Gq activity- and β-arrestin-1 scaffolding-mediated ADGRG2/CFTR coupling are required for male fertility

Dao-Lai Zhang¹,²,³†, Yu-Jing Sun¹,²†, Ming-Liang Ma¹,²†, Yi-jing Wang¹,²†, Hui Lin¹,², Rui-Rui Li¹,², Zong-Lai Liang¹,², Yuan Gao¹,², Zhao Yang¹,², Dong-Fang He¹,², Amy Lin⁴, Hui Mo⁴,², Yu-Jing Lu¹,², Meng-Jing Li¹,², Wei Kong⁵, Ka Young Chung⁶, Fan Yi⁶, Jian-Yuan Li⁶, Ying-Ying Qin⁶, Jingxin Li², Alex R B Thomsen⁴, Alem W Kahsai⁴, Zi-Jiang Chen⁹, Zhi-Gang Xu¹⁰, Mingyao Liu¹¹,¹², Dali Li¹¹*, Xiao Yu²*, Jin-Peng Sun¹,⁴*

¹Key Laboratory Experimental Teratology of the Ministry of Education, Department of Biochemistry and Molecular Biology, Shandong University School of Medicine, Jinan, China; ²Department of Physiology, Shandong University School of Medicine, Jinan, China; ³School of Pharmacy, Binzhou Medical University, Yantai, China; ⁴Department of Biochemistry, School of Medicine, Duke University, Durham, United States; ⁵Key Laboratory of Molecular Cardiovascular Science, Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University, Beijing, China; ⁶School of Pharmacy, Sungkyunkwan University, Suwon, Korea; ⁷Department of Pharmacology, Shandong University School of Medicine, Jinan, China; ⁸Key Laboratory of Male Reproductive Health, National Research Institute for Family Planning, National Health and Family Planning Commission, Beijing, China; ⁹National Research Center for Assisted Reproductive Technology and Reproductive Genetics, Shandong University, Jinan, China; ¹⁰Shandong Provincial Key Laboratory of Animal Cells and Developmental Biology, Shandong University School of Life Sciences, Jinan, China; ¹¹Shanghai Key Laboratory of Regulatory Biology, School of Life Sciences, Institute of Biomedical Sciences, East China Normal University, Shanghai, China; ¹²Department of Molecular and Cellular Medicine, Institute of Biosciences and Technology, Texas A&M University Health Science Center, Houston, United States

Abstract

Luminal fluid reabsorption plays a fundamental role in male fertility. We demonstrated that the ubiquitous GPCR signaling proteins Gq and β-arrestin-1 are essential for fluid reabsorption because they mediate coupling between an orphan receptor ADGRG2 (GPR64) and the ion channel CFTR. A reduction in protein level or deficiency of ADGRG2, Gq or β-arrestin-1 in a mouse model led to an imbalance in pH homeostasis in the efferent ductules due to decreased constitutive CFTR currents. Efferent ductule dysfunction was rescued by the specific activation of another GPCR, AGTR2. Further mechanistic analysis revealed that β-arrestin-1 acts as a scaffold for ADGRG2/Gq/β-arrestin-1/CFTR complex formation in apical membranes, whereas specific residues of ADGRG2 confer coupling specificity for different G protein subtypes, this specificity is critical for male fertility. Therefore, manipulation of the signaling components of the ADGRG2-Gq/β-arrestin-1/CFTR complex by small molecules may be an effective therapeutic strategy for male infertility.

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Introduction

Male infertility is transforming from a personal issue to a public health problem because approximately 15% of reproductive-age couples are infertile, and male infertility accounts for approximately 50% of this sterility (Hamada et al., 2012; Jodar et al., 2015). The unique structure of the male reproductive system increases the difficulty of determining the working mechanisms. Among male reproductive system, the efferent ductules of the male testis play important roles during sperm transportation and maturation by reabsorbing the fluid of the rete testis and maintaining the homeostasis of water and ion metabolism (Hess et al., 1997). Whereas a dysfunction of the efferent ductule reabsorption capacity caused by a developmental defect that produces improper signaling results in epididymal obstructions and abnormal spermiostasis, which ultimately lead to infertility in both humans and other mammals (Hendry et al., 1990; Nistal et al., 1999), manipulating the reabsorption function in the efferent ductules could be developed into a useful contraceptive method for males (Gottwald et al., 2006).

Receptors play key roles in the regulation of fluid reabsorption in tissues such as the proximal tubules and alveoli (Haitchcock et al., 1999; Thomson et al., 2006). In contrast, only a few receptor functions in the efferent ductules have been characterized. Nuclear estrogen receptor α (ERα) must be activated for male reproductive tract development and reabsorption function maintenance to occur (Hess et al., 1997). However, the mechanism by which fluid reabsorption is regulated by cell surface receptors in the efferent ductules is only beginning to be appreciated (Shum et al., 2008). Knockout of an orphan G-protein-coupled receptor (GPCR), ADGRG2 (adhesion G-protein-coupled receptor G2), results in male infertility due to dysregulated fluid reabsorption in the efferent ductules, suggesting an active role for this cell surface receptor in regulating these processes (Davies et al., 2004). However, how ADGRG2 regulates water-ion homeostasis and fluid reabsorption remains elusive.

ADGRG2 belongs to the seven transmembrane receptor superfamily (Hamann et al., 2015), which regulates approximately 80% of signal transduction across the plasma membrane and accounts for 30% of current clinical prescription drug targets. Five different types of G proteins and arrestins act as signaling hubs downstream of these GPCRs, mediating most of their functions (Alvarez-Curto et al., 2016; Cahill et al., 2017; Dong et al., 2017; Li et al., 2018; Liu et al., 2017; Nuber et al., 2016; Thomsen et al., 2016; Yang et al., 2015). In the efferent ductules, it remains unclear how G proteins and their parallel signaling molecules, the arrestins, regulate reabsorption as well as fertility.

Here, we developed a new labeling method utilizing specific red fluorescent protein (RFP) expression driven by the ADGRG2 promoter, which enabled a detailed mechanistic study of efferent ductule functions. By exploiting Adgrg2+/−, Gnaq−/−, Arrb1−/− and Arrb2−/− knockout mouse models, together with the combination of pharmacological interventions and electrophysiological approaches, we have identified the importance of the ubiquitous Gq protein and β-arrestin-1, which confer the ADGRG2 constitutive activity to a basic cystic fibrosis transmembrane conductance regulator (CFTR) current, in fluid reabsorption in the efferent ductules. Both specific Gq activity- and β-arrestin-1 scaffolding-mediated ADGRG2/CFTR coupling are required for male fertility and Cl−/acid-base homeostasis in the efferent ductules. Our results not only reveal how fluid reabsorption in the male efferent ductules is precisely controlled by a specific subcellular signaling compartment encompassing ADGRG2, CFTR, β-arrestin-1 and Gq in non-ciliated cells but also provide a foundation for the development of new therapeutic approaches to control male fertility.

Results

**Gq activity is required for fluid reabsorption and male fertility**

Previous studies have found that knockout of the orphan receptor ADGRG2 causes infertility and fluid reabsorption dysfunction in the efferent ductules, indicating important roles for GPCR signaling in male reproductive functions. Downstream of GPCRs, there are 16 Gα proteins that mediate diverse GPCR functions (DeVree et al., 2016). However, the expression of these G protein subtypes and their functions in the efferent ductules have not been investigated. Here, we show that Gs is more enriched, while G11 and Gi3 have expression levels in the efferent ductules similar to those in brain tissue, whereas all other 11 tested G protein subtypes have detectable expression levels in the
ADGRG2 and CFTR coupling in the efferent ductules and its function in fluid reabsorption

Membrane proteins, including bicarbonate and chloride transporters, sodium/potassium pumps and specific ion channels, are potential osmotic drivers for fluid secretion and reabsorption in the efferent ductules (Estévez et al., 2001; Harvey, 1992; Liu et al., 2015; Park et al., 2001; Russell, 2000; Xiao et al., 2012; Xiao et al., 2011; Zhou et al., 2001). Therefore, we examined the expression levels of these membrane proteins in the efferent ductules and ADGRG2 promoter-labeled ductule cells (Figure 4A and Figure 4—figure supplement 1A). Specifically, Na⁺K⁺Cl⁻ cotransporter (NKCC), down-regulated in adenoma (DRA), CFTR, solute carrier family 26 member 9 (SLC26a9), Na⁺/H⁺ exchanger 3 (NHE3) and the L-type voltage dependent calcium channel Cav1.3 levels were readily measured in ADGRG2 promoter-labeled non-ciliated ductule cells; Na⁺/H⁺ exchanger 1 (NHE1), carbonic anhydrase II (CAII), Short transient receptor potential channel 3 (TRPC3), chloride channel accessory 1 (CLCA1) and Cav1.2 had lower but detectable expression levels, whereas anoctamin-1 (ANO1), V-ATPase and Cav2.2 demonstrated very little expression (Figure 4A and Figure 4—figure supplement 1A). Notably, we used the ADGRG2 promoter to label the non-ciliated cells, as the ADGRG2 receptor is specifically expressed on the apical membrane of these cells in

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Figure 1. The expression of G protein subtypes in the efferent ductules and ADGRG2 promoter-labeled non-ciliated cells. (A) qRT-PCR analysis of mRNA transcription profiles of G proteins in brain tissues and the efferent ductules of WT (n = 3) male mice. Expression levels were normalized to GAPDH levels. *p < 0.05, **p < 0.01, ***p < 0.001, efferent ductules compared with brain tissue. (B) Co-localization analysis of ADGRG2 (red fluorescence) and acetylated-tubulin (green fluorescence) in the efferent ductules of WT mice. Scale bars, 50 μm. (C) Co-localization of ADGRG2 (green fluorescence) and RFP (red fluorescence) in the efferent ductules of WT mice. Scale bars, 50 μm. Figure 1 continued on next page.
and RFP (red fluorescence) in the same cells of male murine efferent ductules infected with the ADGRG2 promoter RFP adenovirus in WT mice. Scale bars, 50 µm. (D) qRT-PCR analysis of mRNA transcription profiles of G protein subtypes in brain tissues and isolated ADGRG2 promoter-labeled non-ciliated cells derived from the efferent ductules of WT (n = 3) male mice. Expression levels were normalized to GAPDH levels. *p < 0.05, **p < 0.01, ***p < 0.001, ADGRG2 promoter-labeled efferent ductule cells compared with brain tissues. n.s., no significant difference. At least three independent biological replicates were performed for Figure 1A and D.

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The following figure supplements are available for figure 1:

**Figure supplement 1.** ADGRG2 is specifically expressed in non-ciliated cells.

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**Figure supplement 2.** The construction of the mouse ADGRG2-promoter-RFP used in the labeling of ADGRG2-expressed cells.

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**Figure 1 continued**

Adgrg2-/-Y mice were not reduced compared with those of their WT littermates (Figure 5B and Figure 5—figure supplement 1). Moreover, application of the CFTR inhibitor CFTRinh-172 increased the pH value of the efferent ductules in WT mice by approximately 0.3 but did not have a significant effect in Adgrg2-/-Y mice, suggesting that CFTR dysfunction in Adgrg2-/-Y mice influences pH homeostasis (Figure 5A–B). Importantly, the pH imbalance in Adgrg2-/-Y mice was rescued by bicarb-free media or application of the carbonic anhydrase inhibitor acetazolamide (Figure 5—figure supplement 2B–C).

In particular, unambiguous co-localization of ADGRG2 and CFTR on the apical membrane was detected (Figure 5C–G and Figure 5—figure supplement 3) and ADGRG2 was associated with CFTR in co-immunoprecipitation assays (Figure 5H and Figure 5—figure supplement 4). Taken together, these results suggest a complex formation and functional coupling of ADGRG2 and CFTR in the non-ciliated cells of the efferent ductules.
Figure 2. Gq activity is required for fluid reabsorption. (A) Images of cultured ligated efferent ductules derived from WT male mice, Adgrg2<sup>−/−</sup> mice and Gnaq<sup>−/−</sup> male mice. Ductule segments were selected by examination of the ciliary beat, which is a marker of cell integrity. Ductule pieces from Adgrg2<sup>−/−</sup>, Gnaq<sup>−/−</sup> or WT mice were ligated, microdissected and cultured for up to 72 hr. Scale bars, 200 μm. (B–C, E–H) Effects of pharmacological intervention on the diameters of ligated efferent ductules derived from WT or Adgrg2<sup>−/−</sup> mice. (B) PTX (100 ng/ml), a Gi inhibitory protein. WT (n = 9) or Adgrg2<sup>−/−</sup>
The outwardly rectifying whole-cell Cl⁻ current (I_{ADGRG2-ED}) of ADGRG2 promoter-labeled efferent ductule cells

We then performed whole-cell Cl⁻ recording of primary ADGRG2 promoter-labeled efferent ductule cells derived from WT and Adgrg2⁻/⁻ mice with normal Cl⁻ concentrations or by substituting Cl⁻ with gluconate (Gluc⁻) in the bath solution (Figure 6A–E and Table 1). Patch-clamp recording on ADGRG2 promoter-labeled non-ciliated cells derived from WT mice revealed a reversible whole-cell Cl⁻ current (I_{ADGRG2-ED}), which was significantly diminished in response to substitution of the bath Cl⁻ solution with Gluc⁻ (148.5 mM Cl⁻ was replaced by 48.5 mM Cl⁻ and 100 mM Gluc⁻) (Figure 6A–B). This whole-cell Cl⁻ current (I_{ADGRG2-ED}) was recovered once Gluc⁻ was substituted with Cl⁻ solution (Figure 6A–B). Further I-V analysis identified an outwardly rectifying whole-cell Cl⁻ current (I_{ADGRG2-ED}) of wild type mice, which was significantly reduced in response to Gluc⁻ substitution (Figure 6C–E and Table 1). In contrast, the I_{ADGRG2-ED} of Adgrg2⁻/⁻ mice was substantially lower than the I_{ADGRG2-ED} of their WT littermates, which showed no significant changes in response to substitution of the bath Cl⁻ solution with Gluc⁻ (Figure 6A–E and Table 1). These results suggested that ADGRG2 deficiency in the efferent ductules significantly reduced the whole-cell Cl⁻ current of ADGRG2 promoter-labeled non-ciliated cells.

CFTR mediates the whole-cell Cl⁻ current of ADGRG2 promoter-labeled efferent ductule cells

We next examined the effects of different Cl⁻ channel and transporter inhibitors on the I_{ADGRG2-ED} of efferent ductule cells derived from Adgrg2⁻/⁻ mice and their WT littermates. Although application of the ANO1 inhibitor Ani9 or the chloride-bicarbonate exchanger inhibitor DIDS exerted no significant effects on the I_{ADGRG2-ED} of WT mice, the specific CFTR inhibitor CFTRinh-172 significantly reduced the I_{ADGRG2-ED} current (Figure 7A–B and Figure 7—figure supplement 1). Moreover, the difference in the I_{ADGRG2-ED} between Adgrg2⁻/⁻ mice and their WT littermates was eliminated by the application of CFTRinh-172(Figure 7A–B). After the application of CFTRinh-172, the I_{ADGRG2-ED} showed no significant response to Gluc⁻ substitution in the bath solution (Figure 7—figure supplement 2). Consistently, when we knocked down CFTR expression in efferent ductules (Figure 7C), the whole-cell Cl⁻ current (I_{ADGRG2-ED}) of WT mice was significantly reduced (Figure 7D–E and Figure 7—figure supplement 3). These results suggested that CFTR is essentially activated in ADGRG2 promoter-labeled efferent ductule cells, which mediate the observed outwardly rectifying whole-cell Cl⁻ current, and ADGRG2 is required for the basic activation of CFTR in these cells.

CFTR is activated by FSK and IBMX (Lu et al., 2010). In response to FSK and IBMX stimulation, the I_{ADGRG2-ED} of both Adgrg2⁻/⁻ and WT mice significantly increased to similar levels (Figure 7F–G), consistent with the western blot results, indicating that CFTR expression levels did not change in Adgrg2⁻/⁻ mice. The results also indicated that basic CFTR activation in ADGRG2 promoter-labeled efferent ductule cells does not represent the full activation state (Figure 7F–G).
Figure 3. Gq expression is required for sperm transportation and male fertility. (A) Representative hematoxylin and eosin staining of WT, Adgrg2<sup>−/−</sup> or Gnaq<sup>−/−</sup> mice. Scale bars, 200 μm. (B–D) Corresponding bar graphs demonstrating the accumulation of spermatozoa according to the hematoxylin and eosin staining of WT (n = 8), Adgrg2<sup>−/−</sup> (n = 9) or Gnaq<sup>−/−</sup> (n = 9) mice. ED: efferent ductules; IS: epididymal initial segment; CA: caput epididymis. (E) Representative photographs of caudal sperm preparation from the caudal epididymis of WT, Adgrg2<sup>−/−</sup> or Gnaq<sup>−/−</sup> mice. Scale bars, 50 μm. (F) Bar Figure 3 continued on next page
Gq activity is required for ADGRG2/CFTR coupling in the efferent ductules

Similar to Adgrg2⁻/⁻ mice, the efferent ductules derived from Gnaq⁺⁺/⁻ mice exhibited imbalances in pH homeostasis (Figure 8A). We utilized Gnaq⁺⁺/⁻ mice because Gnaq⁻⁻ mice were not available due to the infertility of the Gnaq⁺⁺/⁻ mice. Consistently, we observed a significantly decreased whole-cellCl⁻ IADGRG2,ED current of the ADGRG2 promoter-RFP-labeled primary non-ciliated cells in Gnaq⁻⁻/⁻ mice compared with that observed in their WT littermates (Figure 8A–C and Figure 8—figure supplement 1A–B). The application of Ro 31–8220, an inhibitor of the Gq downstream effector PKC, further inhibited the observed IADGRG2,ED and showed much stronger effects than the PKA inhibitor PKI 14–22 (Figure 8—figure supplement 1D–G). These results indicated that the Gq-PKC pathway plays critical roles in basic CFTR activation in the efferent ductules, which controls Cl⁻ and pH homeostasis for efficient fluid reabsorption.

We next investigated whether Gq activation by ADGRG2 is required for CFTR function, as both Gq and ADGRG2 are required for normal CFTR currents in the efferent ductules. In the efferent ductules, the Gq was localized in ADGRG2-expressing cells but not acetylated tubulin-labeled cells (Figure 8E–F and Figure 8—figure supplement 2). Consistently, Gq was readily detected in ADGRG2 antibody immuno-precipitated complexes, whereas Gi was not detectable, suggesting a physical interaction of ADGRG2 with Gq in the efferent ductules (Figure 5H and Figure 5—figure supplement 4). Moreover, the endogenous resting IP1 and cAMP levels of the ligated efferent ductules derived from the Adgrg2⁻/⁻ mice were significantly lower than those of their WT littermates (Figure 8G and H). These decreases were not caused by changes in the expression of the Gs-Adenyl-cyclase or Gq-PLC (Phospholipase C) system because Gs and Gq protein levels were similar (Figure 8—figure supplement 3), and the application of ATP induced similar levels of IP3 accumulation in the Adgrg2⁻/⁻ mice and their WT littermates (Figure 8G). Taken together, these data indicate that Gq regulates fluid reabsorption by mediating ADGRG2/CFTR coupling, and both the Gq-IP3-PKC pathway and the Gs-cAMP pathway were activated in ADGRG2 promoter-labeled efferent ductule cells.

Previous studies have shown that the activation of Angiotensin II receptor, type 2(AGTR2) increases proton secretion (Shum et al., 2008). We therefore stimulated the efferent ductules with different concentrations of angiotensin II and evaluated whether they rescued the fluid reabsorption dysfunction in Adgrg2⁻/⁻ mice by restoring pH homeostasis in the efferent ductules. Although applying 1 μM angiotensin II had no significant effect, administering 100 nM angiotensin II restored fluid reabsorption in the efferent ductules derived from Adgrg2⁻/⁻ mice (Figure 4L–M). This rescue was blocked by only the AGTR2 antagonist PD123319 (Figure 4L) but not by the Angiotensin II receptor, type 1(AGTR1) antagonist candesartan (Figure 4M). In summary, Gq and ADGRG2 regulated fluid reabsorption by maintaining pH and chloride homeostasis. The pharmacological activation of AGTR2 rescued the ADGRG2 or Gq dysfunction involved in fluid reabsorption in the efferent ductules.

ADGRG2/CFTR complex formation mediated by β-arrestin-1 but not β-arrestin-2 is essential for fluid reabsorption in the efferent ductules

In parallel with G protein signaling, arrestins mediate important functions downstream of many GPCRs, including the connection of GPCR activation to channel functions (Alvarez-Curto et al., 2016; Dong et al., 2017; Liu et al., 2017; Thomsen et al., 2016). We therefore examined the fluid reabsorption in Arrb1⁻⁻ and Arrb2⁻⁻ knockout mice. Whereas the efferent ductules derived from Arrb2⁻⁻ knockout mice showed normal fluid reabsorption as well as pH homeostasis compared to their WT littermates, these functions of the efferent ductules derived from Arrb1⁻⁻ knockout mice
Figure 4. Inhibition of CFTR activity in the efferent ductules pheno-copied the activity in Adgrg2−/− mice. (A) qRT-PCR analysis of the mRNA transcription profiles of potential osmotic drivers including selective ion channels and transporters in ADGRG2 promoter-labeled cells, non-ADGRG2 promoter-labeled cells and brain tissues of WT (n = 3) male mice. Expression levels were normalized to GAPDH levels. *p < 0.05, **p < 0.01, ***p < 0.001, ADGRG2 promoter-labeled cells were compared with brain tissues. #p < 0.05, ##p < 0.01, ###p < 0.001, non-ADGRG2 promoter-labeled cells were compared with brain tissues.

Figure 4 continued on next page.

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were significantly impaired (Figure 9A–C and Figure 9—figure supplement 1). Moreover, whereas ADGRG2 and CFTR co-localized in the apical membrane regions of the non-ciliated cells of the efferent ductules derived from Arrb2−/− or WT mice, they were separated in Arrb1−/− mice (Figure 9D–K). In β-arrestin-1-deficient efferent ductules, CFTR localized away from ezrin (Figure 9F–K), an apical membrane marker, suggesting that β-arrestin-1 is required for the correct localization of CFTR. Consistently, whereas CFTR was co-immunoprecipitated with ADGRG2 in WT and Arrb2−/− mice, it was not found in ADGRG2-immunoprecipitated complexes from the efferent ductules derived from Arrb1−/− mice, further suggesting that β-arrestin-1 is an essential component in a signaling complex encompassing ADGRG2 and CFTR in the efferent ductules (Figures 5H and 9L and Figure 9—figure supplement 2).

We therefore used HEK293 cells to investigate the in vitro role of β-arrestins in ADGRG2/CFTR complex formation. Overexpression of β-arrestin-1 but not β-arrestin-2 promoted the interaction between ADGRG2 and CFTR (Figure 9—figure supplement 3), confirming the essential role of β-arrestin-1 in assembly of ADGRG2/CFTR coupling.

**Molecular determinants of ADGRG2 coupling with G protein subtypes and their contribution to the regulation of CFTR activity in vitro**

ADGRG2 belongs to the adhesion GPCR group of the GPCR superfamily (Purcell and Hall, 2018; Monk et al., 2015). Whereas the endogenous ligand of ADGRG2 in the testis is unknown, several members of the same adhesion GPCR subfamily, such as VLGR1 and GRPR56, showed constitutive activity via overexpression in a heterologous system (Purcell and Hall, 2018; Hu et al., 2014; Paavola et al., 2011). To dissect the molecular mechanism underlying ADGRG2 signaling in the modulation of CFTR functions, we overexpressed ADGRG2 and CFTR in HEK293 cells (Figure 10—figure supplement 1). In vitro, the overexpression of ADGRG2 causes constitutive Gs and Gq coupling activity; a stronger effect is observed with ADGRG2β (Figure 10—figure supplements 2–5). Whole-cell recordings were performed to examine the effects of ADGRG2 and CFTR co-expression on membrane currents by using an I-V analysis (Figure 10A). The co-expression of ADGRG2 and CFTR significantly increased the amplitude and slope of the current responses, which were significantly reduced by the CFTR inhibitor CFTRinh-172, compared with cells transfected with CFTR alone, indicating that CFTR channels are activated by ADGRG2 in a recombinant system (Figure 10B–D).

Similarly to primary efferent ductule cells (Figure 7F–G), the application of FSK and IBMX further increased the whole-cell Cl− current in the presence of both ADGRG2 and CFTR, confirming that ADGRG2 increased the basal activity of CFTR but did not stimulate CFTR to a full activation state (Figure 10B–C and Figure 10—figure supplement 5A).

Importantly, increased CFTR activity induced by ADGRG2 was significantly diminished by the PKC inhibitor Ro 31-8220 (Figure 10D and Figure 10—figure supplement 5B–D). Taken together, these
Figure 5. Functional coupling and co-localization of CFTR and ADGRG2 on the apical membrane in the efferent ductules. (A) Intracellular pH (pHi) of the ligated efferent ductules from WT (n = 9) mice and Adgrg2<sup>−/−</sup> (n = 9) mice were measured by carboxy-SNARF (5 μM), with or without incubation with the CFTR inhibitor CFTRinh-172. (B) qRT-PCR analysis of CFTR levels in the efferent ductules of WT (n = 3) or Adgrg2<sup>−/−</sup> (n = 3) mice. (C) Co-localization of ADGRG2 (red fluorescence) and CFTR (sc-8909, Santa Cruz, green fluorescence) in the male efferent ductules of WT mice. Scale bars, 50 μm. (D) Figure 5 continued on next page
Immunofluorescence staining of ADGRG2 (red fluorescence) and CFTR (sc-8909, Santa Cruz, green fluorescence) in the efferent ductules of Adgrg2+/Y mice. Scale bars, 50 μm. (F) Co-localization of ADGRG2 (red fluorescence) and ezrin (green fluorescence) in the male efferent ductules of WT mice. Scale bars, 50 μm. (G) Analysis of ADGRG2 and ezrin fluorescence intensities by Pearson’s correlation analysis. The Pearson’s correlation coefficient was 0.69. (H) ADGRG2 was immunoprecipitated with an anti-ADGRG2 antibody from the male efferent ductules of WT mice or Adgrg2+/Y mice, and co-precipitated CFTR, Gs, Gq, β-arrestin-1, β-arrestin-2 and Gi-1/2/3 levels were examined by using specific corresponding antibodies (CFTR antibody:20738–1-AP, Proteintech). (5A-5B) *p < 0.05, **p < 0.01, ***p < 0.001, Adgrg2+/Y mice compared with WT mice. #p < 0.05, ##p < 0.01, ###p < 0.001. Treatment with selective inhibitors or stimulators was compared with control vehicles. n.s., no significant difference. At least three independent biological replicates were performed for Figure 5A–B.

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The following figure supplements are available for figure 5:

**Figure supplement 1.** Representative agrose gel for the reverse transcription PCR analysis of CFTR mRNA level in efferent ductules of WT or Adgrg2+/Y mice.

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**Figure supplement 2.** pH homeostasis in the efferent ductules was impaired in Adgrg2+/Y mice.

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**Figure supplement 3.** Immunostaining experiments for CFTR location in efferent ductules.

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**Figure supplement 4.** Bar graph representation and statistical analyses of Figure 5H.

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Data demonstrate that ADGRG2 increases CFTR Cl− currents through the activation of Gq-PLC-PKC signaling.

Previous crystallographic studies have shown that the intracellular loop 2 of the β2-adrenergic receptor is important for Gs coupling, and mutations in the intracellular loop three affect G protein coupling activity by receptors (Hu et al., 2014; Rasmussen et al., 2011). We therefore selected mutations in intracellular loops 2 and 3 and examined their effects on the constitutive activity of ADGRG2 in Gs or Gq signaling, as detected by cAMP or NFAT-dual-luciferase reporter (DLR) luciferase measurements (Figure 11A–C and Figure 11—figure supplement 1) in HEK293 cells. Under the equal expression of these mutants in the cell membrane, a double mutation in the ‘DRY’ motif H696A/M697A of ADGRG2 eliminated coupling activity with both Gs and Gq (Figure 11B–C and Figure 11—figure supplement 2). Three mutations in intracellular loop 2, specifically Y698A and F705A, significantly impaired the Gs coupling activity of ADGRG2 but did not exert significant effects on NFAT-DLR activity (Figure 11B–C). However, Y708A in intracellular loop 2 and R803E/K804E in intracellular loop 3 nearly abolished the Gq coupling activity of ADGRG2 but did not have significant effects on intracellular cAMP levels compared with the WT ADGRG2. Thus, the ‘DRY/HMY’ motif mutant is a G-protein dysfunctional mutant for both Gs and Gq signaling. Y698A and F705A are specific Gs-defective mutants, and Y708A and R803E/K804E are specific Gq-defective mutants of ADGRG2 (Figure 11B–C).

The coupling of these ADGRG2 mutants to CFTR activity was then examined using the whole-cell recording technique. Voltage clamps were used to generate the I-V relationships of the CFTR currents in cells co-transfected with CFTR and ADGRG2 (Figure 11D–F and Figure 11—figure supplement 3). Interestingly, although the mutant with a specific Gs signaling defect showed decreased coupling of ADGRG2 to CFTR, the Gq-dysfunctional mutant and the H696A/M697A double Gs/Gq signaling-defective mutant did not demonstrate coupling between ADGRG2 and CFTR (Figure 11D–F). Taken together, these results demonstrate that specific residues in intracellular loops 2 and 3 are determinants of the G protein subtype coupling of ADGRG2. Furthermore, downstream of ADGRG2, Gq signaling is essential for CFTR activation in recombinant in vitro systems.

**Effects of the conditional expression of WT-ADGRG2 or its selective G-subtype signaling mutants on the rescue of reproductive defects in Adgrg2+/Y mice**

We next examined how the molecular determinants of ADGRG2/G protein subtype interactions contribute to the function of ADGRG2 infertility in vivo. Both ADGRG2 WT and G protein subtype
Figure 6. The whole-cell Cl⁻ current recording of ADGRG2 promoter-labeled efferent ductule cells. (A) Time course of whole-cell Cl⁻ current ($I_{\text{ADGRG2-ED}}$) at +100 and −100 mV in ADGRG2 promoter-labeled efferent ductule cells derived from Adgrp2⁻/⁻ mice or their littermates. An ‘a’ or ‘d’ indicates the substitution of the Cl⁻ bath solution with Gluc (148.5 mM Cl⁻ was replaced by 48.5 mM Cl⁻ and 100 mM Gluc); and ‘b’ or ‘e’ indicates the substitution of the Gluc bath solution with Cl⁻ (148.5 mM Cl⁻). ‘a’, ‘b’ and ‘c’ belong to WT mice. ‘d’, ‘e’ and ‘f’ belong to Adgrp2⁻/⁻ mice. (B) The current-voltage relationship of whole-cell Cl⁻ current at −100 mV in WT and Adgrp2⁻/⁻ mice. (C) The current-voltage relationship of whole-cell Cl⁻ current at +100 mV in WT and Adgrp2⁻/⁻ mice. (D) The permeability ratio of Cl⁻/Cs⁺ and Cl⁻/Gluc in WT and Adgrp2⁻/⁻ mice. (E) The reversal potential of whole-cell Cl⁻ current in WT and Adgrp2⁻/⁻ mice.
Figure 6 continued

relationship of $I_{\text{ADGRG2-ED}}$ at specific time points (from 6A) is shown. (C) The whole cell Cl$^-$ current of $I_{\text{ADGRG2-ED}}$ elicited by voltage steps between $-100$ mV and $+100$ mV in a representative ADGRG2-promoter-RFP labeled efferent ductule cells derived from the Adgrg2$^{-/-}$ mice and their wild type littersates. The outwardly rectifying $I_{\text{ADGRG2-ED}}$ was significantly diminished when bath Cl$^-$ was substituted for gluconate (Gluc). (D) Representative whole-cell Cl$^-$ current of ADGRG2 promoter-labeled efferent ductule cells; $I_{\text{ADGRG2-ED}}$ versus voltage (I–V) relationships in response to voltage ramps recorded with a CsCl pipette solution in Adgrg2$^{-/-}$ (n = 8) or WT mice (n = 8). The outwardly rectifying $I_{\text{ADGRG2-ED}}$ was significantly diminished, and its reversal potential ($E_{\text{rev}}$) shifted to the positive direction when Cl$^-$ was substituted for Gluc. (E) Average current densities (pA/pF) measured at 100 mV of (C). Inset: average $E_{\text{rev}}$ (±s.e.m., n = 8 for each condition). **p<0.01, $I_{\text{ADGRG2-ED}}$ in Gluc$^-$ solution was compared with $I_{\text{ADGRG2-ED}}$ in Cl$^-$ solution. ns, no significant difference. At least three independent biological replicates were performed.

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Table 1. Average reversal potential calculated at different Cl$^-$ concentrations for Figure 6C.

| Group   | $E_{\text{rev}}$ (mm) | $E_{\text{rev}}$ [Cl]$_{\text{o}}$ (mm) |
|---------|------------------------|----------------------------------------|
| Nernst  | -4.6                   | 148.5 mM (mV)                          |
| WT      | -4.0 ± 0.51            | 20.1 ± 2.52                            |
| Adgrg2$^{-/-}$ | -4.1 ± 0.36             | 19.4 ± 2.47                            |

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Figure 7. CI currents in the non-ciliated cells of the efferent ductules through CFTR. (A, D and F) Corresponding I-V curves of the whole-cell CI- IADGRG2-ED currents recorded in Figure 6 and (A, D and F) Corresponding I-V curves of the whole-cell CI- IADGRG2-ED currents recorded in Figure 7—figure supplement 1(A,F) and Figure 7—figure supplement 3(D): WT (n = 6), Adgrg2−/− (n = 6), WT +CFTRinh-172 (n = 6), Adgrg2−/−+CFTRinh-172 (n = 6), WT +ANI9 (n = 6), Adgrg2−/−+ANI9 (n = 6), WT +DIDS (n = 6), Adgrg2−/−+DIDS (n = 6); WT +Control RNAi (n = 6), WT +CFTR RNAi (n = 6), Figure 7 continued on next page
Adgrg2-/-Control RNAi (n = 6), Adgrg2-/-CFTR RNAi (n = 6), WT + FSK + IBMX (n = 6), Adgrg2-/-FSK+IBMX (n = 6). (B, E and G) Corresponding bar graph depicting the average current densities (pA/pF) measured at 100 mV in (A), (D) and (F). (C) qRT-PCR analysis of CFTR levels in the efferent ductules treated with CFTR siRNA (n = 3) or control RNAi (n = 3). (B, E and G); *p < 0.05, **p < 0.01, ***p < 0.001, Adgrg2-/- mice compared with WT mice. #p < 0.05, ##p < 0.01, ###p < 0.001. Treatment with selective inhibitors, stimulators or CFTR RNAi was compared with control vehicles or control RNAi. n.s., no significant difference. At least three independent biological replicates were performed for Figure 7B, E and G.

DOI: https://doi.org/10.7554/eLife.33432.019

The following figure supplements are available for figure 7:

**Figure supplement 1.** Effects of different stimulators or inhibitors of osmotic drivers on $I_{\text{ADGRG2-ED}}$ Cl currents of efferent ductule cells derived from Adgrg2-/- mice and their wild type littermates.

DOI: https://doi.org/10.7554/eLife.33432.020

**Figure supplement 2.** Effects of Cl concentration change and CFTRinh-172 on the $I_{\text{ADGRG2-ED}}$ Cl currents.

DOI: https://doi.org/10.7554/eLife.33432.018

**Figure supplement 3.** Effects of CFTR knocked down on the $I_{\text{ADGRG2-ED}}$ Cl currents.

DOI: https://doi.org/10.7554/eLife.33432.021

Mutations Y698A and F705A marginally reduced the inflation of the efferent ductules of Adgrg2-/- mice, whereas the Gq signaling mutants did not exert significant effects on the luminal volume (Figure 13A–F). This result is consistent with the effects of these mutants on sperm numbers in the caudal epididymis, thereby suggesting a direct correlation between efferent ductule reabsorption ability and mature sperm numbers (Figures 12C and 13A–F). Taken together, our results demonstrate that Gq activity is required downstream of ADGRG2, and Gs function contributes to fluid reabsorption in the efferent ductules and sperm transportation.

**Discussion**

Fluid reabsorption is the main function of the efferent ductules and is essential for sperm maturation; it therefore serves as a promising target for the development of new contraceptive methods for men (Hess, 2002). The cell surface orphan receptor ADGRG2 is an X-linked gene specifically expressed in the reproductive system, and recent studies have found that its deficiency results in the dysfunction of fluid reabsorption and male fertility. However, the mechanism by which fluid reabsorption is regulated by ADGRG2 in the efferent ductules remains unclear (Davies et al., 2004). ADGRG2 belongs to the adhesion GPCR (aGPCRs) subfamily, whose members are either structurally essential in specific tissues (VLGR1 participates in forming the ankle link) or critical signaling molecules in the nervous and immune systems (GPR56, CD97 and EMRs) (Purcell and Hall, 2018; Sun et al., 2013; Sun et al., 2016). Although the efferent ductules of Adgrg2-/- mice exhibit normal morphology, our results here have identified essential signaling roles for ADGRG2 in non-ciliated cells of the efferent ductules to maintain pH homeostasis as well as the basic CFTR outward-rectifying current, which is required for fluid reabsorption and sperm maturation. Currently, there have been no reported endogenous ADGRG2 ligands. While an unknown ADGRG2 agonist may be responsible for ADGRG2 function in the efferent ductules, it is also likely that the constitutive activity of ADGRG2 in non-ciliated cells is sufficient to maintain the basic CFTR current and pH homeostasis, which is supported by our data using both primary ADGRG2 promoter-labeled efferent ductule cells and a recombinant heterologous HEK293 system (Figures 5–7 and Figure 10). Therefore, our results provide an example of the functional relevance of the constitutive activity of aGPCRs. Moreover, there are several examples indicating that the constitutive activity of aGPCRs is tunable by mechanical stimulation (Purcell and Hall, 2018; Petersen et al., 2015; Scholz et al., 2015). As ADGRG2 was expressed in efferent ductules that were controlled by extensive tension, it will be interesting to investigate the effects of tension on ADGRG2 functions in future studies.

Downstream of GPCRs, 16 different G protein subtypes and arrestins play important roles in almost every aspect of human physiological processes (Liu et al., 2017; Ning et al., 2015; Yang et al., 2015, 2017). However, the expression and function of five different G protein subtypes as well as arrestins in the efferent ductules have never been systematically investigated. Here, we have determined that the majority of G protein subtypes are expressed in the efferent ductules (Figure 1A and D). Gq activity is essential for male fertility by maintaining basic CFTR activity and
Figure 8. Gq activity regulated Cl\textsuperscript{-} current and pH homeostasis in the efferent ductules. (A) Intracellular pH (pHi) of the ligated efferent ductules from WT (n = 9) mice or Gnaq\textsuperscript{+/--} (n = 9) mice was measured by carboxy-SNARF. (B) The whole-cell Cl\textsuperscript{-} current of the I\textsubscript{ADGRG2-ED} elicited by voltage steps between −100 mV and +100 mV in representative ADGRG2 promoter-RFP-labeled efferent ductule cells derived from Gnaq\textsuperscript{+/--} mice, their WT littermates, or WT murine cells incubated with the PKC inhibitor Ro 31–8220 (500 nM). The whole-cell Cl\textsuperscript{-} I\textsubscript{ADGRG2-ED} current was recorded with a CsCl...
pipette solution (101 mM CsCl, 10 mM EGTA, 10 mM Heps, 20 mM TEACl, 2 mM MgATP, 2 mM MgCl₂, 5.8 mM glucose, pH 7.2, with D-mannitol compensated for osm 290) and a bath solution containing 138 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 10 mM HEPES, pH 7.4 with D-mannitol compensated for osm 310. (C) Corresponding I-V curves of the whole-cell Cl⁻ currents recorded in (B). WT (n = 6), Gnaq⁻/⁻ (n = 6), WT +Ro 31–8220 (n = 6). (D) Corresponding bar graph of the average current densities (pA/pF) measured at 100 mV according to (C). (E) Co-localization of ADGRG2 (red) and Gq (green) in the male efferent ductules. Scale bars, 50 µm. (F) Co-localization of Gq (red) and acetylated-tubulin (yellow) in the male efferent ductules. Scale bars, 50 µm. (G) IP1 levels in the brain tissues, ligated efferent ductules, and livers of WT (n = 9) or Adgrg2⁻/⁻ (n = 9) mice in response to ATP (5 mM) or control vehicles, measured by ELISA. (*p < 0.05, **p < 0.01, ***p < 0.001, Adgrg2⁻/⁻ mice or Gnaq⁻/⁻ mice compared with WT mice. #p < 0.05, ##p < 0.01, ###p < 0.001, Adgrg2⁻/⁻ mice or Gnaq⁻/⁻ mice compared with WT mice. n.s., no significant difference. At least three independent biological replicates were performed for Figure 8A,D,G and H.

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The following figure supplements are available for figure 8:

**Figure supplement 1.** Effects of G protein signaling on the I ADGRG2 ED Cl⁻ currents.

DOI: https://doi.org/10.7554/eLife.33432.023

**Figure supplement 2.** Gq is localized in the ADGRG2 expressed cells, but not the acetylated-tubulin-labeled cells in efferent ductules.

DOI: https://doi.org/10.7554/eLife.33432.024

**Figure supplement 3.** The expression of ADGRG2, CFTR, Gs, Gq, β-arrestin-1, β-arrestin-2 in efferent ductules, brain and liver tissue of WT and Adgrg2⁻/⁻ mice.

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indicated that such a receptor/G protein/β-arrestin mega complex plays important roles in the regulation of important physiological processes, such as fluid reabsorption in the efferent ductules.

Finally, our results suggest that the inhibition of either CFTR or ADGRG2 impairs the resorptive function of the efferent ductules, which may confer a contraceptive function. Indeed, anti-spermato-
genic agents, such as indazole compounds, block CFTR activity (Chen et al., 2005; Gong et al., 2002). Compared with CFTR, which is broadly expressed and has important functions in many tissues, ADGRG2 is specifically expressed in the efferent ductules and epididymis. Contraceptive compounds targeting ADGRG2 may have fewer side effects. Moreover, the dysfunction of ADGRG2 or CFTR is rescued by the activation of AGTR2 in the efferent ductules (Shum et al., 2008). Therefore, a specific agonist of AGTR2 should be considered for the development of therapeutic methods to treat male infertility caused by impaired ADGRG2-Gq-CFTR signaling, such as that observed in CF patients.

Materials and methods

| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Chemical compound, drug           | PTX         | Enzo                | Cat#: BML-G100 | 100 ng/ml              |
| Chemical compound, drug           | U0126       | Sigma               | Cat#: U120   | 10 μM                  |
| Chemical compound, drug           | Ro 31–8220  | Adooq               | Cat#: A13514 | 500 nM                 |
| Chemical compound, drug           | NF449       | Tocris              | Cat#: 1391   | 1 μM                   |
| Chemical compound, drug           | PKI14-22    | Adooq               | Cat#: A16031 | 300 nM                 |
| Chemical compound, drug           | H89         | Beyotime            | Cat#: S1643  | 500 nM                 |
| Chemical compound, drug           | bumetanide  | Aladdinn            | Cat#: B129942| 10 μM                  |
| Chemical compound, drug           | Ani9        | Sigma               | Cat#: SML1813| 150 nM                 |
| Chemical compound, drug           | Niflumic acid (NFA) | Aladdinn | Cat#: N129597 | 20 μM                  |
| Chemical compound, drug           | DIDS        | Sigma               | Cat#: D3514  | 20 μM                  |
| Chemical compound, drug           | GlyH-101    | Adooq               | Cat#: A13723 | 10 μM                  |
| Chemical compound, drug           | CFTRinh-172 | Adooq               | Cat#: A12897 | 10 μM                  |
| Chemical compound, drug           | EGTA        | Aladdinn            | Cat#: E104434| 5 mM                   |
| Chemical compound, drug           | SKF96365    | Sigma               | Cat#: S7809  | 10 μM                  |
| Chemical compound, drug           | Ruthenium red | Sigma         | Cat#: R2751  | 10 μM                  |
| Chemical compound, drug           | Nicardipine | Sigma               | Cat#: N7510  | 20 μM                  |
| Chemical compound, drug           | LaCl3       | Sigma               | Cat#: 449830 | 100 μM                 |
| Chemical compound, drug           | IBMX        | Sigma               | Cat#: I7018  | 100 μM                 |
| Chemical compound, drug           | U73122      | Sigma               | Cat#: U6756  | 10 μM                  |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|----------------------------------|-------------|---------------------|-------------|------------------------|
| Chemical compound, drug          | Forskolin   | Beyotime            | Cat#: S1612 | 10 μM                  |
| Chemical compound, drug          | PD123319    | Adooq               | Cat#: A13201| 1 μM                   |
| Chemical compound, drug          | Candesartan | Adooq               | Cat#: A10175| 1 μM                   |
| Chemical compound, drug          | Amiloride   | Aladdinn            | Cat#: A129545| 1 mM                   |
| Chemical compound, drug          | Acetazolamide | Medchem express   | Cat#: HY-B0782| 500 μM                |
| Peptide, recombinant protein     | ANGII       | China Peptides     |             | 100 nM                 |
| Commercial assay or kit          | Carboxy SNARF–1, acetoxymethyl ester | Invitrogen | Cat#: C-1272 | 5 μM                   |
| Commercial assay or kit          | Lipofectamine TM2000 | Invitrogen | Cat#: 11668–019 |             |
| Commercial assay or kit          | Collagenase I | Sigma           | Cat#: C0130 |             |
| Commercial assay or kit          | cAMP ELISA kit | R and D systems | Cat#: KGE012B |             |
| Commercial assay or kit          | IP1 ELISA assay | Shanghai Lanpai Biotechnology Co., Ltd | Cat#: lp034186 |             |
| Commercial assay or kit          | The dual-luciferase reporter assay system | Promega | Cat#: E1960 |             |
| Antibody                         | ADGRG2 antibody (rabbit polyclonal) | Sigma | RRID: AB_1078923 |             |
| Antibody                         | ADGRG2 antibody (rabbit polyclonal) | Sigma | RRID: AB_2722557 |             |
| Antibody                         | ADGRG2 antibody (sheep polyclonal) | R and D systems | RRID: AB_2722556 |             |
| Antibody                         | CFTR antibody (goat polyclonal) | Santa Cruz | RRID: AB_638427 |             |
| Antibody                         | CFTR antibody (rabbit polyclonal) | Proteintech | RRID: AB_2722558 |             |
| Antibody                         | Gq antibody (goat polyclonal) | Santa Cruz | RRID: AB_2279038 |             |
| Antibody                         | Gq antibody (rabbit polyclonal) | Proteintech | RRID: AB_2111647 |             |
| Antibody                         | Flag antibody (mouse monoclonal) | Sigma | RRID: AB_259529 |             |
| Antibody                         | HA antibody (mouse monoclonal) | Santa Cruz | RRID: AB_627809 |             |
| Antibody                         | GAPDH antibody (rabbit monoclonal) | Cell Signaling | RRID: AB_10622025 |             |
| Antibody                         | Gs antibody (rabbit polyclonal) | Proteintech | RRID: AB_2111668 |             |
| Antibody                         | Gi antibody (mouse monoclonal) | Santa Cruz | RRID: AB_2722559 |             |
| Antibody                         | β-arrestin-1 antibody (rabbit polyclonal) | Dr R.J. Lefkowitz | A1CT |             |
| Antibody                         | β-arrestin-2 antibody (rabbit polyclonal) | Dr R.J. Lefkowitz | A2CT |             |

Continued on next page
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|-------------|------------------------|
| Antibody ANO1 antibody (rabbit polyclonal) | | Proteintech | RRID:AB_2722560 | |
| Antibody Ezrin antibody (rabbit polyclonal) | | Proteintech | RRID:AB_2722561 | |
| Antibody Acetylated Tubulin(Lys40) Antibody(mouse monoclonal) | | Proteintech | RRID:AB_2722562 | |
| Antibody Donkey anti-sheep IgG(H + L) (secondary antibody) | | Abcam | RRID:AB_2716768 | |
| Antibody Donkey anti-rabbit IgG(H + L) (secondary antibody) | | Invitrogen | RRID:AB_2534017 | |
| Antibody Donkey anti-mouse IgG(H + L) (secondary antibody) | | Invitrogen | RRID:AB_141607 | |
| Antibody Donkey anti-goat IgG(H + L) (secondary antibody) | | Invitrogen | RRID:AB_142672, RRID:AB_141788 | |
| Antibody HRP-conjugated Affinipure Rabbit Anti-Sheep IgG(H + L) | | Proteintech | RRID:AB_2722563 | |
| Antibody HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H + L) | | Proteintech | RRID:AB_2722564 | |
| Antibody HRP-conjugated Affinipure Goat Anti-Rabbit IgG(H + L) | | Proteintech | RRID:AB_2722565 | |

All other chemicals or reagents were from Sigma unless otherwise specified.

Mice
Mice were individually housed in the Shandong University on a 12:12 light: dark cycle with access to food and water ad libitum. The use of mice were approved by the animal ethics committee of Shandong university medical school (protocol LL-201502036). All animal care and experiments were reviewed and approved by the Animal Use Committee of Shandong University, School of Medicine. Adgrg2<sup>+/Y</sup> mice were obtained from Dr DLL and MYL at East China Normal University, Shanghai, China. Adgrg2<sup>+/Y</sup> mice and WT mice were generated by crossing WT (C57BL/6J) males mice and Adgrg2<sup>+/+</sup> females mice. Arrb1<sup>−/−</sup> and Arrb<sup>2−/−</sup> mice were obtained from Dr RJ Lefkowitz (Duke University, Durham, NC); Arrb1<sup>−/−</sup> and WT mice were generated by crossing Arrb1<sup>+/+</sup> male mice and Arrb1<sup>+/−</sup> female mice. Arrb2<sup>−/−</sup> and WT mice were generated by crossing Arrb2<sup>+/+</sup> male mice and Arrb2<sup>+/−</sup> female mice. Gnaq<sup>+/−</sup> mice were obtained from Dr JL Liu at Shanghai Jiao Tong University. Gnaq<sup>−/−</sup> mice and WT mice were generated by crossing Gnaq<sup>+/+</sup> male mice and Gnaq<sup>−/−</sup> female mice. All C57BL/6J male mice were purchased from Beijing Vital River Laboratory Animal Technology.

Genotyping the Adgrg2<sup>+/Y</sup> KO mice
Genotyping of the intercrossed mice were examined using following primers: Fcon (Forward-control): TTTCATAGCCAGTGCTCACCTG, Fwt (Forward-wild-type): CCTGGTGCACTGGACCTGAAG, Fmut (Forward-mutant): CTGTGGCCAGACCTTTTGATATRC, R (Reverse-general): CTTCCCTAACATG TGGCATGGC. For the wild-type Adgrg2<sup>+/Y</sup> mice, Fcon, Fwt and R primers were used to generate two PCR products (189 bp, 397 bp); and Fcon, Fmut and R primers were used to generate one PCR product (397 bp). For the mutant Adgrg2<sup>+/Y</sup>, Fcon, Fwt and R primers were used to generate one PCR product (405 bp); and Fcon, Fmut and R primers were used to generate two PCR products (196 bp, 405 bp). The female mice were genotyped by the same method. The knockout of ADGRG2 in these mice was confirmed by western blotting.

Preparation of the membrane fraction of the epididymis and efferent ductules
The membrane fraction of the epididymis or efferent ductules was prepared from pooled mouse tissues (n = 4–6). These tissues (epididymis or efferent ductules) were dounced in a glass tube within

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Zhang et al. eLife 2018;7:e33432. DOI: https://doi.org/10.7554/eLife.33432
Figure 9. β-arrestin-1 is required for fluid reabsorption in the efferent ductules via scaffolding ADGRG2/CFTR complex formation. (A) Diameters of the luminal ductules derived from WT (n = 12), Adgrg2^−/− (n = 12) or Arrb1^−/− (n = 15) mice. (B) Diameters of the luminal ductules derived from WT (n = 12), Adgrg2^−/− (n = 12) or Arrb2^−/− (n = 15) mice. (C) Intracellular pH (pHi) of the ligated efferent ductules derived from WT (n = 9), Arrb1^−/− (n = 9) or Arrb2^−/− (n = 9) mice were measured by carboxy-SNARF. (D) Co-localization of ADGRG2 (red fluorescence) and CFTR (sc-8909, Santa Cruz, green fluorescence)

Figure 9 continued on next page
in the male efferent ductules of Arrb2−/− mice. (E) Analysis of ADGRG2 and CFTR fluorescence intensities in ArrbZ−/− mice by Pearson’s correlation analysis. The Pearson’s correlation coefficient was 0.62. (F) Localization of ADGRG2 (red fluorescence) and CFTR (sc-8909, Santa Cruz, green fluorescence) in the male efferent ductules of Arrb1−/− mice. (G) Analysis of ADGRG2 and CFTR fluorescence intensities in Arrb1−/− mice by Pearson’s correlation analysis. The Pearson’s correlation coefficient was −0.15. (H) Co-localization of ezrin (red fluorescence) and CFTR (sc-8909, Santa Cruz, green fluorescence) in the male efferent ductules of Arrb1−/− mice. (I) Analysis of ezrin and CFTR fluorescence intensities in Arrb2−/− mice by Pearson’s correlation analysis. The Pearson’s correlation coefficient was 0.66. (J) Co-localization of ezrin (red fluorescence) and CFTR (sc-8909, Santa Cruz, green fluorescence) in the male efferent ductules of Arrb1−/− mice, and co-precipitates with CFTR, β-arrestin-1, and β-arrestin-2 were examined by using specific corresponding antibodies (CFTR antibody:20738–1-AP, Proteintech). (9A–C) *p<0.05, **p<0.01, ***p<0.001, Adgrg2Z−/− mice compared with WT mice. #p<0.05, ##p<0.01, ###p<0.001, Arrb1−/− mice or Arrb2−/− mice compared with WT mice. ns, no significant difference. At least three independent biological replicates were performed for Figure 9A–C and L.

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The following figure supplements are available for figure 9:

**Figure supplement 1.** Western blot analysis of β-arrestin1/2 expression in the efferent duct tissue.

DOI: https://doi.org/10.7554/eLife.33432.027

**Figure supplement 2.** β-arrestin-1 is an essential component in a signaling complex encompassing the ADGRG2 and CFTR in efferent ductules.

DOI: https://doi.org/10.7554/eLife.33432.028

**Figure supplement 3.** The complex formation between ADGRG2, β-arrestin-1 and CFTR in HEK293 cells.

DOI: https://doi.org/10.7554/eLife.33432.029

ten volumes of homogenization buffer (75 mM Tris-Cl, pH 7.4; 2 mM EDTA, and 1 mM DTT supplemented with protease inhibitor cocktail). The dounced suspension was centrifuged at 1000 rpm for 15 min to discard the unbroken tissues. The collected suspensions were then centrifuged at 17,000 rpm for 1 hr to prepare the plasma membrane fraction. For the western blot or immunoprecipitation assays, the membranes were re-suspended in lysis buffer (50 mM Tris pH 8.0; 150 mM NaCl; 10% glycerol; 0.5% NP-40; 0.5 mM EDTA; and 0.01% DDM supplemented with protease inhibitor cocktail (Roche, Basel Switzerland) for 30 min.

**Isolation and ligation of efferent ductules**

The efferent ductules were microdissected into 1–1.5 mm lengths and incubated for 24 hr in M199 culture medium containing nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), glutamine (4 mM), 5α-dihydrotestosterone (1 nM), 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 34°C in 95% humidified air and 5% CO2. The segments were then ligated on two ends to exclude the entry and exit of fluids. Digital images of the ductules were analyzed at 0, 3, 12, 24, 36, 48, 60 and 72 hr after ligation. Damaged ductal segments were discarded. A rapid ciliary beat and clear lumens were used as evaluation standards for ductile segments that had undergone ligation. Between 9 and 36 total ductal segments from at least three mice were analyzed for each group. The differences between the means were calculated by one-way or two-way ANOVA.

**Recombinant adenovirus construction (Wang et al., 2009)**

The recombinant adenovirus carrying the RFP or ADGRG2 gene with the ADGRG2 promoter (pm-ADGRG2) from the epididymal genome was produced in our laboratory using the AdEasy system for the rapid generation of recombinant adenoviruses according to the established protocol (Luo et al., 2007). An adenovirus carrying green fluorescent protein (GFP) was used as a control. For the in vivo studies, a single exposure to 5 × 108 plaque-forming units (pfu) of pm-RFP or pm-ADGRG2 adenovirus was delivered to isolated efferent ductules and incubated for 24 hr to allow for sufficient infection. Epididymal efferent ductules or epididymal efferent ductule epithelium were prepared for further experiments.

**Measurement of intracellular pH (pHi) with carboxy-SNARF–1**

Digital images of the ductules were analyzed at 36 hr after ligation. Intracellular pH is examined with SNARF-1, a pH-sensitive fluorophore with a pKα of about 7.5. To load SNARF-1, cultured ductules were incubated with 5 μM SNARF-1-AM (diluted from a 1 mM stock solution in DMSO) for 45 min in
Figure 10. ADGRG2 upregulates CFTR Cl\(^-\) currents through G protein signaling. (A) Whole-cell Cl\(^-\) currents recorded with a CsCl pipette solution in HEK293 cells transfected with plasmids encoding ADGRG2 or/and CFTR, with or without CFTR inhibitor CFTRinh-172 (10 \(\mu\)M) or its activator (FSK (10 \(\mu\)M)+IBMX (100 \(\mu\)M)). (B) Corresponding I-V curves of the whole-cell Cl\(^-\) currents recorded in (C). ADGRG2 (n = 6), CFTR (n = 6), CFTR + CFTRinh-172 (n = 6), CFTR + FSK + IBMX (n = 6), CFTR + ADGRG2 (n = 6), CFTR + ADGRG2+CFTRinh-172 (n = 6), CFTR + ADGRG2+FSK + IBMX (n = 6), CFTR + ADGRG2+Rc 31-8220 (n = 6). (C and D)

Figure 10 continued on next page
Bar graph representation of average current densities (pA/pF) measured at 100 mV according to (B) and Figure 9; (C and D) Bar graph representation of average current densities (pA/pF) measured at 100 mV according to (B) and Figure 10–figure supplement 5C. (10C-10D) *p<0.05, **p<0.01, ***p<0.001, HEK293 cells transfected with CFTR compared with cells transfected with pCDNA3.1. #p<0.05, ##p<0.01, ###p<0.001, HEK293 cells transfected with ADGRG2 compared with non-ADGRG2 transfected cells. $p<0.05, $$p<0.01, $$$p<0.001, CFTRinh-172, FSK, NF449, U73122 or Ro 31–8220 compared with control vehicle. n.s., no significant difference. At least three independent biological replicates were performed for Figure 10C–D.

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The following figure supplements are available for figure 10:

Figure supplement 1. Co-localization analysis of ADGRG2 and CFTR in HEK293 cells.
DOI: https://doi.org/10.7554/eLife.33432.031

Figure supplement 2. Construction and expression of ADGRG2-full length (ADGRG2FL) and a truncated form ADGRG2β.
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Figure supplement 3. Overexpression of ADGRG2FL and ADGRG2β lead to constitutive increased cellular cAMP levels.
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Figure supplement 4. Overexpression of ADGRG2FL and ADGRG2β have constitutive Gq-NFAT signaling activities.
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Figure supplement 5. ADGRG2 upregulates CFTR Cl– currents and Cl– efflux through G protein signaling.
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culture medium at 37°C, 5% CO2. The cells are washed twice with buffer containing 110 mM NaCl, 5 mM KCl, 1.25 mM CaCl2, 1.0 mM MgSO4, 0.5 mM Na2HPO4, 0.5 mM KH2PO4, and 20 mM HEPES, pH 7.4, then placed on the microscope stage in buffer containing 5 mM KCl, 110 mM NaCl, 1.2 mM NaH2PO4, 25 mM NaHCO3, 30 mM glucose, 10 U/ml penicillin, 10 μg/ml streptomycin, and 25 mM HEPES, pH 7.30. The fluorescence was examined using an LSM 780 laser confocal fluorescence microscope (Carl Zeiss) with the excitation wavelength at 488 nm. The emissions of SNARF-1 at 590 and 635 nm were captured in the first two consecutive scans.

Intracellular pH calibration (Seksek et al., 1991)

In vivo pH calibration was performed according to the method developed by Seksek et al. Briefly, after incubation with the fluorescent probe, cells were washed in a buffer containing 10 mM Hepes, 130 mM KCl, 20 mM NaCl, 1 mM CaCl2, 1 mM KH2PO4, 0.5 mM MgSO4, at various pH values obtained by addition of small amounts of 0.1 M solutions of KOH or HCl. The pH changes of the external buffer of the cell suspension were followed with a Tacussel Isis 20000 pH-meter. Addition of nigericin (1 pg/ml) and valinomycin (5 pM) allowed an exchange of K+ for H+ which resulted in a rapid equilibration of external and internal pH. The fluorescence of the probe was excited at 488 nm, then the emission of SNARF-1 at 590 and 635 nm were captured in the first two consecutive scans. The fluorescent ratio values obtained for each pH point were used for the calibration curve obtained with Prism software, from which pHi values of the samples (6.0–8.5) were determined. Determinations were performed in quintuplicate. The sensor does not have significant effects on cell viability.

The effect of bicarbonate on intracellular pH was determined by incubating ductules in culture medium containing 25 mM bicarbonate for 40 min at 37°C, and then transferring these ductules into bicarbonate-free salt solution and then the fluorescence of the SNARF-1 probe was examined (Teti et al., 1989). Bicarbonate-free solutions were prepared by substituting NaHCO3 with Na-glucocinate and equilibrating with air.

1 mM amiloride or 500 μM acetazolamide were added 100 s after the beginning of the measurement to examine the effects of acetazolamide and amiloride.

Quantitative real-time PCR

Total RNA from the mouse efferent ductules was extracted using a standard TRIzol RNA isolation method (Invitrogen, Carlsbad, CA) as previously described (Wang et al., 2014). The reverse transcription and PCR experiments were performed with the Revertra Ace qPCR RT Kit (TOYOBO FSQ-101) using 0.5 μg of each sample, according to the manufacturer’s protocols. The quantitative real-time PCR was conducted in the LightCycler apparatus (Bio-Rad) using the FastStart Universal SYBR
Figure 11. Key mutations of ADGRG2 downregulate CFTR Cl\(^{-}\) currents through G protein signaling. (A) Schematic representation of the location of the selected ADGRG2 mutants in intracellular loop 2 and loop 3 of ADGRG2. (B) Effects of the overexpression of ADGRG2 \((n = 6)\) and its mutations \((n = 6)\) on cAMP levels. (C) Effects of the overexpression of ADGRG2 \((n = 6)\) and its mutations \((n = 6)\) on NFAT-DLR activation. (D) Whole-cell Cl\(^{-}\) currents recorded with a CsCl pipette solution in HEK293 cells overexpressing CFTR, CFTR and ADGRG2-WT, CFTR and ADGRG2-HM696AA, CFTR and ADGRG2-RK803EE. Figure 11 continued on next page.
Green Master (Roche). The qPCR protocol was as follows: 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min; and then increasing temperatures from 65°C to 95°C at 0.1°C/s. The mRNA level was normalized to GAPDH in the same sample and then compared with the control. All primers are listed in Supplementary file 1 and Supplementary file 2.

Immunofluorescence staining

The mice were decapitated, and the epididymis and efferent ductules were removed immediately. After dissection, the epididymis and efferent ductules were fixed in 4% paraformaldehyde by immersion overnight at 4°C. The fixed tissues were then rinsed for 4 hr at 4°C in PBS containing 10% sucrose, for 8 hr in 20% sucrose, and then overnight in 30% sucrose. The tissues were embedded in Tissue-Tek OCT compound (Sakura Fintek USA, Inc., Torrance, CA) and then mounted and frozen at −25°C. Subsequently, 8-μm-thick coronal serial sections were cut at the level of the efferent ductules and mounted on poly-D-lysine-coated slides. The slides were incubated in citrate buffer solution for antigen retrieval. Non-specific binding sites were blocked with 2.5% (wt/vol) BSA, 1% (vol/vol) donkey serum and 0.1% (vol/vol) Triton X-100 in PBS for 1 hr. After blocking, the slides were incubated in primary antibody against ADGRG2 (1:300), CFTR (1:50), Gs (1:20), Gq (1:20), ANO1(1:50), Anti-ezrin(1:50) or Anti-Acetylated Tubulin(Lys40)(1:50) at 4°C overnight. Subsequently, the slides were incubated for 1.5 hr with the secondary antibody (1:500, Invitrogen) at room temperature. For nuclear staining, the slides were incubated with DAPI (1:2000, Beyotime) for 15 min at room temperature. The immunofluorescence results were examined using a LSM 780 laser confocal fluorescence microscope (Carl Zeiss). The normal saline group was treated as the control.

Culture of mouse epididymal efferent duct epithelium (Leung et al., 2001)

After opening the lower abdomen, the efferent ductules were isolated under sterile conditions to remove fat or connective tissue. The ductules were severed into small segments and then transferred to Hanks balanced salt solution (HBSS) containing 0.2% (w/v) collagenase I and 0.1% (w/v) trypsin. Subsequently, the ductules were incubated at 34°C for 1 hr with vigorous shaking (150 strokes/min) and then separated by centrifugation at 800 g for 5 min. The pellets were re-suspended in HBSS containing collagenase I 0.2% (w/v) for 30 min at 34°C with vigorous shaking. The solutions were then centrifuged again at 800 g for 5 min, and the cell pellets were re-suspended in HBSS buffer containing 0.2% (w/v) collagenase I and then subjected to repeated pipetting for 15 min. Finally, the cells were centrifuged at 800 × g again for 5 min and resuspended in M199 medium. The cell suspension was incubated at 34°C for 5–6 hr in 5% CO2. The resulting fibroblasts and smooth muscle cells were attached to the bottom of the culture flask, whereas the epithelial cells were in suspension. The suspensions were collected, and the epithelial cells were seeded on culture flasks.
Figure 12. Conditional expression of ADGRG2 wild-type or its selective G-subtype signaling mutants in the efferent ductules in Adgrg2^+/Y mice and their effects on the morphology, sperm maturation of efferent ductules. (A) Schematic representation of the mouse ADGRG2 promoters used in the rescue experiment. (B) Representative hematoxylin-eosin staining of the WT mice, Adgrg2^+/Y mice or Adgrg2^+/Y mice infected with lentivirus encoding ADGRG2-WT or different G-subtype mutants at the efferent ductules, initial segment or caput of the epididymis. Scale bars, 200 µm. (C) Bar graph
Constructs

The wild-type ADGRG2 full-length (ADGRG2FL) plasmid was obtained from Professor Xu Z. G. at Shandong University School of Life Sciences, Jinan, Shandong, China. ADGRG2 was cloned from mouse total cDNA libraries using the following primers: forward, ATTCCTCGAGGATGCTTTTCTCTGGTG; and reverse, ATTAGAATTCCATTGGCTCGATAAAGTG. The sequences were inserted into the mammalian pEGFP-N2 expression vector, and then ADGRG2FL and ADGRG2 C-terminal truncations (ADGRG2β) were subcloned into the pcDNA3.1 expression vector, with the flag sequence added at the N-terminus. The ADGRG2FL mutants (HM696AA, H696A, M697A, Y698A, K703A, V704A, F705A, Y708A, QL798AA, RK803EE) were generated using a QuikChange Mutagenesis Kit (Stratagene). All of the mutations were verified by DNA sequencing. All primers are listed in Supplementary file 3.

Cell culture, transfection, and western blotting

HEK293 cells were obtained from Cell Resource Center of Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, Shanghai, China). The cell line was validated by STR profiling (Shanghai Biowing Applied Biotechnology (SBWAB) Co. Ltd.) and was negative for mycoplasma as measured by MycoAlert Mycoplasma Detection Kit (Lonza). HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Thermo Scientific, Scoresby, Victoria, Australia), penicillin (100 IU/ml), and streptomycin (100 μg/ml) as previously described (Hu et al., 2014; Wang et al., 2014). For receptor or other protein expression, plasmids carrying the desired genes were transfected into cells using Lipofectamine TM 2000 (Invitrogen). To monitor the protein expression levels, cells were collected 48–72 hr post-transfection with lysis buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1 mM NaF; 1% NP-40; 2 mM EDTA; Tris-HCl, pH 8.0; 10% glycerol; 0.25% sodium deoxycholate; 1 mM Na2VO4; 0.3 μM aprotinin; 130 μM bestatin; 1 μM leupeptin; 1 μM pepstatin; and 0.5% IAA). The cell lysates were subjected to end-to-end blot rotation for 20 min and spun at 12,000 rpm for 20 min at 4°C. Then, an equal volume of 2 x loading buffer was added. Proteins were denatured in the loading buffer and subjected to western blot analysis. The protein bands from the western blot were quantified using ImageJ software (National Institutes of Health, Bethesda MD). Each experiment was repeated at least in triplicate. A data analysis was conducted using GraphPad software.

Co-immunoprecipitation

The efferent ductules of WT or Adgrg2−/− mice were dissected into small pieces. The interaction between proteins is stabilized by addition of 1 ml of cross-linker buffer (D-PBS containing 10 mM HEPES and 2.5 mM DSP in 1:1 (v/v) dimethyl sulfoxide (DMSO)) as previously described (Ning et al., 2015; Yang et al., 2015). After continuous slow agitation for 30 min at room temperature, crosslinking was stopped by adding 25 mM Tris-HCl (pH 7.5) and incubated for another 15 min. The tissue were washed with cold PBS and then lysed in cold lysis buffer with protease inhibitors. After centrifugation, the supernatants were incubated with anti-ADGRG2 antibody (AF7977, R and D systems) for at least 2 hr at 4°C. Next, Protein A/G PLUS-Agarose (sc-2003, Santa Cruz) was added, and the complexes were incubated overnight at 4°C. The beads were washed with PBS buffer several times, and

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The following figure supplement is available for figure 12:

Figure supplement 1. Effect of the conditional expression of ADGRG2-WT or its selective G-subtype signaling mutants on the rescue of reproductive defects in Adgrg2−/− mice.

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Figure 12 continued representing the quantitative analysis of the number of sperm shown in Figure 12B from at least four independent experiments. (D–E) The corresponding bar graph of the accumulation of spermatozoa according to the hematoxylin-eosin staining of the WT, Adgrg2−/− mice or Adgrg2−/− mice infected with the lentivirus encoding different ADGRG2 constructs compared with encoding different ADGRG2 constructs compared with the control lentivirus. $, p<0.05, $$, p<0.01, $$$, p<0.001; Adgrg2−/− mice infected with the lentivirus encoding different ADGRG2 constructs compared with Adgrg2−/− mice infected with the ADGRG2-WT lentivirus. n.s., no significant difference. At least three independent biological replicates were performed for Figure 12C–E.

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The following figure supplement is available for figure 12:

Figure supplement 1. Effect of the conditional expression of ADGRG2-WT or its selective G-subtype signaling mutants on the rescue of reproductive defects in Adgrg2−/− mice.
Figure 13. Effects of conditional expression of ADGRG2 wild-type or its selective G-subtype signaling mutants in Adgrg2⁻/⁻ mice on the fluid reabsorption of efferent ductules. 

(A) Effects of the expression of the ADGRG2-WT adenovirus on the diameter of the ligated efferent ductules derived from the WT or Adgrg2⁻/⁻ mice. 

(B–F) Effects of the expression of adenovirus encoding different ADGRG2 mutants on the diameter of the ligated efferent ductules derived from the WT (n = 12) or Adgrg2⁻/⁻ (n = 12) mice. 

(A–F) *p < 0.05, **p < 0.01, ***p < 0.001; Adgrg2⁻/⁻ mice infected with the empty adenovirus.
proteins were denatured in the SDS-PAGE loading buffer and subjected to western blot analysis with the indicated antibodies.

**Whole-cell patch-clamp recording (Guo et al., 2014)**

The efferent ductules infected by adenovirus with the ADGRG2 promoter were isolated, and epithelial cells were purified and cultured on coverslips before the patch-clamp recording. ADGRG2-promoter labeling was achieved by observation of the RFP fluorescence with the microscope. HEK293 cells transfected with plasmids encoding CFTR together with or without the ADGRG2 wild type or its mutants were cultured on coverslips before the patch-clamp recording. Borosilicate glass-made patch pipettes (Vitrex, Modulohm A/S, Herlev, Denmark) were pulled with a micropipette puller (P-97, Sutter Instrument Co.) to a resistance of 5–7 MΩ after they were filled with pipette solution. The ionic current was recorded with a data acquisition system (DigiData 1322A, Axon Instruments) and an amplifier (Axopatch-200B, Axon Instruments, Foster City, CA). The command voltages were controlled by a computer equipped with pClamp Version nine software. For the whole cell Cl⁻ current measurement, cells were bathed in a solution of NaCl at 130 mM, KCl at 5 mM, MgCl₂ at 1 mM, CaCl₂ at 2.5 mM, and HEPES 20 mM, and D-mannitol was added to an osmolarity of 310 (pH 7.4). Pipettes were filled with a solution of 101 mM CsCl, 10 mM EGTA, 10 mM Heps, 20 mM TEACl, 2 mM MgATP, 2 mM MgCl₂, 5.8 mM glucose, pH7.2, with D-mannitol compensated for osm 290. When the whole-cell giga-seal was formed, the capacitance of the cell was measured. The whole-cell current was obtained by a voltage clamp with the commanding voltage elevated from −100 mV to +100 mV in 20 mV increments (Yu et al., 2011). Further validation of these observed currents were Cl⁻ selective was provided by experiments in which 100 mM of the extracellular Cl⁻ was replaced by gluconate.

**cAMP ELISA**

The efferent ductules were carefully microdissected under sterile conditions to remove fat or connective tissue and then were ligated on two ends to exclude the entry and exit of fluids. After 24 hr, these tissues were rinsed with PBS and homogenized with a tissue homogenizer in cold 0.1 N HCl containing 500 μM IBMX at a 1:5 ratio (w/v). The supernatants were collected after the centrifugation of the tissue lysates at 10,000 × g and then neutralized with 1 N NaOH. The supernatant was collected for the cAMP determination by ELISA according to the manufacturer’s instructions.

**IP1 ELISA**

The efferent ductules were ligated on two ends for 24 hr, and then were added 5 mM ATP or control vehicles to the tissues for 30 min. After half an hour, the tissues were homogenized with a tissue homogenizer in an assay buffer (10 mM HEPES, 1 mM CaCl₂, 0.5 mM MgCl₂, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, 50 mM LiCl, pH 7.4). The 50 mM LiCl was added to block the IP1 degradation. The lysates were centrifuged at 10,000 × g to remove insoluble components, and the supernatant was then collected for IP1 determination by ELISA (lp034186) according to the manufacturer’s instructions.

**GloSensor cAMP assay**

The GloSensor cAMP assay was performed as previously described (Binkowski et al., 2009; Fan et al., 2008; Hu et al., 2014; Kimple et al., 2009). HEK293 cells were transfected with the GloSensor plasmid and the desired expression plasmids (0.8 μg of total DNA) with Lipofectamine 2000 in 24-well dishes. Twenty-four hours after transfection, the cells were plated on 96-well plates at a cell density of 20,000 cells/well. The cells were maintained in DMEM for another 24 hr, washed with PBS and then incubated with 100 μl of solution containing 10% FBS, 2% (v/v) GloSensor cAMP.
Figure 14. Schematic diagram depicting the GPCR signaling pathway in the regulation fluid reabsorption in the efferent ductules. The ADGRG2 and CFTR localized at cell plasma membrane, whereas Gs and Gq localize at the inner surface of non-ciliated cells. Deficiency of ADGRG2 in Adgrg2/−/− mice, reducing the Gq protein level by half in Gnaq+/− mice or PKC inhibitor Ro 31–8220 significantly destroyed the coupling of ADGRG2 to CFTR, thus impaired Cl− and H+ homeostasis and fluid reabsorption of efferent ductules. Structurally, residues in intracellular loops 2 and 3 of ADGRG2 are required for the specific interactions between ADGRG2 and Gq, which are required for CFTR and ADGRG2 coupling and fluid reabsorption. In addition Figure 14 continued on next page
to G protein signaling, β-arrestin-1 is also required for fluid reabsorption in efferent ductules by scaffolding the ADGRG2 and CFTR coupling and complex formation. Therefore, a signaling complex including ADGRG2, Gq, β-arrestin-1 and CFTR that specifically localizes in non-ciliated cells is responsible for the regulation of Cl⁻ and H⁺ homeostasis and fluid reabsorption in the efferent ductules; thus, these functions are important for male fertility. Moreover, activation of the AGTR2 could rescue the H⁺ metabolic disorder caused by ADGRG2 deficiency, which restored the ability of fluid reabsorption in efferent ductules, providing a potential therapeutic strategy in treatment of male infertility caused by dysfunction of GPCR-CFTR signaling in non-ciliated cells.

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Reagent and 88% CO₂-independent medium in each well for 2 hr. The cAMP signal was examined using a luminescence counter (Mithras LB 940).

NFAT dual-luciferase reporter(DLR) assay (Hu et al., 2014)

HEK293 cells in 24-well dishes were co-transfected with plasmids encoding ADGRG2 or its mutants, pGL4.16-NFAT luciferase or pGL4.16-basic luciferase, and pRL-TK Renilla using Lipofectamine 2000. These cells were cultured for approximately 48 hr and then harvested by the addition of 1× passive lysis buffer. After incubation for 15 min at room temperature with shaking, the cell lysates were centrifuged for 10 min at 12,000 rpm at 4°C. NFAT-DLR activity was quantified by a standard luciferase reporter gene assay and then normalized to Renilla luciferase activity (Promega) as previously described (Wang et al., 2014). At least three independent experiments were executed for each dual-luciferase reporter (DLR) assay.

Recombinant lentivirus construction and lentivirus injection

Recombinant lentiviruses containing the ADGRG2 gene and its mutants (HM696AA, H696A, M697A, Y698A, K703A, V704A, F705A, Y708A, QL798AA, RK803EE) under the ADGRG2 promoter were produced according to standard procedures (Tiscornia et al., 2006; Ye et al., 2008). The lentivirus titer was 1 × 10⁹ TU/ml. Mice were anesthetized with 10% chloral hydrate and then the conditional expression of ADGRG2-WT or its selective G-subtype signaling mutants’ lentivirus were microinjected into the interstitial space of the efferent ductules and the initial segment of epididymis at a multiplicity of infection of 100. After 14–21 days, the epididymis transfected with lentivirus were collected for use in further experiments.

Histology (Mendive et al., 2006)

The epididymis and efferent ductules were removed and fixed overnight at 4°C in 4% paraformaldehyde and stored in 70% ethanol until further use. The tissues were dehydrated, embedded in paraffin, and then sectioned into 10 μm slices. In most cases, the whole epididymis was sectioned, and representative samples throughout the organ were mounted on slides for hematoxylin and eosin staining. Hematoxylin and eosin staining was performed according to standard procedures.

Analysis of spermatozoa (Davies et al., 2004)

Spermatozoa from the caudal epididymis of the wild-type (n = 13) or Adgrg2⁻/⁻ knockout (n = 12) mice (ages between 15 and 20 weeks) were collected. The caudal region from the epididymis was open and incubated for 10 min in PBS at 34°C to allow the spermatozoa to appear. The spermatozoa were counted and analyzed by spreading the diluted homogenous suspension over a microscope slide.

Treatment of mice efferent ductules with CFTR siRNA dicer

CFTR siRNA was designed as described before (Ruan et al., 2012; Wang et al., 2006) and chemically modified by the manufacturer (GenePharma). Sequences corresponding to the siRNA of scrambled were: sense, 5’-CUUCCUCUCU UUCUCUCCU UGUGA-3’; and antisense, 5’- TCACA AGGGAGAAA AGAGAGAAG-3’ or CFTR-specific siRNA-CFTR, dicer-1: sense, 5’-GUGCAA UUCAGAGCUUUGUGGAAACG-3’; and antisense, 5’- CUGUCCACAAA GCUCTGAACUUUGCAGC-3’; CFTR-specific siRNA-CFTR, dicer-2: sense, 5’-GACAACUUGUUAGUCUUCUUUUGG-3’; and anti-sense, 5’- UUGGAAAGAGACUAACAGUGUC-3’; CFTR-specific siRNA-CFTR, dicer-3: sense, 5’-
GAGAUUGAU GGUGUCUCAUGGAAUU-3'; and antisense, 5'-AAUUCCAUGAGACACCAUCAAUC UC-3'; For in vivo studies, 15 μg of the siRNA dissolved in 30% pluronic gel (Pluronic F-127, Sigma) solution was delivered to the mice effenter ductules immediately as previously described (Wang et al., 2009). After 7 days, the epididymis transfected with siRNA were collected for further experiments.

**Statistics**

All the western blots were performed independently for at least three times, and the representative experimental results were shown in the main or supplementary figure. All the data are presented as the mean ±SD from at least three independent experiments. Statistical comparisons were performed using an ANOVA with GraphPad Prism5. Significant differences were accepted at p<0.05. The sequence alignments were performed using T-coffee.

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**Author contributions**

Dao-Lai Zhang, Data curation, Software, Formal analysis, Funding acquisition, Investigation, Visualization, Methodology, Writing—original draft; Yu-Jing Sun, Data curation, Software, Formal analysis, Investigation, Visualization, Methodology, Writing—original draft; Ming-Liang Ma, Yi-jing Wang, Software, Investigation, Visualization, Methodology; Hui Lin, Software, Investigation, Methodology; Rui-Rui Li, Zong-Lai Liang, Amy Lin, Investigation, Methodology; Yuan Gao, Hui Mo, Yu-Jing Lu, Meng-Jing Li, Investigation; Zhao Yang, Formal analysis; Dong-Fang He, Supervision; Wei Kong, Ka Young Chung, Fan Yi, Jian-Yuan Li, Ying-Ying Qin, Jingxin Li, Methodology; Alex R B Thomsen, Alem W Kahsai, Zhi-Gang Xu, Data curation; Zi-Jiang Chen, Mingyao Liu, Dali Li, Resources; Xiao Yu, Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Methodology, Project administration; Jin-Peng Sun, Conceptualization, Data curation, Formal analysis, Supervision, Funding acquisition, Validation, Methodology, Writing—original draft, Project administration, Writing—review and editing.
**Author ORCIDs**

Dao-Lai Zhang [http://orcid.org/0000-0003-4428-8731](http://orcid.org/0000-0003-4428-8731)

Amy Lin [http://orcid.org/0000-0001-6723-5443](http://orcid.org/0000-0001-6723-5443)

Jin-Peng Sun [http://orcid.org/0000-0003-3572-1580](http://orcid.org/0000-0003-3572-1580)

**Ethics**

Animal experimentation: Mice were individually housed in the Shandong university on a 12:12 light:dark cycle with access to food and water ad libitum. The use of mice was approved by the animal ethics committee of Shandong university medical school (protocol LL-201502036). All animal care and experiments were reviewed and approved by the Animal Use Committee of Shandong University School of Medicine.

**Decision letter and Author response**

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**Additional files**

**Supplementary files**

- Supplementary file 1. Primers for the Quantitative RT-PCR (qRT-PCR) analysis of mRNA transcription profiles of G protein subtypes and β-arrestins.

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- Supplementary file 2. Primers for the Quantitative RT-PCR (qRT-PCR) analysis of mRNA transcription profiles of potential osmotic drivers including selective ion channels and transporters.

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- Supplementary file 3. Primers for the construction of ADGRG2FL mutants (HM696AA, H696A, M697A, Y698A, K703A, V704A, F705A, Y708A, QL798AA, RK803EE).

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