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

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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/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.

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 (Haithcock 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, 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 7 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 (G protein-coupled receptors), 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., 2017a; Nuber et al., 2016; Thomsen et al., 2016; Yang et al., 2015a). 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 RFP (Red
fluorescent protein) 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 CFTR (Cystic fibrosis transmembrane 
conductance regulator) 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 Ga 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 efferent ductules (Figure 1A). ADGRG2 localizes in cells devoid of 
acetylated-tubulin staining, suggesting that it is specifically expressed in non-ciliated 
cells (Figure 1B and Figure 1; Supplemental Figure 1A-C). We next used the 
promoter region of ADGRG2 to direct the expression of the fluorescent protein RFP, 
which enabled the specific labeling of ADGRG2-expressing non-ciliated cells in the 
efferent ductules (Figure 1C and Figure 1; Supplemental Figure 2A-C). After FACS 
(Fluorescence-activated cell sorting), quantitative RT-PCR (qRT-PCR) results 
indicated that ADGRG2-expressing non-ciliated cells have expression levels of Golf, 
Gi2, Gq, G11, and G13 that are higher than those in brain tissue and expression levels
of Gs, G12 and Gz that are similar to those in brain tissue (Figure 1D).

We next investigated the contribution of different G protein subtype signaling pathways to fluid reabsorption in the efferent ductules using specific pharmacological interventions and knockout models. An ADGRG2 knockout mouse was produced by introducing an 11-nucleotide sequence into the first exon of the ADGRG2 gene (Figure 2; Supplemental Figure 1), thereby creating a positive control for fluid reabsorption dysfunction in the efferent ductules (Davies et al., 2004). The wild-type (WT) mice did not show size alterations due to the normal reabsorption of luminal fluid, but the ligated efferent ductules derived from the ADGRG2 knockout mice displayed a 40% increase in luminal area after 72 h of in vitro culture (Figure 2A).

Application of the Gi inhibitor pertussis toxin (PTX) or the MEK-ERK signaling inhibitor U0126 did not have a significant effect on the efferent ductules (Figure 2B and 2C). In contrast, a 50% reduction in Gq protein levels in Gnaq<sup>+/−</sup> mice or the application of the protein kinase C(PKC) inhibitor Ro 31-8220 significantly impaired fluid reabsorption in the efferent ductules, which mimicked the phenotype of the ductules derived from Adgrg2<sup>−/−</sup> mice (Figure 2A, 2D-E and Figure 2; Supplemental Figure 1F-G). The contribution of Gs-PKA (protein kinase A) signaling to fluid reabsorption of the efferent ductules is confounded. While the application of the Gs inhibitor NF449 or the PKA inhibitors PKI14-22 or H89 to the efferent ductules derived from WT mice slightly increased the volume of the efferent ductules (Figure 2F-H), cAMP regulators, such as the adenyl cyclase activator forskolin (FSK) and the phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine(IBMX), increased the volume of the efferent ductules in an acute manner in both Adgrg2<sup>−/−</sup> mice and WT littermates (Figure 2; Supplemental Figure 2A-B). These results suggested that Gs-PKA signaling is finely tuned in the efferent ductules to maintain its fluid reabsorption function because both increasing and decreasing its activity caused detrimental effects. In conclusion, Gi and MEK-ERK signaling exerted no significant effects, whereas Gq-PKC signaling was required for efficient fluid reabsorption in the efferent ductules.

The efferent ductules of the Gnaq<sup>+/−</sup> animals consistently showed the accumulation of obstructed spermatozoa compared with those of WT mice, whereas the lumen of the initial segment and caput region in Gnaq<sup>+/−</sup> mice contained significantly reduced sperm levels (Figure 3A-D). Sperm numbers prepared from the caudal epididymis and the birth rate of the Gnaq<sup>+/−</sup> mice were also significantly
decreased compared with their WT littermates (Figure 3E-G). Taken together, these data demonstrated that among different G protein subtypes, Gq activity is required for fluid reabsorption and male fertility.

**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 (Estevez 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; Supplemental Figure 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; Supplemental Figure 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 efferent ductules (Figure 1B-C and Figure 1; Supplemental Figure 1-2). A higher expression level of a particular membrane protein, such as CFTR, in the ADGRG2 promoter-labeled cells indicated that these membrane proteins are enriched in non-ciliated cells in efferent ductules but does not indicate that the expression of these proteins is dependent on ADGRG2. For example, the CFTR expression level in ADGRG2 promoter-labeled efferent ductule cells derived from Adgrg2\(^{-/-}\) mice did not differ significantly from that in the cells derived from their WT littermates (Figure 4; Supplemental Figure 1D).

We next used a panel of pharmacological blockers to examine whether the inappropriate regulation of these membrane protein functions was involved in the ADGRG2- or Gq-mediated regulation of fluid reabsorption in the efferent ductules.
Importantly, application of the NKCC blocker bumetanide, the ANO1 inhibitor Ani9, the calcium-dependent chloride channel (CaCC) inhibitor niflumic acid (NFA), TRP channel inhibitors including ruthenium red, SKF96365 and LaCl$_3$, the L-type calcium channel blocker nicardipine or chelating extracellular calcium with EGTA showed no significant effects on fluid reabsorption in the efferent ductules in ligation experiments (Figure 4B-H and Figure 4; Supplemental Figure 1B). Application of 4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) to block the chloride-bicarbonate exchanger exerted a small effect only after 60 hours (Figure 4I), and the application of amiloride to inhibit sodium/hydrogen antiporter NHE1 activity exerted an acute effect on fluid reabsorption (Figure 4; Supplemental Figure 1C), an outcome different from that observed in Adgrg2$^{-/-}$ or Gnaq$^{+/+}$ mice (Figure 2D). In contrast, blocking CFTR activity either with GlyH-101 or CFTRinh-172 had significant effects on fluid reabsorption in the efferent ductules and pheno-copied the Adgrg2$^{-/-}$ mice (Figure 4J-K). Collectively, the phenotype caused by inactivating ADGRG2 and administering a CFTR channel blocker in WT mice suggested that CFTR and ADGRG2 may be functionally connected to the regulation of fluid reabsorption.

CFTR is the key regulator of pH homeostasis and chloride in the reproductive and renal systems and has important functions in fluid reabsorption (Chen et al., 2012). Therefore, we measured the pH value of the efferent ductules. The pH homeostasis was impaired in Adgrg2$^{-/-}$ mice, with a pH value of 7.6 for the inner solution in the efferent ductules, compared to a pH of 7.2 in WT littermates (Figure 5A and Figure 5; Supplemental Figure 2). This dysfunction was not caused by decreased CFTR expression because the mRNA levels of CFTR in the Adgrg2$^{-/-}$ mice were not reduced compared with those of their WT littermates (Figure 5B and Figure 5; Supplemental Figure 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$^{-/-}$ mice, suggesting that CFTR dysfunction in Adgrg2$^{-/-}$ mice influences pH homeostasis (Figure 5A-B). Importantly, the pH imbalance in Adgrg2$^{-/-}$ mice was rescued by bicarb-free media or application of the carbonic anhydrase inhibitor acetazolamide (Figure 5; Supplemental Figure 2B-C).

In particular, unambiguous co-localization of ADGRG2 and CFTR on the apical membrane was detected (Figure 5C-G and Figure 5; Supplemental Figure 3) and ADGRG2 was associated with CFTR in co-immunoprecipitation assays (Figure 5H).
and Figure 5; Supplemental Figure 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.

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^{-/Y} 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). The change in the reversal potential (E_{rev}) with Gluc⁻ replacement followed the Nernst equation (Figure 6C and Table 1). In contrast, the I_{ADGRG2-ED} of Adgrg2^{-/Y} 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-6E 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^{-/Y} mice and their WT littermates. Whereas 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; Supplemental Figure 1). Moreover, the difference in the I_{ADGRG2-ED} between Adgrg2^{-/Y} 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 7A-B). 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; Supplemental Figure 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).

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-cell Cl$^-$ $I_{ADGRG2-ED}$ current of the ADGRG2 promoter-RFP-labeled primary non-ciliated cells in $Gnaq^{+/+}$ mice compared with that observed in their WT littermates (Figure 8B-D and Figure 8; Supplemental Figure 1A-B). The application of Ro 31-8220, an inhibitor of the Gq downstream effector PKC, further inhibited the observed $I_{ADGRG2-ED}$ and showed much stronger effects than the PKA inhibitor PKI 14-22 (Figure 8; Supplemental Figure 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 is localized in ADGRG2-expressing cells but not acetylated tubulin-labeled cells (Figure 8E-F and Figure 8; Supplemental Figure 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; Supplemental Figure 4). Moreover, the endogenous resting IP1 and cAMP levels of the ligated efferent ductules derived from the Adgrg2<sup>+/Y</sup> mice were significantly lower than those of their WT littermates (Figure 8G and 8H). 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; Supplemental Figure 3), and the application of ATP induced similar levels of IP3 accumulation in the Adgrg2<sup>+/Y</sup> 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<sup>+/Y</sup> mice by restoring pH homeostasis in the efferent ductules. Whereas applying 1 μM angiotensin II had no significant effect, administering 100nM angiotensin II restored fluid reabsorption in the efferent ductules derived from Adgrg2<sup>+/Y</sup> 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., 2017a; Thomsen et al., 2016). We therefore examined the fluid reabsorption in Arrb1<sup>−/−</sup> and Arrb2<sup>−/−</sup> knockout mice. Whereas the efferent ductules derived from Arrb2<sup>−/−</sup> 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 were significantly impaired (Figure 9A-C and Figure 9; Supplemental Figure 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 (Figure 5H and 9L and Figure 9; Supplemental Figure 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; Supplemental Figure 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 (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 GPR56, showed constitutive activity via overexpression in a heterologous system (Hall, 2018; Hu et al., 2014a; 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; Supplemental Figure 1). In vitro, the overexpression of ADGRG2 causes constitutive Gs and Gq coupling activity; a stronger effect is observed with ADGRG2β (Figure 10; Supplemental Figures 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). Similar 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; Supplemental Figure 5A).

Importantly, increased CFTR activity induced by ADGRG2 was significantly diminished by the PKC inhibitor Ro 31-8220 (Figure 10D and Figure 10; Supplemental Figure 5B-D). Taken together, these 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 3 affect G protein coupling activity by receptors (Hu et al., 2014b; 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; Supplemental Figure 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; Supplemental Figure 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; Supplemental Figure 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 GRP64, 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\(^{-/-}\) 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 mutants were conditionally expressed in the efferent ductules via virus infection under the 1-kb ADGRG2 promoter (Figure 12A). Similar to ADGRG2 WT mice, exogenously introduced ADGRG2 WT and mutants specifically localized to the inner surface of the non-ciliated cells of the efferent ductules (Figure 12; Supplemental Figure 1A).

The efferent ductules of Adgrg2\(^{-/-}\) animals frequently exhibited the accumulation of obstructed spermatozoa compared with observations in WT mice (Figure 12B). The conditional expression of ADGRG2 in non-ciliated cells in Adgrg2\(^{-/-}\) mice significantly reduced this obstruction, whereas the expression of G protein signaling-deficient mutants of ADGRG2, including Y698A, F705A, Y708A, RK803EE and HM696AA, significantly reduced this rescue effect (Figure 12B-E and Figure 12; Supplemental Figure 1B-C). Specifically, conditional infection of the Gs/Gq double signaling-deficient mutant ADGRG2-HM696AA or the Gq signaling-deficient mutants Y708A and RK803EE did not result in differing levels of accumulation in the efferent ductules compared with those in Adgrg2\(^{-/-}\) mice infected with a control virus. The Gs signaling-deficient mutants Y698A and F705A exhibited improved rescue activity compared with the Gq mutants (Figure 12B-E and Figure 12; Supplemental Figure 1B-C).

Consistent with observations in the efferent ductules, the lumen of the initial segment and caput region in Adgrg2\(^{-/-}\) mice showed reduced sperm numbers compared with those in WT mice (Figure 12B, D-E and Figure 12; Supplemental Figure 1C). The exogenous introduction of WT ADGRG2 to non-ciliated cells nearly
restored the appearance of sperm in the initial segment and significantly increased sperm numbers in the caput (Figure 12B and 12E and Figure 12; Supplemental Figure 1C). However, introducing any of the Gs or Gq signaling-deficient mutants into Adgrg2<sup>−/−</sup> mice did not induce a significant effect on sperm number restoration in these regions (Figure 12B and 12E and Figure 12; Supplemental Figure 1C).

Sperm prepared from the caudal epididymis were then examined. Adgrg2<sup>−/−</sup> mice exhibited significantly reduced sperm numbers and presented morphologically abnormal sperm compared with those of WT mice (Figure 12C and Figure 12; Supplemental Figure 1B). Conditional expression of WT ADGRG2 in the efferent ductules restored sperm numbers in the caudal epididymis by more than half, whereas exogenous introduction of the two Gs-deficient mutants Y698A and F705A into Adgrg2<sup>−/−</sup> mice increased sperm numbers by 5-10% compared with those in WT mice. Expression of the other 4 Gs-, Gq- or double-deficient mutants did not rescue the phenotype (Figure 12C).

To investigate whether the sperm production phenotype was related to fluid reabsorption, we isolated the efferent ductules after virus infection with the WT ADGRG2 or one of the mutants and measured the luminal area after ligation. Interestingly, conditional expression of the Gs-deficient mutations Y698A and F705A marginally reduced the inflation of the efferent ductules of Adgrg2<sup>−/−</sup> 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 (Figure 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 (Hall, 2018; Sun et al., 2013; Sun et al., 2016) (GPR56, CD97 and EMRs). 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 (Figure 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 the constitutive activity of aGPCRs is tunable by mechanical stimulation (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., 2017b; Ning et al., 2015; Yang et al., 2015a; Yang et al., 2017b). However, the expression and function of 5 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 1D). Gq activity is essential for male fertility by maintaining basic CFTR activity and pH homeostasis in the efferent ductules (Figure 14). In particular, specific residues in intracellular loops 2 and 3 are structural determinants of the ADGRG2/Gq interaction (Figure 11-14), which mediates the constitutive activity of Gq-PLC-IP3 signaling in non-ciliated cells of the efferent ductules.

Notably, we found that ADGRG2 and Gq regulate fluid reabsorption in the
efferent ductules via the activation of CFTR, an important ion channel whose mutation leads to cystic fibrosis (CF). One of the hallmarks of CF is infertility (Cutting, 2015; Massie et al., 2014), which has a 97-98% incidence rate in male CF patients (Chen et al., 2012). CFTR knockout and the application of specific CFTR inhibitors in animal models indicate that CFTR plays important roles in spermatogenesis and sperm capacitation (Chen et al., 2012). Here, we demonstrated the specific co-localization of ADGRG2 and CFTR in the apical membrane in the non-ciliated cells of the efferent ductules (Figure 5 and Figure 9). CFTR was basically active in ADGRG2 promoter-labeled efferent ductule cells, and this activity was significantly decreased by ADGRG2 or Gq deficiency. The application of a specific CFTR inhibitor, CFTRinh-172, consistently pheno-copied the ligated efferent ductules of Adgrg2^−/− mice (Figure 4K). Further pharmacological intervention in the efferent ductules and recombinant experiments in vitro confirmed the coupling of ADGRG2 and CFTR activity through Gq. Moreover, previous studies have shown that PKC phosphorylation is required for subsequent PKA phosphorylation to fully activate CFTR (Chappe et al., 2004; Jia et al., 1997). Our study not only agreed with the observation that PKC and PKA lie downstream of Gq and Gs, respectively, but also suggested that ADGRG2-activated Gq primes the full activation of CFTR in the efferent ductules. Therefore, our results demonstrate that the physiological and functional coupling of ADGRG2 and CFTR mediated by Gq in the non-ciliated cells of the efferent ductules primes the basic activity of CFTR, which is essential for fluid reabsorption. The ADGRG2-Gq-CFTR signaling axis is important to maintain male reproductive functions (Figure 14).

Parallel to G protein signaling, β-arrestins are known to play important roles in almost all GPCR functions (Cahill et al., 2017; Dong et al., 2017; Liu et al., 2017a; Yang et al., 2017a). Knockout of β-arrestin-1 but not β-arrestin-2 abolished the co-localization of ADGRG2 and CFTR, demonstrating the essential role of β-arrestin-1 in assembling ADGRG2/CFTR/Gq signaling compartmentalization to regulate Cl− and pH homeostasis during fluid reabsorption in the efferent ductules. For decades, GPCR/β-arrestin complexes were thought to play fundamental roles in the internalization and desensitization of G protein signaling. Recently, a mega complex encompassing the GPCR, G trimer proteins and β-arrestins was identified by using an in vitro reconstruction system in HEK293 cells to provide a new paradigm of GPCR signaling (Thomsen et al., 2016). Consistently, we identified the ability of β-arrestin-1...
to facilitate ADGRG2/Gq/CFTR signaling compartmentalization, which 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-spermatogenic 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.

### Key Resources Table

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

**Materials and methods**

**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>−/−</sup> mice were obtained from Dr. DL.L. and MY.L. at East China Normal University, Shanghai, China. *Adgrg2*<sup>+/−</sup> mice and WT mice were generated by crossing WT (C57BL/6J) males mice and *Adgrg2*<sup>+/−</sup> females mice. *Arrb1*<sup>−/−</sup> and *Arrb2*<sup>−/−</sup> mice were obtained
from Dr. R.J. Lefkowitz (Duke University, Durham, NC, USA); \( Arrb1^{+/−} \) and WT mice
mice were generated by crossing \( Arrb1^{+/−} \) male mice and \( Arrb1^{+/+} \) female mice. \( Arrb2^{+/−} \)
and WT mice were generated by crossing \( Arrb2^{+/−} \) male mice and \( Arrb2^{+/+} \) female
mice. \( Gnaq^{+/−} \) mice were obtained from Dr. JL. Liu at Shanghai Jiao Tong University.
\( Gnaq^{+/−} \) mice and WT mice were generated by crossing \( Gnaq^{+/−} \) male mice and \( Gnaq^{+/+} \)
female mice. All C57BL/6J male mice were purchased from Beijing Vital River
Laboratory Animal Technology.

**Genotyping the \( Adgrg2^{−/−} \) KO mice**

Genotyping of the intercrossed mice were examined using following primers: Fcon
(Forward-control): TTTCATAGCCAGTGCTCACCTG, Fwt (Forward-wild-type):
CCTGTTGGCAGACCTGAAG, Fmut (Forward-mutant):
CTGGTTGGCAGACCTTGATAC, R (Reverse-general):
CTTCCTAAATGTGCATGCG. For the wild-type \( Adgrg2^{+/+} \) 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
\( Adgrg2^{−/−} \), 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 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
h 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)
Isolation and ligation of efferent ductules

The efferent ductules were microdissected into 1-1.5-mm lengths and incubated for 24 h 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 h 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 3 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 h 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 36h after ligation. Intracellular pH is examined with SNARF-1, a pH-sensitive fluorophore with a pKa of about 7.5. To load SNARF-1, cultured ductules were incubated with 5μM SNARF-1-AM (diluted from a
1mM stock solution in DMSO) for 45 min in 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 Mg₂SO₄, 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 a 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 pH 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-gluconate
and equilibrating with air.

1mM amiloride or 500μM 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) 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 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 h at 4°C in PBS containing 10% sucrose, for 8 h in 20% sucrose, and then overnight in 30% sucrose. The tissues were embedded in Tissue-Tek OCT compound (Sakura Fintek USA, Inc., Torrance, CA, USA) 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 h. 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 h 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 h 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 h 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.

**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, ATTCTCGAGGA TGCTTTTCTCTGGG; and reverse, ATTGAATTCATTTGCTCGATAAGTG. 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., 2014b; 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 48-72h 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 Na₃VO₄; 0.3 μM aprotinin; 130 μM bestatin; 1 μM leupeptin; 1 μM repstatin; and 0.5% IAA). The cell lysates were subjected to end-to-end rotation for 20 min and spun at 12,000 rpm for 20 min at 4°C. Then, an equal volume of 2x 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 10mM HEPES and 2.5mM DSP in 1:1 (v/v) dimethyl sulfoxide (DMSO)) as previously described (Ning et al., 2015; Yang et al., 2015b). After continuous slow agitation for 30 min at room temperature, crosslinking was stopped.
by adding 25mM Tris-HCl (pH 7.5) and incubated for another 15 minutes. 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&D systems) for at least 2 h 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 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., USA) 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, USA). The command voltages
were controlled by a computer equipped with pClamp Version 9 software. For the
whole cell Cl\(^-\) current measurement, cells were bathed in a solution of NaCl at 130
mM, KCl at 5 mM, MgCl\(_2\) at 1 mM, CaCl\(_2\) 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 Hepes, 20 mM TEACl, 2 mM
MgATP, 2 mM MgCl\(_2\), 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 hours, 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 hours, and then were added 5mM ATP or control vehicles to the tissues for 30 minutes. 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 50mM 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., 2014b; 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 h, washed with PBS and then incubated with 100 μl of solution containing 10% FBS, 2% (v/v) GloSensor cAMP reagent and 88% CO₂-independent medium in each well for 2h. The cAMP signal was examined using a luminescence counter (Mithras LB 940).
NFAT dual-luciferase reporter(DLR) assay (Hu et al., 2014b)

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 h 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 $1 \times 10^9$ 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 UUCUCUCCCU UGUGA-3’; and antisense, 5’- TCACA AGGGAGAGAA AGAGAGGAAG-3’ or CFTR-specific siRNA-CFTR, dicer-1 : sense, 5’-GUGCAAAUUCAGACUUUGUGGAACAG-3’; and antisense, 5’- CUGUCCACAAA GCUCTGAUUUGUCAC-3’; CFTR-specific siRNA-CFTR, dicer-2: sense, 5’-GACAACUUGUAGUCUUCUUUCCAA-3’ ; and antisense, 5’- UUGGAAAGACUAACAAGUUUGUC-3’ ; CFTR-specific siRNA-CFTR, dicer-3 : sense, 5’-GAGAUUGGGA GGUGUCUCAUGGAAUU-3’ ; and antisense, 5’-AAUUCCAUGAGACACCAUAAUCUC-3’ ; For in vivo studies, 15μg of the siRNA dissolved in 30% pluronic gel (Pluronic® F-127, sigma) solution was delivered to the mice efferent ductules immediately as previously described (Wang et al., 2009). After 7 days, the epididymis transfected with siRNA were collected for further experiments.

Statistics

All of the western blots were performed independently for at least 3 times, and the representative experimental results were shown in the main or supplementary figure. All of 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.

**Additional information**

**Competing interests**

The remaining authors have no competing interests to declare.

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**Author contributions**

JP.S and X.Y conceived the whole project. X.Y. and JP.S supervised the overall project design and execution. JP.S designed all GPCR signaling experiments and X.Y.
designed all ion channel experiments. JP.S, X.Y., DL.Z. and Y.J.S. designed all the
detail of the experiments. DL.Z., Y.J.S., ML.M., Y.J.W., H.L., Z.Y., ZG.X., X.Y. and
JP.S. participated in data analysis and interpretation. DL.Z., ML.M., Y.J.S., Y.J.W.,
DF.H. and A.L. performed cell biology, molecular biology, biochemistry experiments
and recombinant adenovirus or lentivirus construction. JY.L., K.Y.Z., W.K., JX.L.,
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H.L., and ZL.L performed electrophysiology experiments. DL.Z., Y.J.S., Y.J.W. and
F.Y. performed all immunofluorescence staining. DL.Z., Y.J.W., YY.Q. and ZJ.C.
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DL.Z., Y.J.S., Y.G., H.M., Y.J.L and MJ.L. performed all animal experiments and
isolation and ligation of efferent ductules. RR.L. performed new qRT-PCR
experiments for G protein and arrestins of the efferent ductule cells derived from WT
and Adgrg2^{-/-} mice. F.Y. helped in design of the animal models. W.K helped in design
of the in vivo RNAi experiments and the KY. C. helped in fluid reabsorption and
design of ADGRG2 mutants for G protein coupling. YY.Q, F.Y. and ZJ.C participated
in immunohistochemical analysis and discussion. X.Y. and JP.S wrote the manuscript.
All of the authors have seen and commented on the manuscript.

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Figure legends

**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 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.

**Figure 2. Gq activity is required for fluid reabsorption.**

(A) Images of cultured ligated efferent ductules derived from WT male mice, *Adgrg2*\(^{−/−}\) mice and *Gnaq*\(^{−/−}\) male mice. Ductule segments were selected by examination of the ciliary beat, which is a marker of cell integrity. Ductule pieces from *Adgrg2*\(^{−/−}\), *Gnaq*\(^{−/−}\) or WT mice were ligated, microdissected and cultured for up to 72 h. Scale bars, 200 μm.

(B-C, E-H) Effects of pharmacological intervention on the diameters of ligated efferent ductules derived from WT or *Adgrg2*\(^{−/−}\) mice.

(B) PTX (100 ng/ml), a Gi inhibitory protein. WT (n=9) or *Adgrg2*\(^{−/−}\) (n=8); (C) U0126 (10 μM), a MEK inhibitor (ERK pathway blockade), WT (n=12) or *Adgrg2*\(^{−/−}\)
(n=12). (E) Ro 31-8220 (500nM), a protein kinase C (PKC) inhibitor, WT (n=12) or Adgrg2\(^{-/+}\) (n=10); (F) NF449 (1 \(\mu\)M), a Gs inhibitor, WT (n=9) or Adgrg2\(^{-/+}\) (n=9); (G) PKI14-22 (300 nM), a PKA inhibitor, WT (n=9) or Adgrg2\(^{-/+}\) (n=9); (H) H89 (500 nM), a non-selective PKA inhibitor, WT (n=9) or Adgrg2\(^{-/+}\) (n=9).

(D) Diameters of the luminal ductules derived from WT (n=27) mice remained unchanged over 72 h, whereas the lumens of the ductules derived from Adgrg2\(^{-/+}\) (n=21) mice and Gnaq\(^{+/+}\) (n=16) mice were significantly increased, indicating fluid reabsorption dysfunction.

(2B-2H) *, \(p<0.05\), **, \(p<0.01\), ***, \(p<0.001\), Adgrg2\(^{-/+}\) mice or Gnaq\(^{+/+}\) mice were 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 2B-H.

**Figure 3. Gq expression is required for sperm transportation and male fertility.**

(A) Representative hematoxylin & eosin staining of WT, Adgrg2\(^{-/+}\) or Gnaq\(^{+/+}\) mice. Scale bars, 200 \(\mu\)m.

(B-D) Corresponding bar graphs demonstrating the accumulation of spermatozoa according to the hematoxylin & eosin staining of WT (n=8), Adgrg2\(^{-/+}\) (n=9) or Gnaq\(^{+/+}\) (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\(^{-/+}\) or Gnaq\(^{+/+}\) mice. Scale bars, 50 \(\mu\)m.

(F) Bar graph depicting the quantitative analysis of the number of sperm shown in Figure 1E of WT (n=8), Adgrg2\(^{-/+}\) (n=10) or Gnaq\(^{+/+}\) (n=10) mice.

(G) Line graph depicting the fertility of Gnaq\(^{+/+}\) (n=6) and WT (n=6) male mice at various ages, as measured by the median number of embryos.

(3B-D and 3F-G): *, \(p<0.05\), **, \(p<0.01\), ***, \(p<0.001\), Adgrg2\(^{-/+}\) mice or Gnaq\(^{+/+}\) mice were compared with WT mice. #, \(p<0.05\), ##, \(p<0.01\), ###, \(p<0.001\). Gnaq\(^{+/+}\) mice were compared with Adgrg2\(^{-/+}\) mice. n.s., no significant difference.
At least three independent biological replicates were performed for Figure 3B-D and 3F-G.

Figure 4. Inhibition of CFTR activity in the efferent ductules pheno-copied the activity in Adgrg2<sup>−/−</sup> 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.

(B-M) Effects of different channel blockers on the diameters of luminal ductules derived from WT or Adgrg2<sup>−/−</sup> mice. (B) Bumetanide (10 μM), an NKCC blocker, WT (n=9) or Adgrg2<sup>−/−</sup> (n=10); (C) Ani9 (150 nM), an ANO1 inhibitor, WT (n=9) or Adgrg2<sup>−/−</sup> (n=9); (D) NFA (20 μM), a CaCC inhibitor, WT (n=9) or Adgrg2<sup>−/−</sup> (n=10); (E) ruthenium red (10 μM), a non-specific TRP channel blocker, WT (n=12) or Adgrg2<sup>−/−</sup> (n=12); (F) SKF96365 (10 μM), a TRPC channel inhibitor, WT (n=12) or Adgrg2<sup>−/−</sup> (n=9); (G) nicardipine (20 μM), an L-type calcium channel blocker, WT (n=12) or Adgrg2<sup>−/−</sup> (n=12); (H) EGTA (5 mM), an extracellular calcium chelator, WT (n=9) or Adgrg2<sup>−/−</sup> (n=9); (I) DIDS (20 μM), a chloride-bicarbonate exchanger blocker, WT (n=9) or Adgrg2<sup>−/−</sup> (n=10); (J) GlyH-101 (25 μM), a non-specific CFTR inhibitor, WT (n=17) or Adgrg2<sup>−/−</sup> (n=15); (K) CFTRinh-172 (10 μM), a specific CFTR inhibitor, WT (n=12) or Adgrg2<sup>−/−</sup> (n=10). (L) Effects of an angiotensin receptor agonist (angiotensin II, 100 nM) and PD123319 (1 μM, an AT2 receptor antagonist) on the diameters of luminal ductules derived from WT or Adgrg2<sup>−/−</sup> mice (n≥12). (M) Effects of angiotensin II (100 nM) and candesartan (1 μM, an AT1 receptor antagonist) on the diameters of luminal ductules derived from WT or Adgrg2<sup>−/−</sup> mice (n≥12).

Application of GlyH-101 and CFTRinh-172 to ligated ductules derived from WT mice recapitulated the phenotype of the ductules derived from Adgrg2<sup>−/−</sup> mice.
(4A-M)*, p<0.05, **, p<0.01, ***, p<0.001; Adgrg2<sup>-/Y</sup> 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 4A-M.

**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>-/Y</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>-/Y</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) Analysis of ADGRG2 and CFTR fluorescence intensities by Pearson’s correlation analysis. The Pearson's correlation coefficient was 0.76.

(E) Immunofluorescence staining of ADGRG2 (red fluorescence) and CFTR (sc-8909, Santa Cruz, green fluorescence) in the efferent ductules of Adgrg2<sup>-/Y</sup> 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<sup>-/Y</sup> 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<sup>-/Y</sup> 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.

**Figure 6. The whole-cell Cl⁻ current recording of ADGRG2 promoter-labeled efferent ductule cells**

(A). Time course of whole-cell Cl⁻ current (I_{ADGRG2-ED}) at +100 and -100 mV in ADGRG2 promoter-labeled efferent ductule cells derived from Adgrg2^{−/−} 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 “e” belong to WT mice. “d”, “e” and “f” belong to Adgrg2^{−/−} mice.

(B) The current-voltage relationship of I_{ADGRG2-ED} at specific time points (from 6A) is shown.

(C) The whole cell Cl⁻ current of I_{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 littermates. The outwardly rectifying I_{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_{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_{ADGRG2-ED} was significantly diminished, and its reversal potential (E_{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_{rev} (± s.e.m., n=8 for each condition). **, p<0.01, I_{ADGRG2-ED} in Gluc⁻ solution was compared with I_{ADGRG2-ED} in Cl⁻ solution. ns, no significant difference. At least three independent biological replicates were performed.

**Figure 7. Cl⁻ currents in the non-ciliated cells of the efferent ductules through CFTR.**

(A, D and F) Corresponding I-V curves of the whole-cell Cl⁻ I_{ADGRG2-ED} currents
recorded in Figure 6 and Supplemental Figure 2. WT (n=6), /Y (n=6); WT+CFTRinh-172 (n=6), /Y+CFTRinh-172 (n=6), WT+ANI9 (n=6), Adgrg2/Y+ANI9 (n=6), WT+DIDS (n=6), /Y+DIDS (n=6); WT+Control RNAi (n=6), WT+CFTR RNAi (n=6), /Y+CFTR RNAi (n=6); WT+Control RNAi (n=6), /Y+CFTR RNAi (n=6); WT+FSK+IBMX (n=6), /Y+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).

(7B, E and G) *, p<0.05, **, p<0.01, ***, p<0.001, /Y 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. ns, no significant difference. At least three independent biological replicates were performed for Figure 7B, E and G.

Figure 8. Gq activity regulated Cl- current and pH homeostasis in the efferent ductules.

(A) Intracellular pH (pHi) of the ligated efferent ductules from WT (n=9) mice or Gnaq+/-(n=9) mice was measured by carboxy-SNARF.

(B). The whole-cell Cl- current of the I_{ADGRG2-ED} elicited by voltage steps between -100 mV and ±100 mV in representative ADGRG2 promoter-RFP labeled efferent ductule cells derived from Gnaq+/− mice, their WT littermates, or WT murine cells incubated with the PKC inhibitor Ro 31-8220 (500 nM). The whole-cell Cl- I_{ADGRG2-ED} current was recorded with a CsCl pipette solution (101 mM CsCl, 10 mM EGTA, 10 mM Hepes, 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 (D).

(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<sup>+/−</sup> (n=9) mice in response to ATP (5 mM) or control vehicles, measured by ELISA.

(H) cAMP concentrations in the brains, ligated efferent ductules, and livers of WT (n=9) or Adgrg2<sup>+/−</sup> (n=9) mice were measured using ELISA.

(8A,8D,8G-H) *, p<0.05, **, p<0.01, ***, p<0.001, Adgrg2<sup>+/−</sup> mice or Gnaq<sup>−/−</sup> compared with WT mice. #, p<0.05, ##, p<0.01, ###, p<0.001, ATP- or Ro 31-8220-treated cells were compared with control vehicles. ns, no significant difference.

At least three independent biological replicates were performed for Figure 8A, D, G and H.

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<sup>+/−</sup> (n=12) or Arrb1<sup>−/−</sup> (n=15) mice.

(B) Diameters of the luminal ductules derived from WT (n=12), Adgrg2<sup>+/−</sup> (n=12) or Arrb2<sup>−/−</sup> (n=15) mice.

(C) Intracellular pH (pHi) of the ligated efferent ductules derived from WT (n=9), Arrb1<sup>−/−</sup> (n=9) or Arrb2<sup>−/−</sup> (n=9) mice were measured by carboxy-SNARF.

(D) Co-localization of ADGRG2 (red fluorescence) and CFTR (sc-8909, Santa Cruz, green fluorescence) in the male efferent ductules of Arrb2<sup>−/−</sup> mice.

(E) Analysis of ADGRG2 and CFTR fluorescence intensities in Arrb2<sup>−/−</sup> 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 Arrb2\(^{-/-}\) 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.

(K) Analysis of ezrin and CFTR fluorescence intensities in Arrb1\(^{-/-}\) mice by Pearson’s correlation analysis. The Pearson's correlation coefficient was -0.15.

(L) ADGRG2 was immunoprecipitated by an anti-ADGRG2 antibody in the male efferent ductules of Arrb1\(^{-/-}\) mice or Arrb2\(^{-/-}\) 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) *, \(p<0.05\), **, \(p<0.01\), ***, \(p<0.001\), Adgrg2\(^{-/-}\) 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.

**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).
(C and D) Bar graph representation of average current densities (pA/pF) measured at 100 mV according to (D) and Figure 9; Supplemental Figure 6.

(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.

**Figure 11. Key mutations of ADGRG2 downregulates 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-Y698A, CFTR and ADGRG2-F705A, CFTR and ADGRG2-Y708A or CFTR and ADGRG2-RK803EE.

(E) Corresponding I-V curves for the whole-cell Cl⁻ currents recorded in (D).

(F) Bar graph representation of average current densities (pA/pF) measured at 100 mV according to (E).

(11B-11C and 11F) *, \( p < 0.05 \), **, \( p < 0.01 \), ***, \( p < 0.001 \), cells transfected with ADGRG2-WT or mutants compared with the control plasmid (pCDNA3.1). #\( p < 0.05 \), ##, \( p < 0.01 \), ###, \( p < 0.001 \), cells overexpressing ADGRG2 mutants compared with ADGRG2-WT. n.s., no significant difference.

At least three independent biological replicates were performed for Figure 11B-C, F.
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 GRP64-WT or different G-subtype mutants at the initial segment of the epididymis. Scale bars, 200 μm.

(C) Bar graph representing the quantitative analysis of the number of sperm shown in Figure 12B from at least 4 independent experiments.

(D-E) The corresponding bar graph of the accumulation of spermatozoa according to the hematoxylin-eosin staining of the WT, Adgrg2^−/Y mice or Adgrg2^−/Y mice infected with lentivirus encoding GRP64-WT or different G-subtype mutants at the efferent ductules (D) or initial segment (E) of epididymis.

(C-E) *, 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; Adgrg2^−/Y mice infected with the adenovirus encoding different ADGRG2 constructs compared with Adgrg2^−/Y mice infected with the control adenovirus. $, p<0.05, $$$, p<0.001; ADGRG2 mutant virus compared with ADGRG2-WT virus. n.s., no significant difference.

At least three independent biological replicates were performed for Figure 12C-E.

Figure 13. Effects of conditional expression of ADGRG2 wild-type or its selective G-subtype signaling mutants in Adgrg2^−/Y 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^−/Y 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^−/Y (n=12) mice.

(A-F) *, p<0.05, **, p<0.01, ***, p<0.001; Adgrg2^−/Y mice infected with the empty
adenovirus compared with WT mice infected with the empty adenovirus. \#p<0.05, \##, 
p<0.01, ###, p<0.001; Adgrg2^{+/Y} mice infected with the adenovirus encoding different 
ADGRG2 constructs compared with Adgrg2^{+/Y} mice infected with the control
adenovirus. n.s., no significant difference.

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^{+/Y} 
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 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.

Figure 1–figure supplement 1

ADGRG2 is specifically expressed in non-ciliated cells.

(A) Control experiments: Direct immunofluorescence staining of secondary 
antibodies used in the manuscript (including donkey anti-sheep, red fluorescence; and 
donkey anti-rabbit, green fluorescence) in WT male mice efferent ductules. Scale bars,
(B) Microscope analysis of efferent ductules and the nucleus in WT male mice, including a light image. Scale bars, 50 μm.

(C) Bar graph representation and statistical analyses of co-localization of ADGRG2 and acetylated-tubulin in WT male mice efferent ductules (corresponding to Figure 1B in the main manuscript), n = 3 mice per group; 4-10 random areas were selected from each section, and 6 sections were randomly selected from each mouse.

**Figure 1—figure supplement 2**

The construction of the mouse ADGRG2-promoter-RFP used in the labeling of ADGRG2 expressed cells.


(C) Isolated epididymal efferent duct epithelium infected with the ADGRG2-promoter RFP adenovirus specifically labeled the ADGRG2-expressing non-ciliated cells. Scale bars, 50 μm.

**Figure 2—figure supplement 1**

The ADGRG2 protein knockout strategy, PCR strategy and Western blot results of *Adgrg2<sup>-<sub>Y</sub></sup>* and *Gnaq<sup>++</sub>* mice.

(A) Schematic representation of the ADGRG2 knockout strategy for the *Adgrg2<sup>-<sub>Y</sub></sup>* mice. 2bp nucleotides were removed and 10bp nucleotides were inserted in the first exon of the ADGRG2 gene in the ADGRG2 mutant mice by the CRISP-CAS9 approach. In the ADGRG2-deficient mice, the translation of ADGRG2 was terminated at the 7th amio acid after the signal peptide.

(B) Schematic representation for the primers used in the genotyping of the ADGRG2 mutant mice or their wild type littermates.

(C) Schematic description of the PCR strategy and expected results for genotyping. The genotyping of mice was determined by PCR and visualized by bromide staining of agarose Gels.

(D) Western blot analysis of ADGRG2 expression in efferent duct tissue of WT and
Adgrg2/Y mice. All blots were normalized to GAPDH.

(E) Bar graph representation and statistical analyses of (D). At least three independent experiments were carried out.

(F) Western blot analysis of Gq expression in efferent duct tissue of WT and Gnaq+/− mice. All blots were normalized to GAPDH.

(G) Bar graph representation and statistical analyses of (F). At least three independent experiments were carried out.

(E,G) *, p<0.05, **, p<0.01, ***, p<0.001, Adgrg2/Y mice or Gnaq+/− mice were compared with WT mice. n.s., no significant difference.

**Figure 2—figure supplement 2**

Effects of Forskolin and IBMX on the diameters of ligated efferent ductules derived from WT or Adgrg2/Y mice.

(A) Effects of forskolin (10μM), an adenylyl cyclase (AC) activator, on the diameters of ligated efferent ducts; WT(n=11) or Adgrg2/Y (n=10);

(B) Effects of IBMX(100μM), a non-specific inhibitor of cAMP and cGMP phosphodiesterases (PDEs), on the diameters of ligated efferent ducts. WT(n=9) or Adgrg2/Y (n=9).

(A-B)*, p<0.05, **, p<0.01, ***, p<0.001, Adgrg2/Y mice were compared with WT mice. #, p<0.05, ##, p<0.01, ###, p<0.001. Selective inhibitors or stimulators treated were compared with control vehicles.

**Figure 4—figure supplement 1**

Expression and functional analysis of potential osmotic drivers in efferent ductules

(A) Quantitative RT-PCR (qRT-PCR) analysis of mRNA transcription profiles of potential osmotic drivers including selective ion channels and transporters in efferent ductules, brain and liver of wild type (WT) (N=3) male mice. Expression levels were normalized with GAPDH levels. *, p<0.05, **, p<0.01, ***, p<0.001, brain were compared with efferent ductules. #, p<0.05, ##, p<0.01, ###, p<0.001. Liver were compared with efferent ductules. n.s., no significant difference.
(B-C) Effects of different channel or transporter blockers on the diameters of luminal ductules derived from WT or Adgrg2<sup>-/Y</sup> mice. (B) LaCl<sub>3</sub> (100 μM), a non-selective TRPC3/6/7 blocker, WT(n=12) or Adgrg2<sup>-/Y</sup>(n=12); (C) Amiloride(1mM), a sodium/hydrogen antiporter NHE1 inhibitor, WT(n=10) or Adgrg2<sup>-/Y</sup>(n=12).

(D) Quantitative RT-PCR (qRT-PCR) analysis of the ADGRG2, CFTR, Gαs, Gαq, β-arrestin-1 and β-arrestin-2 expression level in ADGRG2 promoter-labeled efferent ductule cells derived from Adgrg2<sup>-/Y</sup> mice(n=3) and their WT littermates(n=3). Expression levels were normalized with GAPDH levels. (B-D) **, p<0.01, ***, p<0.001; Adgrg2<sup>-/Y</sup> mice compared with WT mice. ##, p<0.01, Selective inhibitors or stimulators treated were compared with control vehicles. n.s., no significant difference.

**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<sup>-/Y</sup> mice.

Representative agrose gel for the reverse transcription PCR analysis of CFTR mRNA level in efferent ductules of WT or Adgrg2<sup>-/Y</sup> mice. The upper band (220bp PCR product) in each lane represents CFTR, whereas the lower band (100bp product) represents GAPDH (This figure was related to Figure 5B).

**Figure 5—figure supplement 2**

pH homeostasis in the efferent ductules was impaired in Adgrg2<sup>-/Y</sup> mice.

(A) The relationship between R value (fluorescence emission intensity at 635nm/fluorescence emission intensity at 590nm) and pH<sub>i</sub> for the indicator 5'(and 6')-carboxy-10-dimethylamino-3-hydroxy-spiro[7H-benzo[c]xanthene-7,1'(3H)-isobenzofuran]-3'-one (carboxy SNARF-1 ).

(B-D) Intracellular pH (pH<sub>i</sub>) of the ligated efferent ductules with or without bicarbonate(25mM)(B), Acetazolamide(500μM)(C), Amiloride(1mM)(D) from WT mice and Adgrg2<sup>-/Y</sup> mice.

(B-D)*, p<0.05, Selective inhibitors or stimulators treated were compared with control vehicles.
**Figure 5-figure supplement 3**

**Immunostaining experiments for CFTR location in efferent ductules.**

(A) Co-Immunofluorescence staining of ADGRG2 (red fluorescence) and ANO1 (green fluorescence) in WT male mice efferent ductules. Scale bars, 50 μm.

(B) Immunofluorescent staining of CFTR (sc-8909, Santa Cruz, green fluorescence) and acetylated-tubulin (yellow fluorescence) in WT male mice efferent ductules. Scale bars, 50 μm. A representative image was selected from at least three independent experiments.

(C) Bar graph representation and statistical analyses of co-localization cells of CFTR and acetylated-tubulin in WT male mice efferent ductules (related to (B)).

**Figure 5-figure supplement 4**

Bar graph representation and statistical analyses of Figure 5H.

***, p<0.001, Adgrg2⁻/⁻ lysates or IP protein were compared with WT lysates or IP protein respectively. n.s., no significant difference.

**Figure 7-figure supplement 1**

**Effects of different stimulators or inhibitors of osmotic drivers on I\_ADGRG2-ED Cl⁻ currents of efferent ductule cells derived from Adgrg2⁻/⁻ mice and their wild type littermates.**

(A) The whole cell Cl⁻ current of I\_ADGRG2-ED elicited by voltage steps between -100mV and +100 mV in a representative ADGRG2-promoter-RFP labeled efferent ductule cells derived from the Adgrg2⁻/⁻ mice and their wild type littermates with or without selective inhibitors or stimulators.

(B) Corresponding bar graph of average reversal potential (E\_rev) (±s.e.m., n=6 for each condition) in (A) and calculated Nernst potential at according Cl⁻ concentrations. n.s., no significant difference; compared to calculated Nernst potential.

**Figure 7-figure supplement 2**

**Effects of Cl⁻ concentration change and CFTRinh-172 on the I\_ADGRG2-ED Cl⁻ currents.**

(A) The whole cell Cl⁻ current of I\_ADGRG2-ED elicited by voltage steps between -100mV
and +100 mV in a representative ADGRG2-promoter-RFP labeled efferent ductule cells derived from the Adgrg2<sup>+/−</sup> mice and their wild type littermates with or without CFTR selective inhibitors CFTRinh-172, and in response to bath Cl<sup>−</sup> concentration change (Cl<sup>−</sup> was substituted for gluconate (Gluc<sup>−</sup>)).

(B) Representative whole cell Cl<sup>−</sup> current of I<sub>ADGRG2<sub>-ED</sub></sub> versus voltage (I-V) relationships in response to voltage ramps recorded in (A) with a CsCl pipette solution.

(C) Corresponding bar graph of average current densities (pA/pF) measured at 100 mV. **, p<0.01, ***, p<0.001, compared with WT mice in 148.5 Cl<sup>−</sup> condition. #, p<0.05, WT mice in 148.5 Cl<sup>−</sup> condition treated with CFTRinh-172 was compared with WT mice in 48.5 Cl<sup>−</sup> condition treated with CFTRinh-172.

(D) Corresponding bar graph of average reversal potential(E<sub>rev</sub>) (±s.e.m., n=8 for each condition) in (A-B).

**Figure 7-figure supplement 3**
Effects of CFTR knocked down on the I<sub>ADGRG2<sub>-ED</sub></sub> Cl<sup>−</sup> currents.

(A) The whole cell Cl<sup>−</sup> current of I<sub>ADGRG2<sub>-ED</sub></sub> elicited by voltage steps between -100mV and +100 mV of primary efferent ductule cells after CFTR-siRNA or Scramble-siRNA treatment.

(B) Corresponding bar graph of average reversal potential(E<sub>rev</sub>) (±s.e.m., n=6 for each condition) in (A). n.s., no significant difference; compared to calculated Nernst potential.

**Figure 8-figure supplement 1**
Effects of G protein signaling on the I<sub>ADGRG2<sub>-ED</sub></sub> Cl<sup>−</sup> currents.

(A) The whole cell Cl<sup>−</sup> current of I<sub>ADGRG2<sub>-ED</sub></sub> elicited by voltage steps between -100mV and +100 mV in a representative ADGRG2-promoter-RFP labeled efferent ductule cells derived from the Gnaq<sup>+/−</sup> mice and their wild type littermates, in control bath solution or in response to substitution of Cl<sup>−</sup> with the gluconate (Gluc<sup>−</sup>).

(B) Representative whole cell Cl<sup>−</sup> current of ADGRG2-promoter-labeled efferent ductule cells I<sub>ADGRG2<sub>-ED</sub></sub> versus voltage (I-V) relationships in response to voltage ramps recorded in (A) with a CsCl pipette solution.

(C) Corresponding bar graph of average current densities (pA/pF) measured at 100
mV. Inset: Average $E_{\text{rev}}$ (±s.e.m., $n=8$ for each condition).

(D) The whole cell Cl\(^-\) current of $I_{\text{ADGRG2-ED}}$ elicited by voltage steps between -100mV and +100 mV in a representative ADGRG2-promoter-RFP labeled efferent ductule cells derived from the $Adgrg2^{+/Y}$ mice and their wild type littermates, with or without specific G protein signaling pathway inhibitors (including the Gs-PKA inhibitor PKI14-22 or the Gq-PKC inhibitor Ro 31-8220).

(E) Corresponding I-V curves of the whole-cell Cl\(^-\) currents $I_{\text{ADGRG2-ED}}$ recorded in (D).

(F) Corresponding bar graph of average current desnities (pA/pF) measured at 100 mV in (D-E).

(G) Corresponding bar graph of average reversal potential($E_{\text{rev}}$) and calculated Nernst potential (±s.e.m., $n=6$ for each condition) in (D-E). n.s., no significant difference; the $E_{\text{rev}}$ were compared with calculated Nernst potential.

(C,F)* $p<0.05$, **,$p<0.01$, $Gnaq^{+/}$ or $Adgrg2^{+/Y}$ mice were compared with their wild type littermates. #, $p<0.05$, substitution of Cl\(^-\) with the gluconate (Gluc\(^-\)) or cells treated with different inhibitors were compared with control solution.

Figure 8-figure supplement 2

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

(A) Bar graph representation and statistical analyses of co-localization of Gq and ADGRG2 in WT male mice efferent ductules (corresponding to Figure 8E), $n = 3$ mice per group; 4-10 random areas were selected from each section, and 6 sections were randomly selected from each mouse.

(B) Bar graph representation and statistical analyses of co-localization of Gq and acetylated-tubulin in WT male mice efferent ductules (corresponding to Figure 8F), $n = 3$ mice per group; 4-10 random areas were selected from each section, and 6 sections were randomly selected from each mouse.

Figure 8-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 $Adgrg2^{+/Y}$ mice.

(A) Western blot analysis of ADGRG2, CFTR, Gs, Gq, β-arrestin-1, β-arrestin-2
expression in efferent ductules, brain and liver tissue of WT and Adgrg2^{+/Y} mice. A representative western blot from at least three independent experiments was shown. (CFTR antibody:20738-1-AP, Proteintech).

(B) Bar graph representation and statistical analyses of (A). All blots were normalized to GAPDH. ns: no significant difference; Adgrg2^{+/Y} mice compared with WT mice in the same tissue.

**Figure 9-figure supplement 1**

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

(A,C) Western blot analysis of β-arrestin1/2 expression in the efferent duct tissue of WT and Arrb2^{-/-}(A) or Arrb1^{-/-}(C) mice. A representative western blot from at least three independent experiments was shown.

(B,D) Bar graph representation and statistical analyses of (A,C). All blots were normalized to GAPDH.*** p<0.001, Arrb2^{-/-} mice or Arrb1^{-/-} mice were compared with WT mice respectively. n.s., no significant difference.

**Figure 9-figure supplement 2**

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

(A) Co-localization of Ezrin (red fluorescence) and CFTR (sc-8909, Santa Cruz, green fluorescence) in male efferent ductules of the WT mice and Adgrg2^{+/Y} mice. Scale bars, 50 μm. Analysis of Ezrin and CFTR fluorescence intensities by perason’s correlation. The pearson's correlation coefficient is 0.6 for WT mice and 0.65 for Adgrg2^{+/Y} mice.

(B) Bar graph representation and statistical analyses of Figure 9L. *** p<0.001, Arrb2^{-/-} lysates or IP protein were compared with Arrb1^{-/-} lysates or IP protein respectively. n.s., no significant difference.

**Figure 9-figure supplement 3**

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

(A) HEK293 cells were transfected with equal amount plasmids encoding ADGRG2, CFTR, β-arrestin-1 or β-arrestin-2 plasmids. The Flag- ADGRG2 were pulled down
by M2-Flag beads and the associated CFTR, β-arrestins were detected by western blot. Representative images from at least 3 independent experiments are shown (CFTR antibody:20738-1-AP, Proteintech).

(B) Bar graph representation and statistical analyses of (A).***, p<0.001, IP protein of ADGRG2 overexpressed cells were compared with control plasmids transfected cells respectively. n.s., no significant difference.

Figure 10-figure supplement 1
Co-localization analysis of ADGRG2 and CFTR in HEK293 cells.
(A) Co-localization of ADGRG2 (red fluorescence) and CFTR (green fluorescence) in HEK293 cells. Scale bars, 20 μm. A representative figure from at least three independent experiments was shown.
(B) Analysis of ADGRG2 and CFTR fluorescence intensities by person’s correlation. The pearson's correlation coefficient is 0.56.

Figure 10-figure supplement 2
Construction and expression of ADGRG2-full length (ADGRG2FL) and a truncated form ADGRG2β.
(A-C) Construction and expression of ADGRG2-full length (ADGRG2FL) and a truncated form ADGRG2β. (A) Schematic illustration of the structure of the ADGRG2FL and the ADGRG2β used in the current study. The full length ADGRG2 encompasses a GPS site, which was cleaved after its transportation to the plasma membrane through an auto-hydrolysis mechanism. Therefore, we made a truncation version of the ADGRG2β, which starts at the auto-cleaved site, T584. A signal peptide and a flag-tag were added at the N-terminal for both ADGRG2FL and ADGRG2β. (B) Confocal images showed that the ADGRG2FL and ADGRG2β were primarily localized at the plasma membrane. Scale bars, 10 μm. (C) The expression of ADGRG2FL and ADGRG2β were detected by Western blot. The observed 180kDa band of the ADGRG2FL is the N-terminal part of the ADGRG2 protein due to the auto-hydrolysis. The apparent 200kDa band of the ADGRG2β is due to the glycosylation. Representative western blots from at least 3 independent experiments were shown.
**Figure 10-figure supplement 3**  

Overexpression of ADGRG2FL and ADGRG2β lead to constitutive increased cellular cAMP levels.

(A-C) Overexpression of ADGRG2FL leads to constitutively increased intracellular cAMP levels. HEK293 cells were co-transfected with the GloSensor plasmid and the control pcDNA3.1 vector or the ADGRG2FL expression vector at the indicated concentrations. (A) Representative curve of the intracellular cAMP level measured by the Glosensor assay. (B) A dose-dependent cAMP increase by overexpression of ADGRG2FL was detected. (C) The corresponding ADGRG2FL protein expression level was examined by western blot. **, $p<0.01$, ***, $p<0.001$, the ADGRG2FL-transfected cells were compared to pcDNA3.1-transfected cells.

(D-F) Overexpression of ADGRG2β leads to constitutively increased intracellular cAMP levels. HEK293 cells were transfected with the GloSensor plasmid and the control pcDNA3.1 vector or the ADGRG2β expression vector at the indicated concentrations. (D) Representative curve of the intracellular cAMP level measured by the Glosensor assay. (E) A dose-dependent cAMP increase by overexpression of ADGRG2β was detected. (F) The corresponding ADGRG2β protein expression level was examined by western blot. ***, $p<0.001$, the ADGRG2β transfected cells were compared to pcDNA3.1-transfected cells.

(G-H) ADGRG2FL overexpressed cells showed higher cAMP levels than the control cells. (G) The constitutive cAMP accumulation in HEK293 cells transfected with ADGRG2FL was further enhanced by the overexpression of Gs in a dose-dependent manner. (H) The corresponding ADGRG2FL and Gs protein expression levels were examined by western blot. ***, $p<0.001$, the Gs and ADGRG2 transfected cells were compared to only ADGRG2 transfected cells. #, $p<0.05$, ##, $p<0.01$, ###, $p<0.001$, the Gs and pcDNA3.1 transfected cells were compared to only pcDNA3.1 transfected cells. $$, p<0.01$$, $$$, p<0.001$, The ADGRG2FL overexpressed cells were compared to the corresponding vector pcDNA3.1 transfected cells with equal Gs protein amount.

(I-J) ADGRG2β overexpressed cells showed higher cAMP levels than the control cells. (I) The constitutive cAMP accumulation in HEK293 cells transfected with ADGRG2β was further enhanced by the overexpression of Gs in a dose-dependent manner. (J) The corresponding ADGRG2β and Gs protein expression levels were
examined by a western blot. ***, p<0.001, the Gs and ADGRG2β transfected cells were compared to only ADGRG2β transfected cells. #, p<0.05, ##, p<0.01, ###, p<0.001, the Gs and pcDNA3.1 transfected cells were compared to only pcDNA3.1 transfected cells. $$, p<0.01,$$$, p<0.001, The ADGRG2β overexpressed cells were compared to the corresponding vector pcDNA3.1 transfected cells with equal Gs protein amounts.

(K-M) Comparison of The cAMP levels in the cells with similar expression levels of ADGRG2FL and ADGRG2β. (K-L) The ADGRG2β exhibits higher constitutive cAMP activity compared to ADGRG2FL. (M) The corresponding ADGRG2FL and ADGRG2β protein expression levels were examined by the ELISA assay. ***, p<0.001, ADGRG2FL or ADGRG2β transfected cells were compared to control vector transfected cells ###, p<0.001, ADGRG2β transfected cells were compared to ADGRG2FL transfected cells.

Figure 10-figure supplement 4

Overexpression of ADGRG2FL and ADGRG2β have constitutive Gq-NFAT signaling activities.

(A-B) Dose-dependent effect of ADGRG2FL (A) or ADGRG2β (B) overexpression on the luciferase activity of the NFAT-DLR. *, p<0.05, **, p<0.01, ***, p<0.001, the ADGRG2 transfected cells were compared to non-transfected cells.

(C-D) ADGRG2FL overexpressed cells showed higher NFAT signaling activity than the control cells. (C) The luciferase activity of the NFAT-DLR in HEK293 cells transfected with ADGRG2FL was further enhanced by the overexpression of Gq in a dose-dependent manner. (D) The corresponding ADGRG2FL and Gq protein expression levels were examined by western blot. ***, p<0.001, the Gq and ADGRG2FL transfected cells were compared to only ADGRG2FL transfected cells. #, p<0.05, ##, p<0.01, ###, p<0.001, the Gq and pcDNA3.1 transfected cells were compared to only pcDNA3.1 transfected cells. $$, p<0.01,$$$, p<0.001, The ADGRG2FL overexpressed cells were compared to the corresponding vector pcDNA3.1 transfected cells with equal Gq protein amounts.

(E-F) ADGRG2β overexpressed cells showed higher NFAT signaling activity than the control cells. (E) The luciferase activity of the NFAT-DLR in HEK293 cells transfected with ADGRG2β was further enhanced by the overexpression of Gq in a dose-dependent manner. (F) The corresponding ADGRG2β and Gq protein expression
levels were examined by western blot. *, p<0.05, **, p<0.01, ***, p<0.001, the Gq and ADGRG2β transfected cells were compared to only ADGRG2β transfected cells. 
#, p<0.05, ##, p<0.01, ###, p<0.001, the Gq and pcDNA3.1 transfected cells were compared to only pcDNA3.1 transfected cells. $$$, p<0.01,$$$$, p<0.001. The ADGRG2β overexpressed cells were compared to the corresponding vector pcDNA3.1 transfected cells with equal Gq protein amounts.

(G) Comparison of the luciferase activity of the NFAT-DLR in the cells with similar expression levels of ADGRG2FL and ADGRG2β. The ADGRG2β exhibits higher constitutive activity in NFAT luciferase assay when compared to ADGRG2FL. ***, p<0.001, ADGRG2FL or ADGRG2β transfected cells were compared to control vector transfected cells. ###, p<0.001, ADGRG2β transfected cells was compared to ADGRG2FL transfected cells.

**Figure 10-figure supplement 5**

ADGRG2 upregulates CFTR Cl⁻ currents and Cl⁻ efflux through G protein signaling.

(A) Corresponding bar graph of average reversal potential(E_{rev}) (±s.e.m., n=6 for each condition) in HEK293 cells transfected with plasmids encoding ADGRG2 or/and CFTR, with or without CFTR inhibitor CFTRinh-172(10μM) or its activator (FSK(10μM)+IBMX(100μM)) and calculated Nernst potential. n.s., no significant difference; the E_{rev} were compared with calculated Nernst potential.

(B) Whole-cell Cl⁻ currents recorded with a CsCl pipette solution in HEK293 cells transfected with plasmids encoding ADGRG2 or/and CFTR, with or without the PKA inhibitor PKI14-22 or the PKC inhibitor Ro 31-8220.

(C) Corresponding I-V curves of the whole-cell Cl⁻ currents (n=6 for each condition) recorded in (B).

(D) Corresponding bar graph of average reversal potential(E_{rev}) (±s.e.m., n=6 for each condition) recorded in (B-C) and calculated Nernst potential. n.s., no significant difference; the E_{rev} were compared with calculated Nernst potential.

**Figure 11-figure supplement 1**

Sequence alignment of the transmembrane domains of ADGRG2 (Homo sapiens, Mus musculus, Rattus norvegicus), β2AR (Homo sapiens, Mus musculus, and
*Rattus norvegicus*, and GPR126 (*Homo sapiens*).

The ADGRG2 mutation sites studied in the current work were highlighted. The green color indicated that the mutants caused both Gs and Gq defects of ADGRG2 coupling; the yellow color indicated that the mutants induced only Gs defects of ADGRG2 coupling, and the blue color indicated that the mutant induced only Gq defects of ADGRG2 coupling.

**Figure 11-figure supplement 2**

*Western blot and ELISA analysis of the expression of these mutants in the cell membrane.*

(A) Western blot of ADGRG2 WT and its mutations (HM696AA, H696A, M697A, Y698A, K703A, V704A, F705A and Y708A in intracellular loop 2; and QL798AA and RK803EE in intracellular loop 3). Representative western blot from at least 3 independent experiments.

(B) ELISA of the expression level of ADGRG2-WT and its mutations in the plasma membrane.

***, p<0.001, the protein level of ADGRG2-WT and its mutations in the plasma membrane were compared to control.

**Figure 11-figure supplement 3**

*Corresponding bar graph of average reversal potential (E\textsubscript{rev}) (±s.e.m., n=6 for each condition) recorded in figure 11D-11E and calculated Nernst potential.*

n.s., no significant difference; the E\textsubscript{rev} were compared with calculated Nernst potential.

**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\textsuperscript{lo}\textsuperscript{Y} mice.*

(A) The enlarged images of the ADGRG2 expression in the epididymal initial segment at three weeks after the injection of the lentivirus of ADGRG2-WT or mutants. Scale bars, 100 μm.

(B) Photographs of caudal sperm preparations from the caudal epididymis of the WT mice, Adgrg2\textsuperscript{lo}\textsuperscript{Y} mice or Adgrg2\textsuperscript{lo}\textsuperscript{Y} mice infected with a lentivirus encoding ADGRG2 or its different mutants. Scale bars, 50 μm.

(C) The corresponding bar graph of the accumulation of spermatozoa according to the
hematoxyline-eosin staining of the WT, Adgrg2\textsuperscript{Y} mice or Adgrg2\textsuperscript{Y} mice infected with lentivirus encoding ADGRG2-WT or different G subtype mutants at the caput of epididymis. *, p<0.05, ***, p<0.001, Adgrg2\textsuperscript{Y} mice and Adgrg2\textsuperscript{Y} mice infected with lentivirus were compared to WT mice. ###, p<0.001, Adgrg2\textsuperscript{Y} mice infected with lentivirus were compared to Adgrg2\textsuperscript{Y} mice. $, p<0.05, $$, p<0.01; ADGRG2 mutant virus compared with ADGRG2-WT virus. n.s., no significant difference.

Table 1: Average reversal potential calculated at different Cl\textsuperscript{-} concentrations for Figure 6C.

<table>
<thead>
<tr>
<th>Group</th>
<th>$\text{E}<em>{\text{rev}}[\text{Cl}\textsuperscript{-}]</em>{o}$ 148.5mM(mV)</th>
<th>$\text{E}<em>{\text{rev}}[\text{Cl}\textsuperscript{-}]</em>{o}$ 48.5mM(mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nernst</td>
<td>-4.6</td>
<td>25.3</td>
</tr>
<tr>
<td>WT</td>
<td>-4.0 ± 0.51</td>
<td>20.1 ± 2.52</td>
</tr>
<tr>
<td>Adgrg2\textsuperscript{Y}</td>
<td>-4.1 ± 0.36</td>
<td>19.4 ± 2.47</td>
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Average reversal potential($\text{E}_{\text{rev}}$) (±s.e.m., n=8 for each condition) in Figure 6C and calculated Nernst potential at different Cl\textsuperscript{-} concentrations. The Nernst equation was:

$$\text{E}_{\text{rev}} = -\frac{RT}{Z} \text{Ln} \left( \frac{\text{Cl}\textsuperscript{-}}{\text{Cl}\textsuperscript{-}\text{out}} \right).$$

Supplementary File 1

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

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.

Supplementary File 3

Figure 6

A

148mM Cl⁻ 48mM Cl⁻ 148mM Cl⁻

pAPF

WT

Adgrp2⁻/⁻

I⁺100mV

10

50

100

150

200

250

300

t(s)

I⁻100mV

-10

0

B

WT

Adgrp2⁻/⁻

P A

0

200

-200

0 20 40 60 80 100

ms

C

200pA

10ms

0 mV

-100 mV

WT 148.5mM Cl⁻

WT 48.5mM Cl⁻ 100mM Gluc⁻

Adgrp2⁻/⁻ 148.5mM Cl⁻

Adgrp2⁻/⁻ 48.5mM Cl⁻ 100mM Gluc⁻

D

WT  Adgrp2⁻/⁻

Na⁺  Cl⁻

Cl⁻  Cs⁺

TEA⁺

Gluc⁻

E

pAPF

(n=6)

(n=6)

E_revmV

WT  Adgrp2⁻/⁻

-40 0 40

-100 0 100

pAPF

** n.s. n.s.

n.s.
Figure 8

A

\[
\begin{array}{c|c}
\text{WT} & 6.70 \\
Gnaq^{+/+} & 6.75 \\
\end{array}
\]

B

\[
\begin{array}{c|c|c}
0 mV & 100 mV & -100 mV \\
WT & Gnaq^{+/+} & WT + Ro 31-8220 \\
\end{array}
\]

C

\[
\begin{array}{c|c}
\text{WT} & 6.70 \\
\text{Gnaq}^{+/+} & 6.75 \\
\text{WT + Ro 31-8220} & 6.70 \\
\end{array}
\]

D

\[
\begin{array}{c|c|c|c}
\text{WT} & 6.70 & 6.75 & 6.70 \\
\text{Gnaq}^{+/+} & 6.70 & 6.75 & 6.70 \\
\text{WT + Ro 31-8220} & 6.70 & 6.75 & 6.70 \\
\end{array}
\]

E

DAPI, ADGRG2, Gq, Merge

F

DAPI, Gq, Acetylated-tubulin, Merge

G

\[
\begin{array}{c}
\text{WT}(9) \\
\text{Adgrg2}^{-/-}(9) \\
\text{n.s.} \\
\end{array}
\]

H

\[
\begin{array}{c}
\text{WT}(9) \\
\text{Adgrg2}^{-/-}(9) \\
\text{n.s.} \\
\end{array}
\]
Figure 9

A

WT(12) Adgr2^{+/−}(12) Arbb1^{−/−}(15)

Width(% of 0h)

0 12 24 36 48 60 72

B

WT(12) Adgr2^{+/−}(12) Arbb2^{−/−}(15)

Width(% of 0h)

0 12 24 36 48 60 72

C

WT Arbb1^{−/−} Arbb2^{−/−}

pH

D

DAPI ADGRG2 CFTR Merge

Enlarged

E

CFTR intensity

ADGRG2 intensity

F

DAPI ADGRG2 CFTR Merge

Enlarged

G

CFTR intensity

ADGRG2 intensity

H

DAPI Ezrin CFTR Merge

Enlarged

I

Ezrin intensity

CFTR intensity

J

DAPI Ezrin CFTR Merge

Enlarged

K

Ezrin intensity

CFTR intensity

L

IB:ADGRG2 180 130 180kDa

IB:CFTR 180 130 -180kDa mature

Lysates 160kDa immature

IB:βarr1 55kDa

IB:βarr2 50kDa

IB:GAPDH 35kDa

180kDa mature

IP-ADGRG2

55kDa

50kDa

40kDa

36kDa

55kDa

40kDa

50kDa

180kDa

IB:ADGRG2

IB:CFTR

IB:βarr1

IB:βarr2

IB:GAPDH
**Figure 10**

A. Graph showing current-voltage relationships for different conditions:
- ADGRG2
- CFTR
- CFTR+ADGRG2
- CFTR+ADGRG2+CFTRinh-172
- CFTR+FSK+IBMX
- CFTR+ADGRG2+FSK+IBMX

B. Graph showing pA/pF vs. mV for different conditions:
- ADGRG2(6)
- CFTR(6)
- CFTR+ADGRG2(6)
- CFTR+FSK+IBMX(6)
- CFTR+ADGRG2+FSK+IBMX(6)
- CFTR+ADGRG2+CFTRinh-172(6)
- CFTR+CFTRinh-172(6)

C. Bar graphs comparing pA/pF for different conditions:
- CFTR(6)
- CFTR+ADGRG2(6)
- CFTR+ADGRG2+FSK+IBMX(6)
- CFTR+ADGRG2+CFTRinh-172(6)
- CFTR+FSK+IBMX(6)

D. Bar graphs comparing pA/pF for different conditions:
- CFTR(6)
- CFTR+ADGRG2(6)
- CFTR+ADGRG2+PKI14-22(6)
- CFTR+ADGRG2+Ro 31-8220(6)

N.S. indicates no significant difference.
Figure 1-figure supplement 2

A

5' upstream sequence
TGAGTGTTAATAACTTTG...GGAGGGACGCGGCCCCGACAGAAA...CCGCAG

ADGRG2 promoter

RFP

ADGRG2-pm-RFP

-1000bp +104bp

B

pAdTrack-ADGRG2-pm-RFP

PAcl LITR PA Notl PA RFP KanR PAcl RITR Left arm Right arm EcoRI

Ori

C

Comparison of DAPI, RFP, and Merge images for WT and Adgrg2^-/- cells.
**Figure 2-figure supplement 1**

**A**
- DNA → RNA → Protein
- Wild type sequence:
  - ATGCTTGTGACACACCTTGAGCAGCT
  - Mutant sequence:
    - ATGCTTGTGACACACCTTGAGCAGCT

**B**
- Wild type sequence:
  - 5′ TGTTCATAGGCACTGTTAAGAG
  - Mutant sequence:
    - ATGATGGGACACATCTATATGG
- Fwt primer: 5′ GTGGCCACTGTTAAGAG
- Fcon primer: 5′ GTGGCCACATCTATATGG
  - 3′GGTACCAATATCTCTTC

**C**

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<tr>
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<th>Fcon/Fmut+R</th>
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<td></td>
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<tr>
<td>Adgr2^{+/y}</td>
<td>397/189</td>
<td>397/-</td>
</tr>
<tr>
<td>Adgr2^{+/y}</td>
<td>405/-</td>
<td>405/196</td>
</tr>
<tr>
<td>Adgr2^{+/y}</td>
<td>397/189</td>
<td>397/-</td>
</tr>
<tr>
<td>Adgr2^{+/y}</td>
<td>397,405/189</td>
<td>397,405/196</td>
</tr>
<tr>
<td>Adgr2^{+/y}</td>
<td>405/-</td>
<td>405/196</td>
</tr>
</tbody>
</table>

**D**

- kDa:
  - IB:ADGRG2
    - 180 kDa
    - 130 kDa
  - IB:GAPDH
    - 36 kDa

**E**

- Protein level
  - WT
  - Adgr2^{+/y}
  - ***
  - Adgr2^{+/y}
  - ***

**F**

- kDa:
  - IB:Gq
    - 45 kDa
  - IB:GAPDH
    - 36 kDa

**G**

- Protein level
  - WT
  - Gnaq^{+/y}
    - n.s.
  - Gnaq^{+/y}
    - **
    - *
Figure 2-figure supplement 2

A

- WT+Forskolin(11)
- Adgrg2-/-+Forskolin(10)
- WT+vehicle(10)
- Adgrg2-/-+vehicle(10)

B

- WT+IBMX(9)
- Adgrg2-/-+IBMX(9)
- WT+vehicle(8)
- Adgrg2-/-+vehicle(9)

Width(% of t=0h)

Time(h)
**Figure 4-figure supplement 1**

**A**

![Relative mRNA level](image)

**B**

![Width (% of t=0h)](image)

**C**

![Width (% of t=0h)](image)

**D**

![Relative mRNA level](image)
Figure 5-figure supplement 1
Figure 5-figure supplement 4

- WT lysates
- Adgrp2±/y lysates
- WT IP
- Adgrp2±/y IP

Protein level

n=3

- ADGRG2
- CFTR
- Gs
- Gq
- βarr1
- βarr2
- Gi1/2/3

Significance:
- n.s.: not significant
- ***: significant
Figure 7-figure supplement 3

A

200pA
10ms
100 mV
0 mV
-100 mV

Scramble-siRNA

CFTR-siRNA

B

E_{rev}(mV)

n.s.

Nernst
Scramble-siRNA
CFTR-siRNA
Figure 8-figure supplement 2

A

- Colocalized cell (Gq and ADGRG2)
- Non-colocalized cell (Gq and ADGRG2)

B

- Co-localized cell (Gq and Acetylated-tubulin)
- Non-colocalized cell (Gq and Acetylated-tubulin)
Figure 9-figure supplement 1

A

IB: βarr2

kDa: 55, 40, 35

WT, WT, Arbb2^−/−, Arbb2^−/−

50kDa

IB: GAPDH

kDa: 35

36kDa

B

protein level

WT, WT, Arbb2^−/−, Arbb2^−/−

n.s.

0, 0.5, 1.0, 1.5

C

IB: βarr1

kDa: 55, 40, 35

WT, WT, Arbb1^−/−, Arbb1^−/−

55kDa

IB: GAPDH

kDa: 35

36kDa

D

protein level

WT, WT, Arbb1^−/−, Arbb1^−/−

n.s.

0, 0.5, 1.0, 1.5

***
Figure 9-figure supplement 3

A

<table>
<thead>
<tr>
<th>ADGRG2</th>
<th>CFTR</th>
<th>βarr1</th>
<th>βarr2</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

Lysates

- IB: CFTR
  - 180 kDa Mature
  - 160 kDa Immature
- IB: HA (βarr1/2)
  - 55 kDa
  - 50 kDa
- IB: FLAG (ADGRG2)
  - 95 kDa
- IB: GAPDH
  - 36 kDa

B

(n=3)

<table>
<thead>
<tr>
<th>ADGRG2</th>
<th>CFTR</th>
<th>βarr1</th>
<th>βarr2</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

Bar graph:
- CFTR
- HA
- FLAG

Legend:
- n.s.
- ***

Graph:
- n.s. n.s. n.s.
- n.s. n.s.
- ***
- ***
- ***
- ***
- ***
Figure 10-figure supplement 5

A

![Graph showing E_{rev} (mV) with n.s. labels for different conditions.]

B

![Graph showing current traces for CFTR and various combinations.]

C

![Graph showing pA/pF vs. mV with different conditions.]

D

![Graph showing E_{rev} (mV) with n.s. labels for different conditions.]
Figure 11-figure supplement 3

![Bar chart showing Erev (mV) for different conditions.]

- Enerst
- ADGRG2(6)
- CFTR-ADGRG2(6)
- CFTR-ADGRG2-Y698A(6)
- CFTR-ADGRG2-F705A(6)
- CFTR-ADGRG2-Y708A(6)
- CFTR-ADGRG2-RK803EE(6)
- CFTR-ADGRG2-HM696AA(6)

n.s. indicates no significant difference.