fig. S6B), this thickening is associated in part with prominent remodeling of Bluo-Gal positive columnar epithelial cells. Comparison of wt and Lrrc26 KO sections from lactating mammary gland at higher resolution (SI Appendix, Fig. S9) revealed staining in cells likely to line the alveoli and also those that contribute to lactiferous ducts.

Many tissues were entirely negative for Bluo-Gal reaction even with prolonged reaction times (SI Appendix, Fig. S10). These tissues included brain regions, pancreas, kidney, glandular stomach, spleen, bladder, vas deferens, cauda epididymis, aorta, and cerebral artery. The absence of a positive Bluo-Gal signal in the glandular
stomach seems surprising, given that the qRT-PCR results suggest a high level of Lrc26 mRNA in this tissue (SI Appendix, Fig. S1), and that the weak presence of LRRC26 protein was confirmed by WB (Fig. 1). The absence of Bluo-Gal signal in acinar pancreas is also somewhat unexpected. Clearly, there is a wide range in the effectiveness of the Bluo-Gal staining protocol among tissues, even among those showing robust message levels, such as lacrimal and parotid glands. Spleen is another loci in which there is no obvious Bluo-Gal reaction, despite qRT-PCR data revealing a higher Lrc26 message level than in some other Bluo-Gal positive tissues (e.g., small intestine and vagina). Although reasons for the weaker than expected Bluo-Gal staining in some tissues are unclear, one possibility might be cell-specific variations in factors regulating promoter activity controlling β-gal expression.

Finally, with ovary, testis, and caput epididymis, sections from both wt and LRRC26 KO mice exhibited similar loci of darker coloration (SI Appendix, Fig. S11). Because of this nonspecific staining, we were unable to assess whether there is any Lrc26 promoter activity in these tissues, although the qRT-PCR estimates also confirm that Lrc26 message levels in these tissues are low.

**LRRC26 Coassembles with SLO1 in Parotid Gland, Lacrimal Gland, and Colon.** At least one important function of LRRC26 is to serve as a regulatory subunit of SLO1/BK channels (12, 14, 28). In addition to the profound functional effects of LRRC26 on BK channels, LRRC26 was shown to coimmunoprecipitate with SLO1 in LNCaP human prostate cancer cells (12). Here we selected three tissues: parotid gland, lacrimal gland, and colon, in which we were able to detect relatively abundant LRRC26 protein, to test whether LRRC26 coassembles with SLO1. First, membrane proteins were prepared from wt, Slo1−/−, and Lrc26−/− mice for the three tissues. The presence of SLO1 protein was then confirmed in wt and Lrc26−/− membrane proteins, but not Slo1−/− proteins (Fig. 3 A, B, and C). The protein samples were then checked for the presence of LRRC26 protein following an initial IP step with the

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**Fig. 2.** Bluo-Gal staining is observed in glandular acinar cells and goblet and Paneth cells of gastrointestinal tract. In A–D, tissues were developed for Bluo-Gal staining and counterstained with eosin. (A) LRRC26 KO tissues show abundant Bluo-Gal reaction product throughout a lacrimal gland section that is absent in wt sections. (B) In parotid, Bluo-Gal reaction product (right) is observed sparsely, but throughout acinar cells, whereas dense staining likely corresponds to intralobular and interlobular parotid ducts. (C) Bluo-Gal staining in submandibular gland is confined to cells likely to be seromucous acinar cells, whereas larger glandular duct cells display little if any staining. (D) Bluo-Gal staining in sublingual gland is distributed throughout acinar cells and ducts within the gland. In E–G, tissues were first processed with Bluo-Gal staining followed by a periodic acid-Schiff (PAS) reaction. (E) Goblet cells in villi of the small intestine are positive for PAS staining at the apical end corresponding to mucus granules and positive for Bluo-Gal staining at the basal end. (F) Crypts in the colon exhibit abundant PAS-positive cells, with the most superficial cells also enriched with Bluo-Gal staining. (G) Crypts of the small intestine reveal positive PAS staining and Bluo-Gal staining in Paneth cells and goblet cells.
LRRC26 polyclonal Ab, confirming the presence of LRRC26 protein in both wt and Slo1−/− proteins from all three tissues, but not in Lrc26−/− proteins (Fig. 3 A2, B2, and C3). Subsequently, the LRRC26-IP products were blotted with the SLO1 Ab, indicating that SLO1 immunoprecipitates with LRRC26, but only in wt protein samples, and not in the samples from Slo1−/− or Lrc26−/− mice (Fig. 3 A3, B3, and C3). For each tissue, an Ab to Na+/K+ATPase α1A was used to confirm that similar amounts of proteins were applied (Fig. 3 A4, B4, and C4). The coassembly of LRRC26 with SLO1 in parotid gland, lacrimal gland, and colon supports the view that LRRC26 is a BK regulatory subunit in these tissues and predicts that BK gating will be shifted leftward in these cells.

LRRC26 KO Abolishes the Profound Leftward Activation Range of BK Currents in Cells from the Lacrimal, Parotid, and Submandibular Glands. Native cell BK currents that display functional properties consistent with the presence of SLO1 α and LRRC26 subunits have, to date, been clearly demonstrated only in prostate tumor cells (12, 17), parotid (18, 29), and submandibular (30, 31) gland cells. To confirm the consequences of LRRC26 KO on BK channel function in cells likely to express LRRC26, we used lacrimal, parotid, and submandibular dissociated acinar cells. For each gland, cells were acutely dissociated and whole-cell patch-clamp recordings were obtained with physiological K+ gradients to compare currents in wt and LRRC26 KO cells. Because earlier work on parotid BK currents revealed some reduced sensitivity to the BK-specific inhibitor, iberiotoxin (32), and the β4 regulatory subunit of BK channels confers reduced iberiotoxin sensitivity on BK channels (33), we also examined currents in β4 KO parotid cells. In all cases, the pipette solution contained buffered 250 nM Ca2+, which should produce minimal activation of any IK1 current present in such cells, while allowing robust voltage- and time-dependent BK activation. In wt lacrimal gland cells, depolarizing voltage steps result in activation of a time- and voltage-dependent outward current (Fig. 4A1, with appreciable activation even at voltages negative to 0 mV. In contrast, in lacrimal cells from the LRRC26 KO animals, even voltage steps to +100 mV produce minimal current activation (Fig. 4A2). KO of the BK β4 subunit does not appear to alter the lacrimal gland BK current properties (Fig. 4A3). Currents activated by identical protocols but in cells from the parotid gland exhibited almost identical properties. In wt cells, time- and voltage-dependent currents were activated at voltages negative to 0 mV (Fig. 4B1), whereas in LRRC26 KO cells, steps to +100 mV barely activated any current (Fig. 4B2), with no obvious effect of β4 subunit KO (Fig. 4B3). Currents obtained from submandibular acinar cells also showed robust activation in wt cells at voltages where little current was activated in LRRC26 KO cells (SI Appendix, Fig. S12A). Conductance vs. voltage (GV) curves generated showed that KO of the LRRC26 subunit resulted in an ∼145 mV rightward gating shift for lacrimal gland cells (Fig. 4C), a rightward 120 mV gating shift in the parotid acinar cells (Fig. 4D), and a rightward 145 mV gating shift in the submandibular acinar cells (SI Appendix, Fig. S12B). The magnitude of the shift in gating with or without LRRC26 is consistent with the known effects of LRRC26 subunit when it is heterologously expressed with BK α-subunits (12, 28). Similarly, comparison of BK single channel activity in inside-out patches from wt and LRRC26 KO parotid gland cells reveals a similar gating shift to that observed in whole-cell recordings (SI Appendix, Fig. S13). In parotid cells (Fig. 4 E and F) cells, the time- and voltage-dependent currents were almost completely inhibited by either 5 mM tetraethylammonium (TEA) or 100 nM paxilline both in wt and LRRC26 KO cells. These sensitivities confirm that the currents both in wt and Lrc26−/− cells are BK currents (Fig. 4G). Together these properties identify these currents as BK current and establish that LRRC26 is the critical partner of the BK pore-forming subunits that is essential for the left-shifted gating range in lacrimal, parotid, and submandibular gland acinar cells.

For the set of parotid cells, we observed no differences in average cell capacitance, BK current density, or IK current density (taking the leak current at −30 mV as an indication of IK) (SI Appendix, Fig. S14A). Given that β4 might have effects on channel kinetics that might not be revealed solely by examination of GV curves, we also measured activation time constants for wt, β4 KO, and LRRC26 KO parotid cells. No differences were observed between wt and β4 KO cells (SI Appendix, Fig. S14B), whereas activation time constants in LRRC26 KO parotid cells were slower at all voltages.

LRRC26 KO Reproduces the Effect of SLO1 KO in Reducing the Concentration of K+ in Parotid and Submandibular Gland Salivary Secretions. Bulk saliva largely reflects contributions of secretion from three distinct paired salivary glands, the parotid, the submandibular, and the sublingual. The saliva composition varies to some extent among each gland type, differing in the extent to which glands that are largely composed of serous cells primarily secrete fluid and electrolytes or mucous cells that also secrete large amounts of glycosylated proteins such as mucins. An assay previously used to examine the role of KO of SLO1 α-subunits on salivary gland function is to monitor the salt composition of saliva collected either via an in vivo assay of parotid or submandibular glands or via an ex vivo assay of isolated, perfused

Fig. 3. Anti-LRRC26 antibody pulls down SLO1 protein in parotid, lacrimal gland, and colon. (A) Confirmation of LRRC26 association with SLO1 protein in mouse parotid. (A1) Total membrane proteins from parotid wt, Slo1+/−, and Lrc26−/− mice were blotted with anti-SLO1 Ab (L6/60, Antibodies, Inc.), identifying the SLO1 protein in wt and Lrc26+/− mice, but not Slo1−/− mouse. (A2) Proteins immunoprecipitated by the ProSci LRRC26 Ab were Western blotted, showing that LRRC26 is present in both wt and Slo1−/− mice, but not in the LRRC26 KO mice. (A3) Following immunoprecipitation of parotid membrane proteins with the LRRC26 Ab, SLO1 protein is identified in wt immunoprecipitated proteins, but not in Slo1−/− or Lrc26−/− proteins. (A4) Aliquots of the parotid membrane protein preparations were blotted with a NaK ATPase1A1 Ab to confirm that similar amounts of proteins were applied in all cases. (B1) Lacrimal gland total membrane proteins were blotted with the anti-SLO1 Ab. (B2) wt and Slo1−/−, but not Lrc26−/−, lacrimal gland proteins contain LRRC26 protein. (B3) SLO1 protein is found in lacrimal gland membrane proteins immunoprecipitated with the LRRC26 Ab. (B4) Aliquots of lacrimal gland membrane proteins were blotted for ATP1A1. Note the markedly lower amounts of ATP1A1 in lacrimal gland, compared with parotid. (C1–C4) SLO1 protein in colon is also immunoprecipitated with the LRRC26 Ab. LRC26 KO Reproduces the Effect of SLO1 KO in Reducing the Concentration of K+ in Parotid and Submandibular Gland Salivary Secretions. Bulk saliva largely reflects contributions of secretion from three distinct paired salivary glands, the parotid, the submandibular, and the sublingual. The saliva composition varies to some extent among each gland type, differing in the extent to which glands that are largely composed of serous cells primarily secrete fluid and electrolytes or mucous cells that also secrete large amounts of glycosylated proteins such as mucins. An assay previously used to examine the role of KO of SLO1 α-subunits on salivary gland function is to monitor the salt composition of saliva collected either via an in vivo assay of parotid or submandibular glands or via an ex vivo assay of isolated, perfused

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submandibular glands (30, 31). In one set of wt and LRRC26 KO mice, the carbachol-evoked secretion from the submandibular gland was used in an ex vivo assay to assess effects on secretion independent of potential systemic effects of the agonist. In the ex vivo assay, the K⁺ concentration in saliva secreted from the submandibular was reduced to a similar extent as observed in vivo (Fig. 5B). This decrease was comparable to earlier results in SLO1 KO mice (30, 31).

**Discussion**

The present results show that Lrrc26 message and LRRC26 protein appear to be specifically associated with secretory epithelial cells, in some cases organized into subunits, and most likely including both those secreting primarily fluid (serous) and those secreting mucus (mucous). This finding is supported by the distribution of Lrrc26 message, the Bluo-Gal–defined loci of LRRC26 promoter activity, and the presence of verified LRRC26 protein in many relevant tissues. For three cases in which LRRC26 protein can be most readily detected: lacrimal gland, parotid gland, and colon, comminunoprecipitation of LRRC26 protein with BK channel pore-forming subunits was demonstrated. Furthermore, in three cases: lacrimal, parotid, and submandibular glands, changes in BK channel properties in the LRRC26 KO animal reveal that BK channels in such wt cells are normally associated with LRRC26. The fact that LRRC26 is a normal part of BK channels has also previously been established for prostate tumor cells (12). Finally, we show that, in salivary gland, the previously established effect of BK channel KO on salivary K⁺ concentration (30, 31) can be entirely mimicked by LRRC26 KO. Because of the remarkable left-shifted gating behavior of LRRC26-containing BK channels, we suggest that the activity of such BK channels near normal cell resting potentials plays a unique role in maintaining high K⁺ fluxes that are essential to secretion in all secretory epithelial cells. At present, whether this is true in all cellular loci in which Lrrc26 message and LRRC26 protein are found remains to be established. For those tissues for which we do not have functional data, e.g., trachea, bronchioles, small intestine, mammary gland, and uterine and vaginal epithelium, the present results only suggest that specific attention to the role of LRRC26-containing BK channels in the regulation of secretion in such cells is warranted.
Loci of Expression of LRRC26-Containing BK Channels in Native Tissues. The marked diversity in BK channel properties among different cells depends in large measure on the identity of particular regulatory subunits in a given BK channel complex (15, 34). LRRC26-containing BK channels, with their enormously shifted activation range, are probably those BK channels most suited for unique roles in nonexcitable cells. Based on previous results and results reported here: Where have BK channels with properties consistent with the presence of LRRC26 subunits definitively been found?

Previous functional data have suggested the presence of LRRC26-containing BK channels in prostate tumor cells (12, 17) and parotid acinar cells (18, 19). Other tissues in which BK channels appear to be activated at unusually negative voltage ranges include lacrimal gland (2, 35) and pancreatic acinar cells (36). The present results confirm that the gating range of parotid, submandibular, and lacrimal gland BK channels is defined by LRRC26. There is also some pharmacological and functional evidence supporting the presence of LRRC26-containing BK channels in tracheal epithelium (37), based on the presence of a mallotoxin-resistant component of a BK-dependent process. Mallotoxin has been shown to be an activator of BK channels at low nanomolar concentrations (38), but heterologously expressed BK channels containing LRRC26 are resistant to mallotoxin as are the native BK channels in parotid (19). The specific identity of the tracheal cell type that participates in this phenomenon has not been identified. However, based on the distribution of Bluo-Gal staining, we propose that LRRC26-containing BK channels in the trachea are specifically located in the bronchiolar exocrine cells known as club cells and also goblet cells, but additional work will be required to clarify this. In regards to pancreas, although we detected low levels of Lrnc26 message in pancreatic samples, we were unable to detect LRRC26 protein. Based on early recordings from pancreatic acinar cells (39, 40) in which half activation of BK channels occurred at very negative voltages, we think it likely that LRRC26-containing BK channels are present in such cells, albeit at low current density. In fact, probably the best criterion for the presence of LRRC26-containing BK channels in a given cell type will rest on demonstration of appreciable fractional activation of BK channels at membrane potentials negative to 0 mV with 0 cytosolic Ca2+. When LRRC26 protein is clearly coassembled with BK channels, the extreme gating shift at 0 Ca2+ of such BK channels is unambiguous (28). LRRC26 has also been proposed to contribute to BK channels in brain arteriole smooth muscle (41), but BK channels and currents with the functional properties characteristic of those associated with LRRC26 subunits were not observed. Here we found no evidence for the presence of Lrnc26 message or LRRC26 protein in various neuronal cells, electrically excitable endocrine cells, or aortic smooth muscle cells.

One interesting cell type that exhibits BK currents with properties that seem indicative of the potential presence of LRRC26 subunits is that of hair cells of the cochlea. These cells are, in fact, epithelial-derived cells (42). In such cells, with 0 cytosolic Ca2+, BK currents are activated at very negative potentials relative to other BK currents (43, 44), very similar to currents produced by the presence of LRRC26. Although this possibility will require further attention, we were unable to obtain any indication of Lrnc26 message in the cochlea.

The Role of LRRC26 in Exocrine Gland Secretory Function and Epithelial Cell Function. Although the physiological role of LRRC26-containing BK channels in most tissues remains largely unaddressed, previous work has established that BK channels in parotid and submandibular glands are essential for normal secretory function. BK KO results in decreased K+ concentrations in secreted fluid from mouse submandibular (31) and parotid (1) glands. The results here confirm that, in parotid, submandibular, and lacrimal gland, the shifted gating of BK channels in those cells arises from the presence of LRRC26, as originally suggested by Begenisch and colleagues in regards to parotid (18, 19). Here we tested the possibility that the simple absence of the LRRC26 subunit might be sufficient to mimic the effects of BK α-subunit KO. Consistent with this idea, we observed a decrease in concentration of secreted [K+] from both the parotid and submandibular glands of LRRC26 KO animals similar to previous observations from SLO1 KO animals (1, 31). This observation supports the view that the presence of the LRRC26 subunit in BK channels in these cells is the key determinant that defines the physiological role of BK channels in maintaining normal secretion of K+. We hypothesize that the presence of LRRC26-containing BK channels in other secretory epithelial cells will exert similar functional roles in maintaining secretory function.

The present results offer a number of intriguing hypotheses for future work. In the case of lacrimal glands, it would be expected that the [K+] in the tears of LRRC26 KO mice would be reduced. Furthermore, in intestine and colon, given the importance of goblet and Paneth cells in maintaining the normal mucus layer and protection against bacterial infection (45, 46), it might be expected that LRRC26 KO mice may manifest gastrointestinal and colon dysfunctions with appropriate triggers. We anticipate that the LRRC26 KO mice will be useful in assessing important physiological roles of secretory epithelial cells in a variety of cells.

Potential Roles of LRRC26-Containing BK Channels in Tumor Growth Regulation. Before its recognition as a likely BK regulatory subunit, message or protein for human LRRC26 was initially observed in a variety of cancer cell lines and human cancer samples (CAPC) (16). CAPC was found to inhibit tumor cell proliferation and tumor growth by regulating NF-κB and its target genes (20). These observations raise two questions: First, is there a specific role of LRRC26-containing BK channels in tumor growth regulation and, second, might LRRC26 have functions unrelated to BK channels? Although the present findings are not adequate to answer these questions, in those tissues where we have directly recorded BK currents or tested for coassembly between LRRC26 and BK α-subunits, LRRC26 appears to be a critical regulatory component of BK channels. Given that a large number of ion channels have been implicated in tumor growth regulation, including KCa3.1 (47, 48), calcium channels (49), sodium channels (50), and a variety of K+ channels (51, 52), it is worthwhile to consider the possibility that specific ion channels per se may not be intrinsically related to tumor growth regulation, but that some aspect of membrane potential regulation, perhaps linked to cell cycle regulation, is the determinant of whether a given ion channel may promote or impede tumor growth.

Overview. The present results suggest that the large gating shift produced by LRRC26 serves specific physiological roles in a specific type of epithelial cell, perhaps related to the ability of LRRC26-containing BK channels to be activated near normal cell resting potentials at basal or low cytosolic Ca2+ levels (12). However, LRRC26 is one of a family of LRR proteins, including LRRC52, LRRC38, and LRRC55, which together have been designated as γ-subunit family (γ1–γ4) that associates with SLO family channels (14). Might other γ-subunits play equally specific roles in regulating BK channels in other loci? At present, only LRRC52 (γ2) appears to be exclusively expressed in mammalian sperm (13) and is a critical regulatory subunit of the alkalization-activated SLO3 K+ channel (53), the closest homolog of the SLO1/BK channel. Specific interaction partners for LRRC38 and LRRC55 in native tissues have not been identified.
The results presented here highlight the important point that LRRC26-containing BK channels play a unique physiological role distinct from BK channels composed of other regulatory subunits, e.g., αβ2 channels in endocrine cells or neurons, α+β4 subunits in smooth muscle, or α+β4 subunits in various brain loci. We observed that the K⁺ concentration of saliva arising from parotid and submandibular acinar cells is reduced in LRRC26 KO mice in a fashion identical to that resulting from complete SLO KO. Thus, LRRC26 is essential to defining the physiological role of BK channels in such cells. By providing a major pathway for K⁺ efflux close to normal cell resting potentials even in the absence of increases in cytosolic Ca²⁺, LRRC26-containing BK channels likely play similar roles in maintaining K⁺ efflux in all secretory epithelial cells. Because of the markedly distinct functional properties of BK channels in different subunit composition, these results suggest that, in discussions of the physiological roles of BK channels, particular attention must be given to identifying the regulatory subunit composition and its biophysical effects on BK channels in any given cell type.

Materials and Methods

Animal Care. Animals were handled and housed according to the National Institutes of Health Committee on Laboratory Animal Resources guidelines. All experimental protocols were approved by the Institutional Animal Care and Use Committees of Washington University (protocol 20150258) and the National Institute of Dental and Craniofacial Research, National Institutes of Health (protocol ASP 16-802).

Source of LRRC26 KO Mice. The LrRc26 KO mouse strain used for this research project was created from ES cell clone 10167A-DS, generated by Regeneron Pharmaceuticals, Inc. and made into live mice by the KOMP Repository (https://www.komp.org) and the Mouse Biology Program (https://mmp. mousebiology.org) at the University of California, Davis. Methods used to create the VelociGene targeted alleles have been published (54). The VelociGene-targeted allele was replaced with lacZ and a floxed reporter cassette (ZEN-UBI-hyg) (velociGene/komp/detail/10167).

Pariotid, Submandibular, and Lacrimidal Gland Dissociations. Parotid cells were isolated following the enzymatic digestion protocol previously described (1). Briefly, after mice were killed by CO₂ inhalation, the parotid glands were carefully removed and placed in Earle's minimum essential medium solution containing 1% BSA (Sigma) and 2 mM of Δ-glutamine (BSA-GLN-MEM). The parotid glands were minced ∼300 times with fine scissors and digested in this solution containing 0.01% trypsin and 0.5 mM EDTA, at 37 °C, for 5 min. Trypsin digestion was stopped by addition of 0.2% of trypsin inhibitor (Sigma T90003) and the tissue was further digested for another 20 min with 0.17 mg/mL of Liberase TL (Roche) dissolved in BSA-GLN-MEM containing 0.2% of trypsin inhibitor, at 37 °C, with constant stirring at very low speed. After 10 min of Liberase digestion, the cells were mechanically dispersed by pipetting three to four times and allow it to sit at 37 °C. Then, the cells were washed with BSA-GLN-MEM and centrifuged at 800 × g for 1 min, repeating this washing step once. A final wash was performed using Earle's minimum essential medium containing only 2 mM of glutamine (GLN-MEM). Then, after centrifugation the cells were suspended in GLN-MEM, plated on coverslips previously treated with poly-D-lysine, allowed to attach during at least 45 min, and then maintained at room temperature until used (usually within 3 h after dissociation). Individual coverslips were subsequently transferred to a recording chamber continuously perfused with extracellular solution for electrophysiological recordings. Lacrimidal and submandibular gland cells were dissociated using a similar procedure with the following minor differences: Lacrimal gland dissociation omitted the initial trypsin treatment (Liberase treatment only), whereas submandibular glands required Liberase treatment for 30 min.

Basic Recording Methods. Standard whole-cell recording methods were done using an Axopatch 200B amplifier (Molecular Dynamics). Data acquisition was performed using a 16-bit analog/digital converter and voltage stimulation protocols were accomplished by using Clampex 8.0 (Molecular Dynamics) with analysis of waveforms done via Clampfit. Patch-clamp pipettes were made from borosilicate glass and coated with Sylgard. Typical pipette resistances after heat polishing typically were of 1.5–2.5 MΩ. Following whole-cell access, cells were used if the series resistance (Rₛ) was less than 10 GΩ. Rₛ was compensated 85%. Current records were filtered at 10 kHz. For whole-cell recordings, solutions were identical to those used in previous work on Ca²⁺-dependent K⁺ currents in parotid acinar cells in which extracellular and internal anions were largely replaced with glutamate, to remove contributions of any Cl⁻ currents (1). The bath solution (extracellular) consisted of (in millimoles): 135 NaCl, 5 K-glu- matate, 2 CaCl₂, 2 MgCl₂, 10 Hepes (pH 7.2). The pipette solution (intracellular) consisted of (in millimoles): 135 mM K-glu matate, 10 Hepes and, 5 mM EGTA + 3 mM Ca²⁺, which results in a free Ca²⁺ solution of 250 nM (pH 7.2). For these nominal ionic gradients, Eₘ = −84.5 mV. Tetraethylammonium or paxilline was added to the external solutions at final concentrations given in the text. Experiments were done at room temperature, typically 22–24 °C.

In Vivo and ex Vivo Saliva Collection and [K⁺] Determination. In vivo secretion was stimulated by i.p. injection of the cholinergic receptor agonist pilocarpine-HCl (10 mg/kg body weight) from mice anesthetized by i.p. injection of chloral hydrate (400 mg/kg body weight). Gland-specific ductal saliva was collected for 30-min stimulation by dissecting the ducts from the parotid and submandibular glands and inserting their distal ends into individual calibrated glass capillary tubes (Sigma-Alrich). Body temperature was maintained at 37 °C using a regulated blanket (Harvard Apparatus). Ex vivo saliva collection perfusion was performed at room temperature as previously reported (30). In brief, following ligation of all branches of the common carotid artery except the submandibular artery, the submandibular gland was removed, cannulated, perfused, and the main duct inserted into a calibrated glass capillary tube. Salivation was stimulated for 10 min by addition of the cholinergic receptor agonist carbachol (0.5 μM). The ex vivo perfusion solution contained (in millimoles): 4.3 KCl, 120 NaCl, 25 NaHCO₃, 5 glucose, 10 Hepes, 1 CaCl₂, 1 MgCl₂, pH 7.4. The saliva collected was stored at −20 °C. The potassium concentration was analyzed by atomic absorption spectroscopy (PerkinElmer Life Sciences 3030 spectrophotometer).

Statistical Analysis. The Kolmogorov-Smirnov (KS) test was used to generate the KS statistic. For cases in which the number of entries in one or both sample populations was less than 10, a two-tailed, unpaired Student’s t test was used. Data are presented as mean ± SEM. Bar graphs for the K⁺ secretion data display the mean and SE. Significance was determined using Student’s t test with P < 0.05 considered to be statistically significant.

ACKNOWLEDGMENTS. We thank Ramon Lorca (University Colorado-Denver) for assistance in staging of pregnant females and Todd Begennis for guidance and insightful comments on the nuances of salivary gland physiology. This work was supported in part by NIH Grant GM 118114 (to C.J.L.). NIH DE000738 from the Intramural Research Program of the National Institute of Dental and Craniofacial Research (NIDCR), National Institutes of Health (to J.E.M.), the Department of Anesthesiology; Washington University School of Medicine; the Intramural Research Program of the NIH-NIDCR; and the NIDCR Veterinary Research Core DE000740.


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Supporting Information
Yang et al.

SI Materials and Methods

Generation of Lrrc26 KO mice
Mice obtained from KOMP were bred with CMV-Cre deleter mice (B6.C-Tg(CMV-cre)1Cgn/J, The Jackson Laboratory) to delete the selection cassette. The Lrrc26"" strain has been maintained in a C57BL/6 background out to N=6. All procedures related to animal care and treatment conformed to institutional and NIH guidelines. Genotyping of all animals was accomplished with PCR reactions on mouse tail samples, through a pair of PCR reactions. For the KO allele, the primer pairs were 5’AGAACTGGTGGCTTTGATCG and 5’GTCTGTCCTAGCTTCCTCACTG, resulting in a 385 bp product (Fig. S2B), while, for the WT allele, the primer pairs were 5’-TCCGGATGCGCCTTCAACAGTG and 5’-GGAGCACTAGAAACACCTGCAGT, resulting in a 290 bp product.

Bluo-Gal staining
Fresh tissues from Lrrc26"" mice were dissected and quickly immersion-fixed with 0.2% glutaraldehyde in PBS buffer at 4°C for 24 hours. Fixed tissues were transferred to ice-cold 30% sucrose in PBS buffer and kept at 4°C for 24 hours. 20-25 µm frozen tissue sections were then prepared with cryostat at -20°C. Slides were washed once briefly with PBS and then once with 37°C Bluo-Gal dilution buffer (5 mM Potassium Ferricyanide, 5 mM Potassium Ferrocyanide, 2 mM Magnesium Chloride, 0.1% Tween-20 in PBS(pH 7.4)). Just before staining, Bluo-Gal stock solution (4% (w/v) Bluo-Gal (Invitrogen, 15519-028) in dimethylformamide) was diluted at 1:40 in 37°C Bluo-Gal dilution buffer to prepare the fresh Bluo-Gal working solution. Tissue sections were then incubated in the Bluo-Gal working solution at 37°C in a humidity box. In order to obtain clear and specific staining results, the staining times have been optimized as follows: 1-2 hours for lacrimal gland; 2-4 hours for submandibular gland, prostate and trachea; 3-4 hours for vomeronasal organ; 24 hours for all other tested tissues. Wild-type tissue sections were prepared and stained side by side with the same protocol as negative controls. Finally, sections were rinsed in PBS, counterstained with Eosin Y Solution (HT110116-500 ml; Sigma-Aldrich, St. Louis MO USA) or Periodic Acid-Schiff (PAS) Staining System (395B-1KT; Sigma-Aldrich) and mounted with aqueous mounting medium.

RNA extraction and quantitative RT-PCR
Tissues from C57BL/6J mice (8-12 weeks old) were dissected, quickly frozen in liquid nitrogen and, then saved at -80°C. Total RNA from each tissue was isolated using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA USA). cDNA was synthesized using the BioRad iScript cDNA Synthesis Kit (170-8891; BioRad, Hercules, CA USA). For the negative control groups, all components except the reverse transcriptase were included in the reaction mixtures. Real-Time PCR was performed with specific primers (Table S1) and Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham MA, USA; 4367659) under reaction conditions identical to that described previously (1). PCR specificity was verified by the dissociation curve from a single peak which was run following the real-time PCR reaction. Message levels were normalized to
the abundance of β-actin message. The mean value was averaged from 3 separately prepared RNA samples.

**Table S1  Primers used for real-time PCR**

<table>
<thead>
<tr>
<th>gene</th>
<th>Cat.No of QuantiTect Primer Assay from Qiagen</th>
<th>amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kcnma1</td>
<td>Forward: 5′-TCTCAGCATTGGTGCCCTCGTAAT-3′</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GTAGAGGAAGAAGACGTGGAA-3′</td>
<td></td>
</tr>
<tr>
<td>Lrrc26</td>
<td>Forward: 5′-TGTGCCTGCGCTCCCCCTTTGCACT-3′</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CGGATCCGAAAGACTGCTAGTA-3′</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward: 5′-TGGAGAAGAGCTATGAGCTGCTG-3′</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GTATTTGCGATGCCACAGGAT-3′</td>
<td></td>
</tr>
</tbody>
</table>

Note: All the above primers amplify regions crossing adjacent exons.

**Protein preparation, Immunoprecipitation, Protein Deglycosylation and Western Blotting**

Mouse tissues from 8-12 week old mice were frozen in liquid nitrogen, smashed into powder with the liquid nitrogen pulverizer and then homogenized on ice with a Teflon-glass pestle in 0.32 M sucrose in PBS solution, including 10 mM PMSF in acetone and 1%(v/v) Protease Inhibitor Cocktail (P8465-25ml, Sigma-Aldrich). The tissue suspension was centrifuged at 300 g for 10 minutes at 4°C and the supernatant was then transferred to an ultra-speed centrifuge tube. The membrane fraction was isolated by centrifuging the tube at 150,000 g for 60 minutes and the resulting membrane pellet was resuspended in 1% dodecyl maltoside (DDM) lysis buffer (50 mM Na phosphate, 150 mM NaCl, 10 mM KCl, 1% DDM, pH 7.2) with 10 mM PMSF in acetone and 1% (v/v) Protease Inhibitor Cocktail. The suspension was rocked in a 4°C cold room for 2 hours and then centrifuged at 18,000 g for 10 minutes. The supernatant was saved as the membrane protein sample. 1.5 ml of membrane proteins was mixed with 4 μg of a custom polyclonal rabbit anti-mLRRC26 antibody (Prosci Inc., Poway, CA USA) and 30 µl Trueblot Rabbit IP beads (00-8800-25; Rockland Immunochemicals, Limerick, PA USA) at 4°C for overnight. The rabbit anti-LRRC26 antibody was raised to an epitope near the LRRC26 C-terminus corresponding to residues 299-320: RTTVRHLLRRQLDPEGPPSLED. IP Beads were collected by a brief spin at 10,000 g, washed thrice, and finally resuspended in 37.5 µl of 0.5% DDM lysis buffer. To remove the N-linked glycosylated sugars in the IP product, 1 µl of PNGase F (170-6883; Bio-Rad) was used according to manufacturer’s non-denaturing protocol. The deglycosylation reaction was done at 37°C for 1 hour. Proteins were then eluted from beads at room temperature for 30 minutes by the addition of 25 µl Laemml SDS-Sample Buffer (BP-110R, Boston BioProducts, Ashland, MA USA) to each 50 µl reaction mixture. Western blots were performed as previously described (2). For the primary Abs, anti-SLO1 (L6/60; Antibodies Inc., Davis, CA USA) was diluted to 2 µg/ml in 5 ml 0.25% non-fat milk in PBS (0.1% Tween 20); anti-mLRRC26 (ProSci) was diluted to 2.8 µg/ml in 5 ml 0.25% non-fat milk in PBS (0.1% Tween 20); Sodium Potassium ATPase Alpha 1 Antibody (M7-PB-E9) (#NB300-584, Novus Biologicals, Littleton, CO USA ) was diluted to 10 µg/ml in 5 ml 0.25% non-fat milk in PBS (0.1% Tween 20). For secondary antibodies, Mouse TrueBlot Ultra anti-mouse IgG HRP (18-8817-33, Rockland) and Rabbit TrueBlot Ultra anti-rabbit IgG HRP (18-8816-33, Rockland) were 1:2500 diluted in 5 ml 0.25% non-fat milk in PBS (0.1% Tween 20), respectively.
Fig. S1. Quantitative RT-PCR of mouse tissues for presence of *Kcnma1* (*Slo1*) and *Lrrc26* message. (A) For each indicated tissue, three separate RNA preparations (except for whole VNO where n=2) were prepared and each sample run in triplicate. Bars show means and standard errors, while means from individual preparations are given by filled circles. All values for both *Slo1* (*Kcnma1*; gray) and *Lrrc26* (red) were normalized to the abundance of β-actin message. (B) Levels of *Lrrc26* message relative to β-actin are plotted in order of relative abundance with bars shaded for measurements within each 10-fold level of expression.
**Fig. S2. Generation and confirmation of LRRC26 KO.** (A) The wild type Lrcc26 genomic structure is shown. The gene contains two exons with a short intron. 1089 base pairs (nucleotides 25289972-25291060 in mouse chromosome 2) in the wild type gene were replaced with sequence encoding lacZ and a neomycin-resistance (neo) cassette with the latter bracketed by LoxP sites (shown on bottom). The 5’ insertion of the lacZ gene is 17 nucleotides before the original start codon of the Lrcc26 gene and is expected to be under control of the Lrcc26 promoter. Mice heterozygous for the KO allele were obtained following resuscitation of cryopreserved embryos from the KOMP repository. The floxed neomycin cassette was subsequently deleted in crosses with CMV-Cre deleter mice (B6.C-Tg(CMV-cre)1Cgn/J, The Jackson Laboratory). (B) PCR reactions from mouse tails confirm the successful disruption of the Lrcc26 coding region. Each tail sample was used in two separate PCR reactions. Left three lanes show a PCR reaction to test for a 385 bp product corresponding to the Lrcc26 KO allele, while right three lanes test for presence of 290 bp wt allele. (C) Membrane proteins from prostate were purified and subjected to immunoprecipitation with the LRRC26 Ab. (Top) Following western blot, the LRRC26 polyclonal Ab (ProSci) identifies a band of about 40 kD in wt samples, a 35 kD band following deglycosylation with PNGase F, and the absence of any band in the Lrcc26 KO mice. (Bottom) Total membrane protein samples were blotted with Na⁺/K⁺ ATPase Alpha1 Ab (ATP1A1; Novus). Note increase in ATP1A1 in the Lrcc26 KO sample. This was also seen in other tissues (e.g., trachea, mammary gland, lung, and glandular stomach (Fig. 1)). We have no explanation for this. Although ATP1A1 is a typical control marker in membrane protein samples, it is possible that its abundance can be regulated. The increased intensity in the ATP1A1 band does not alter the conclusion that LRR26 protein is absent in the KO tissues.
Figure S3. Representative low resolution views of glandular and intestinal tissues showing positive Bluo-Gal reaction product in the LRRC26 KO mice. In all cases, 20 μm sections were obtained. Tissue-specific details of the fixation and Bluo-Gal reaction incubation conditions are given in the Methods. Tissues include: (A) lacrimal gland, (B) parotid, (C) submandibular gland, (D) sublingual gland, (E) small intestine, and (F) colon.
Figure S4. Comparison of lacrimal and parotid gland Bluo-Gal staining in the same sections highlights tissue-specific variation in effectiveness of Bluo-Gal reaction. (A) Left panel shows a section through both lacrimal (LG) and parotid gland (PG) from a wt mouse following a 2 hour Bluo-Gal incubation, while, on the right, a section from an LRRC26 KO mouse that also shows both lacrimal and parotid glands was also subjected to a 2 hour Bluo-Gal incubation. (B) Left and right hand panels correspond to sections from wt and LRRC26 KO mice, again showing both lacrimal and parotid gland, but incubated in the Bluo-Gal working solution for 24 hours. The factors influencing tissue-specific differences in the Bluo-Gal reaction despite ostensibly similar message levels in wt tissues remain unknown. (C) Sections show comparison of wt (left) and LRRC26 KO (right) Bluo-Gal staining of sublingual gland (SLG) and submandibular gland (SMG) following 2 hours incubation in Bluo-Gal working solution.(D) Sections compare wt and LRRC26 KO staining in sublingual and submandibular glands after 24 hours incubation. Note that ductal cells in the submandibular gland exhibit dark appearance in wt sections after 24 hrs incubation.
Figure S5. PAS-positive, Bluo-Gal positive cells are also found in VNO and conjunctiva. (A) Lower resolution view of VNO section shows that Bluo-Gal positive cells in non-sensory epithelium and also other cells adjacent to lumen in the Lrrc26−/− section (right), but not in wt (left). (B) On the left, higher resolution VNO section shows clumps of PAS-positive cells corresponding to cells of Jacobson’s glands from a wt mouse. On the right, PAS-positive cells overlap with Bluo-Gal positive cells from a section from Lrrc26−/− mouse. Also note Bluo-Gal staining in the non-sensory epithelium (*). (C) Bluo-Gal staining is also observed in the epithelial cell layer of Lrrc26−/− conjunctiva (right), but not in wt. (D) The wt conjunctiva epithelial layer exhibits robust PAS staining, which overlaps with Bluo-Gal staining in the Lrrc26−/− conjunctiva.
Figure S6. Epithelial cell layers in other tissues show dark blue Bluo-Gal staining in the LRRC26 KO mice. As before, tissue-specific details of the fixation and Bluo-Gal reaction incubation conditions are given in the Methods. Tissues positive for Bluo-Gal staining in the LRRC26 KO mice, but not wt, include: (A) trachea (esophagus is negative), (B) bronchioles in lung, (C) oviduct, (D) prostate (urethra is negative), (E) uterus, (F) cervix and vaginal fornix, and (G) lactating mammary gland.
Figure S7. Epithelia cell layers in cervix and vagina exhibit enhanced Bluo-Gal staining during pregnancy. (A) Cervical section from non-pregnant Lrrc26−/− female. (B) Similar cervical section from a pregnant Lrrc26−/− female at P18. (C) Higher magnification section showing vaginal fornix from a non-pregnant Lrrc26−/− female. (D) Section of vaginal fornix from P18 Lrrc26−/− female.
Figure S8. Higher magnification views of pregnant cervix and vagina reveal Bluo-Gal staining in superficial columnar epithelial cells. (A1-A3) Sections from cervix of P18 Lrrc26−/− female showing low resolution view (A1) of Bluo-Gal staining with eosin counterstaining, higher resolution hematoxylin/eosin stained view (A2) showing superficial columnar epithelial cells, and then a similar view of the columnar epithelial layer with strong Bluo-Gal staining (A3). (B1-B3) Sections from vagina of P18 Lrrc26−/− female show lower resolution view (B1), higher resolution view (B2) showing large columnar epithelial cells, and then the Bluo-Gal staining (B3) in the epithelial layer.
Figure S9. Bluo-Gal staining in lactating mammary gland is associated with alveoli and cells lining ducts. (A) On the left, section (10x) from a wt lactating mammary gland is negative for Bluo-Gal reaction product, while, on the right, sections from Lrrc26−/− lactating mammary gland reveal Bluo-Gal positive cells surrounding ducts and also in cells likely to contribute to alveoli. (B) Left and right panels are as in A, but at 20X magnification to better highlight the loci of Bluo-Gal staining.
Figure S10. Tissues from \textit{Lrre26}\textsuperscript{-/-} mice that are negative for Bluo-Gal staining. Tissues for which Bluo-Gal staining was undetectable and indistinguishable between LRRC26 KO and \textit{wt} included: (A) cerebellum and dorsal cochlear nucleus, (B) olfactory bulb, (C) eye, (D) pancreas, (E) kidney, (F) glandular stomach, (G) spleen, (H) bladder, (I) vas deferens, (J) cauda epididymis, (K) aorta, and (L) cerebral aorta.
Figure S11. Tissues for which nonspecific dark staining was observed in both wt and Lrrc26−/− mice. (A) Tissues were first developed for Bluo-Gal staining and then counterstained with eosin. In sections of ovary from both wt and Lrrc26−/− mice, loci of somewhat darkened tissue were observed which did not differ between genotypes. (B) Sections from both wt and lrrc26−/− testis also show similar darkened loci, with no indication of any specific Bluo-Gal staining. (C) Sections from caput epididymis also exhibit similar dark loci that are identical between wt and KO mice.
Figure S12. KO of LRRC26 shifts gating of BK currents in submandibular acinar cells. A) Currents (shown to +200 mV) were activated with the indicated voltage protocol in a wt acinar cell (left) and an LRRC26 KO acinar cell (right) from submandibular gland. Pipette/intracellular Ca\(^{2+}\) was 250 nM. Red trace reflects voltage step to +100 mV. (B) GV curves were generated from tail currents recorded from traces as in panel A. From fits to individual cells, for 5 wt cells, mean $V_h = 25.7 \pm 6.3$ mV and $z=1.4 \pm 0.2$; for 5 LRRC26 KO cells, $V_h = 169.2 \pm 9.6$ mV with $z=1.3 \pm 0.1$. Fits of averaged GV\(s\) yielded similar values: for wt, $V_h = 25.4 \pm 1.7$ mV (±90% c.l.) with $z=1.5 \pm 0.09$ and, for LRRC26 KO, $V_h = 179.4 \pm 1.5$ mV with $z=0.9 \pm 0.1$. Note that the GV from wt cells exhibited an upward climb at the most positive activation potentials. This was not observed in parotid or lacrimal gland cells. A two component Boltzmann function yielded a better fit to the wt GV curves (not shown), with the 2\(^{nd}\) component approximating about 0.05 of the total conductance. We suggest that this may represent a fraction of BK channels without LRRC26 subunits, as has been noted in heterologous expression studies of LRRC26 with SLO1 subunits (3).
Figure S13. Excised inside-out patches of BK channel activity also reveal gating shift differences between wt and LRRC26 KO animals. (A) BK channels were activated in inside-out patches from a wt parotid cell with symmetric K⁺ solutions and 0 cytosolic Ca²⁺. Robust channel activation occurs at voltages negative to +50 mV. Red boxes highlight traces displayed at higher resolution in panels D and E. (B) BK channels from an inside-out patch from a LRRC26 KO parotid cell with 0 cytosolic Ca²⁺ require voltages in excess of +100 mV to produce appreciable channel activation. (C) GV curves were generated from measurements of steady-state current from BK channel activity from inside-out patches from 3 wt and 3 LRRC26 KO BK parotid cells. For wt, $V_h=38.7\pm5.1$ mV, $z=1.01\pm0.03$; for LRRC26 KO, $V_h=156.9\pm7.6$ mV, $z=1.6\pm0.1$. (D) Trace shows characteristic BK channel activity monitored at -40 mV in a patch from a wt parotid cell. (E) Trace shows openings of individual BK channels at +80 mV in a parotid cell patch from a LRRC26 KO mouse.
Figure S14. Comparison of basic properties of wt and LRRC26 KO parotid cells. (A) On the left, cell capacitance (Cm) is compared for 10 wt and 10 LRRC26 KO parotid cells. The KS test statistic yielded P= 0.186. In the middle, maximum BK conductance is compared for wt and LRRC26 KO cells. KS P= 0.066. On the right, IK current density estimated from current amplitude at -20 mV is compared for wt and LRRC26 KO parotid cells. KS P=0.433. (B) Time constants of activation were measured from 12 wt, 12 LRRC26 KO, and 3 β4 KO parotid cells.

References