Supporting Online Material

Materials and Methods

Ex vivo SMG organ culture

SMGs and mesenchyme-free epithelial rudiment culture have been previously described (*S1*). The isolated epithelia were placed in 15 μl of laminin-111 (Trevigen); medium was supplemented with FGF10 (400 ng/ml; R&D Systems) and heparin (0.05 μg/ml; Celsus). In some experiments, carbachol (10 nM, C4382, Sigma), HBEGF (2 ng/ml; R&D Systems), DAMP (10 μM; Tocris), or the EGFR inhibitor PD168393 (10 μM; Calbiochem EMD Biosciences) were added to the media. Epithelial buds were counted using ImageJ software. Experiments were repeated at least three times.

For recombination experiments, E12.5 SMG were dispase-treated, then separated into mesenchyme and epithelia, with or without the PSG, and collected in 5 % BSA. After washing in DMEM/F12, epithelia ±PSG were recombined with 3-4 mesenchymes on a filter and floated on culture medium. In the rescue experiments, carbachol (50 nM) or PD168393 (10 μM) were added to the culture medium after explants had been cultured for at least 4 h at 37°C.

For adult SMG culture, the terminal lobules (~ 1 mm^2) were removed with fine forceps from adult SMGs, thus denervating them, and they were cultured on filters as described above for 44 hrs with CCh or DAMP and PD and immunostained or lysed and analyzed by qPCR.

Keratin 5 lineage tracing

We crossed *K5-rTA* mice, which contain the bovine K5 promoter with *tet-Cre-R26R-lacZ* mice (*S2*). The 5.4 kb bovine K5 promoter, which includes 5' regulatory and promoter regions from -5300 bp to +138 bp, directs lacZ expression to stratified epithelia in a manner analogous to endogenous K5 (*S3*). The pregnant mice were fed doxycycline, 6 gr/kg (6000 ppm; Bio-serve) from E8-E11, and we analyzed LacZ staining of the SMGs after birth (P1). The nerves in P1 SMG have some endogeneous beta-galactosidase activity, resulting in pale blue staining along the primary ducts. To show the specificity of the Cre excision, K5-tetCre-R26R-lacZ mice were fed with doxycycline for 3 days. The lobules of the SMGs were dissociated with fine forcepts after fixation and whole-mount immunofluorescence analysis was performed as described (*S1*), using antibodies to beta-galactosidase (Abcam; ab9361) and K5 (Covance; AF138). The expression of the bovine-K5 promoter was also compared to endogenous K5 expression by staining SMGs from mice expressing the bovine-K5 promoter driving Venus expression, with both anti-K5 and anti-EGFP antibodies, which recognize Venus. The K5-*Venus* transgene was constructed by cloning the Venus (YFP variant) (*S4*) into a pBSKII-derived vector, downstream from the bovine K5 promoter and upstream from a polyadenylation signal. K5-*Venus* mice were produced by injecting a 9.1 kb Asp718-excised fragment into FVB/N blastocytes. Founders were screened for transgene insertion by Southern blot analysis, and subsequent generations were screened by PCR using specific primers.

Prostate organ culture.

Ventral prostates from P6 ICR mice were fixed and stained for in a similar manner to SMGs. Ventral prostates were also cultured for 48h, with or without DAMP and PD and after 48 hrs were immunostained or lysed and analyzed by qPCR.

Immunofluorescence analysis

Wholemount SMG immunofluorescence analysis has been described (*S1*). Primary antibodies: mouse anti-E-cadherin (1:200; BD Biosciences); Tubb3 (clone TUJ1 at 1:400; R&D Systems), EGFR (1:200; BD Biosciences), cytokeratin 5 (AF 138 at 1:1200; Covance Research); muscarinic receptor 1 (451-460 at 1:400; Research and Diagnostic Antibodies); and troma-111 cytokeratin antibody (cytokeratin 19, 1:200; Developmental Studies Hybridoma Bank). Cell proliferation was detected using a Click-it EdU Labeling and Detection Kit (Invitrogen).

Gene expression analysis

Real-time PCR was performed as previously described (*S5*). We also used TaqMan low density arrays with an Applied Biosystems 7900HT Fast Real Time System. Gene expression was normalized to *Rps18* or *Rps29*.

RNA interference

SMGs were transfected with 400 nM siRNA (Dharmacon) in 200 μl of culture medium using RNAiFect (Qiagen, CA), as previously described (*S6*), using at least two siRNAs for Chrm1. Total RNA was purified after 48 hours of culture, and gene knockdown was measured by qPCR.

Fluorescence-activated cell sorting analysis

E13.5 SMGs cultured for 44 h in 10 μM DAMP or 20 μM PD168393 were dissociated by EDTA (5 mM; Gibco 15575) and fixed in acetone-methanol (1:1) at -20°C for 5 min. Typically we obtained ~ 28,000 +/-1600 cells/control gland after culture. Antibodies used for immunostaining (Krt5, Krt19, EGFR, and M1), Ki67 (550609, BD Pharmingen, CA), and caspase3 (9664L, Cell Signaling, MA) were labeled with fluorochromes using Dylight (Thermo Scientific) or LYNX (AbD Serotec) conjugation kits, according to the manufacturer's instructions. Anti-E-cadherin APC (FAB7481A) was purchased from R&D Systems. Single cell suspensions were incubated with the antibodies at 4°C for 30 min. Analysis was performed on a FACS Calibur Flow Cytometer (BD) with at least 50,000 events for each measurement. Data were analyzed by FlowJo software (Tree Star). Gates were set using isotype controls.

S1. Z. Steinberg *et al*., *Development* **132**, 1223 (Mar, 2005).
S2. L. Vitale-Cross, P. Amornphimoltham, G. Fisher, A. A. Molinolo, J. S. Gutkind, *Cancer Res* **64**, 8804 (Dec 15, 2004).
S3. A. Ramírez *et al*., *Differentiation* **58**, 53 (Nov, 1994)
S4. T. Nagai *et al*., *Nat Biotechnol* **20**, 87 (Jan, 2002).
S5. V. N. Patel *et al*., *Development* **134**, 4177 (Dec, 2007).
S6. I. T. Rebustini *et al*., *Dev Cell* **17**, 482 (Oct, 2009).

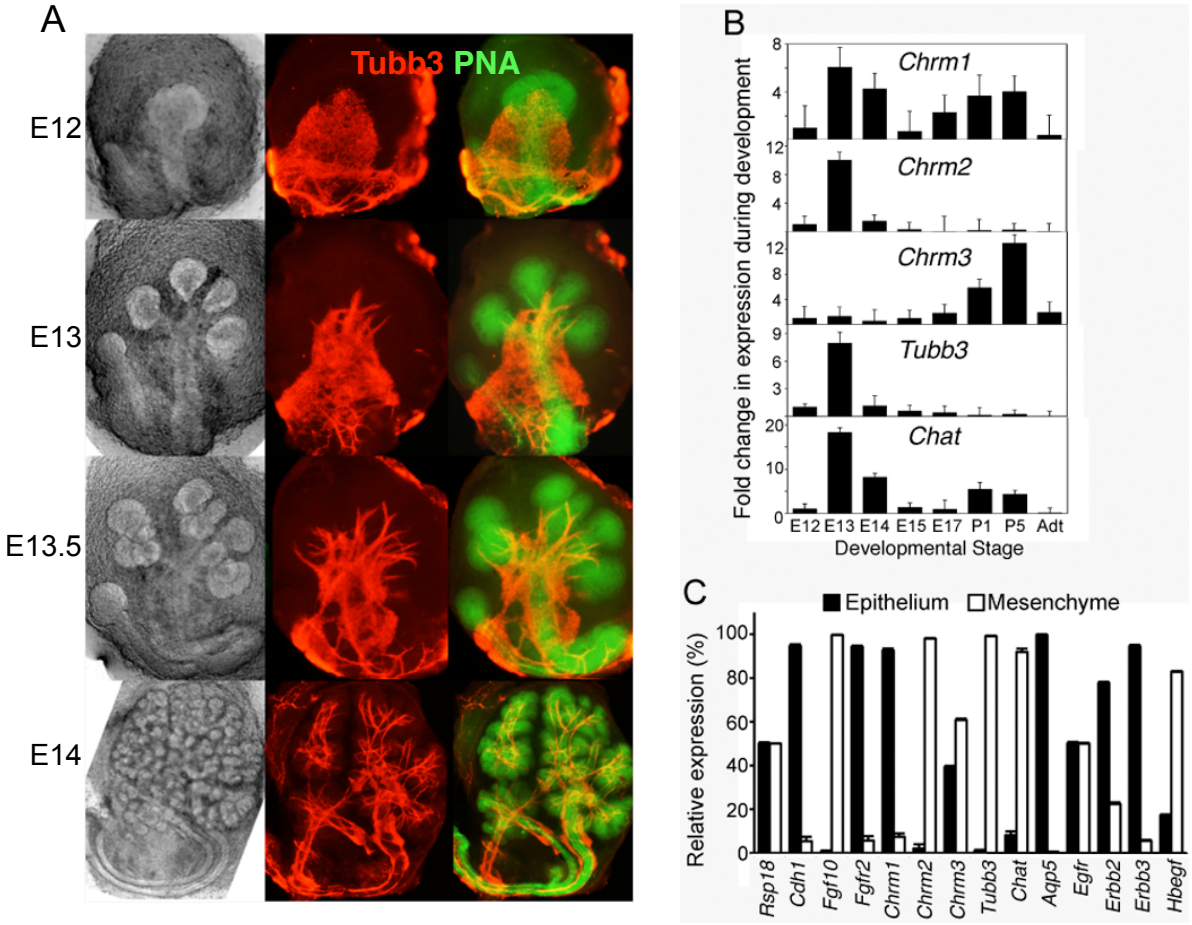

Fig. S1. The SMG and PSG undergo parallel development and M1 is the predominant muscarinic receptor expressed during epithelial branching morphogenesis. (**A**) E12, E13, E13.5, and E14 SMGs were stained for the nerves with beta3-tubulin (red) and the epithelium with PNA (green). Whole-mount epi-fluorescence images are shown. (**B**) Temporal gene expression profile of SMG development from embryonic day (E)12 to adult (Adt) by qPCR. Muscarinic receptors M1(*Chrm1*) and M2 (*Chrm2*), the acetylcholine transferase (*Chat*), and neuronal tubulin (*Tubb3*), were upregulated when branching morphogenesis begins (E12-E13). M3 (*Chrm3*) expression increases in postnatal stages of development. Error bars represent the SEM of three independent experiments. (**C**) The E13 SMG epithelium was separated from the mesenchyme, and gene expression was compared by qPCR. *Fgf10* and *Cdh1* (E-cadherin) were used as markers of mesenchyme and epithelium, respectively. Data were normalized to *Rps18.* P=postnatal; Ecad = E-cadherin.

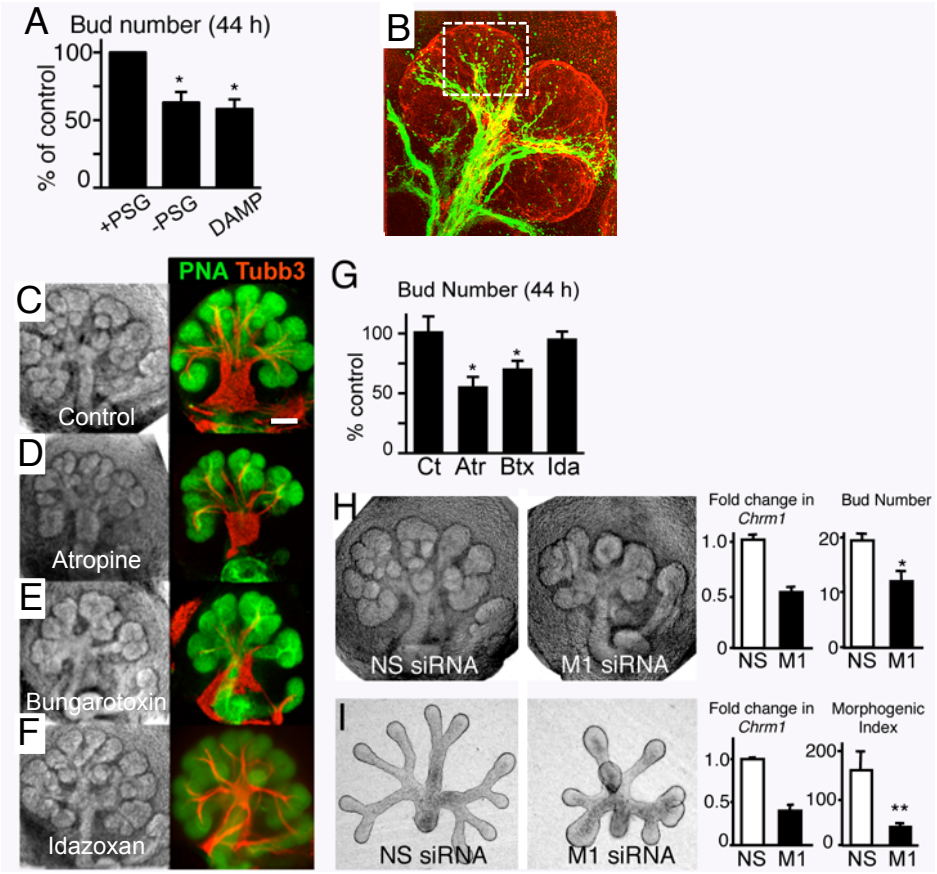

Fig. S2. **Perturbation of acetylcholine and muscarinic receptor function, as well as siRNA knockdown of M1 receptor (*Chrm1*), decrease branching morphogenesis.** (**A**) Epithelial branching was reduced after removal of PSG or inhibition of muscarinic receptors with DAMP (Graph is quantitation of Figure 1A-C). (**B**) Neuronal axons have abundant varicosities (box) around E13.5 epithelial end buds, suggesting that neuronal-epithelial interactions influence epithelial morphogenesis. **(C** to **G**) E12 SMG were treated with atropine (Atr; **B**), beta-bungarotoxin (Btx; **C**), or idazoxan (Ida; **D**) for 44 h. End buds were counted (**G**), the nerves stained withTubb3 (red), and the epithelium was stained with PNA-FITC (green). Images are a single projection of a confocal stack. (**H**) siRNA knockdown of M1 (*Chrm1*) in E12 SMG reduced *Chrm1* expression by ~ 50 % and the number of end buds by ~ 40 % after 48 h. (**I**) siRNA knockdown of M1 (*Chrm1*) in E13 epithelia reduced *Chrm1* expression by ~ 60 % and epithelial morphogenesis by ~75 % after 48 h. The decrease in *Chrm1* expression was measured by qPCR and normalized to expression with non-silencing siRNA. The Morphogenic Index = number of end buds x the length of the ducts (AU).

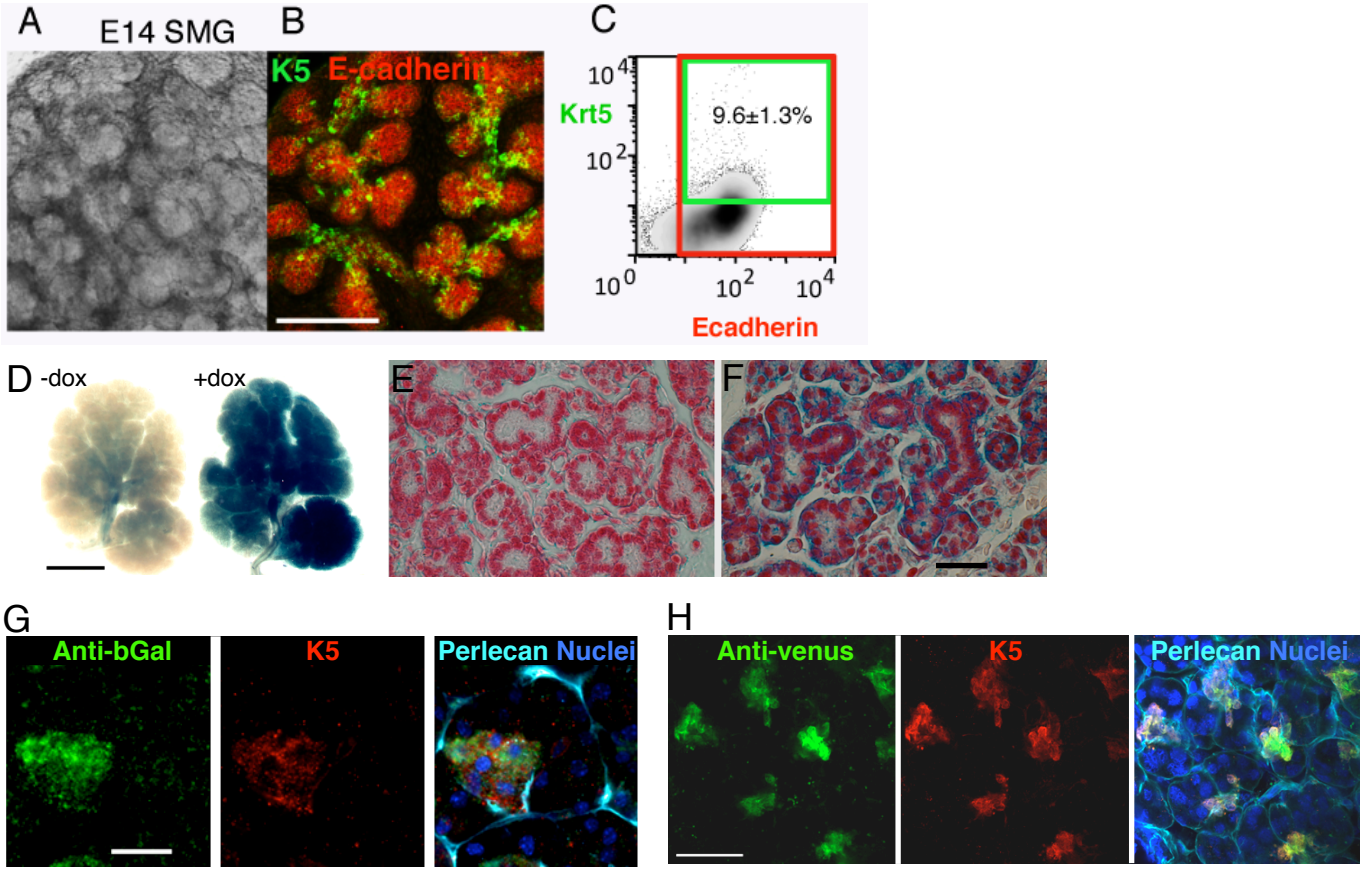

Fig S3. K5 marks an epithelial progenitor cell population in the SMG and sublingual gland. (**A-B**) Light micrograph and confocal analysis of K5+ cells (green) in the E14 SMG illustrate that they are localized in the end buds and ducts of the epithelium (red is E-cadherin). Image is a single confocal section (5µM), scale bar = 100 µm. (**C**) FACS analysis shows that 9.6 ± 1.3% of the epithelial cells in an E14 SMG are K5+. (**D-F**) Lineage tracing shows that K5 cells are progenitor cells of the epithelial compartment of the SMG. We crossed *K5-rTA* mice with *tet-Cre-R26R-lacZ* mice (*13*). The pregnant mice were fed doxycycline from E8-E11, SMG ontogenesis begins at E11, and we analyzed LacZ staining of the SMGs after birth. SMGs were collected from dox- and dox+ treated mice at postnatal day 1 (P1) and stained with X-gal. Whole-mount LacZ staining revealed widespread labeling of the ductal and acinar epithelial compartment of the SMG (**D**). In the control gland the PSG and nerves have some endogeneous beta-galactosidase activity, resulting in pale blue staining along the primary ducts. A section of each gland with nuclei counterstained with fast red is shown below the whole mount X-gal staining (**E and F**). LacZ+ cells (blue) were located throughout the SMG in both the ductal and acinar compartments. Bar = 1mm. (**G**) After induction of Cre excision by doxycycline the cells in the SMGs that express b-galactosidase (anti-bGal, green) are K5+ (red). Scale bar = 20µm. (**H**) The expression of the bovine-K5-promoter used for lineage tracing in the SMG corresponds with endogenous K5+ expression in the SMG. SMGs from mice expressing bovine-K5-*Venus* were stained with anti-Venus (green) and endogenous K5 protein (red). Scale bar = 50µm. (**G-H**) Perlecan (cyan) labels the basement membrane and the nuclei were labeled with Hoescht (blue).

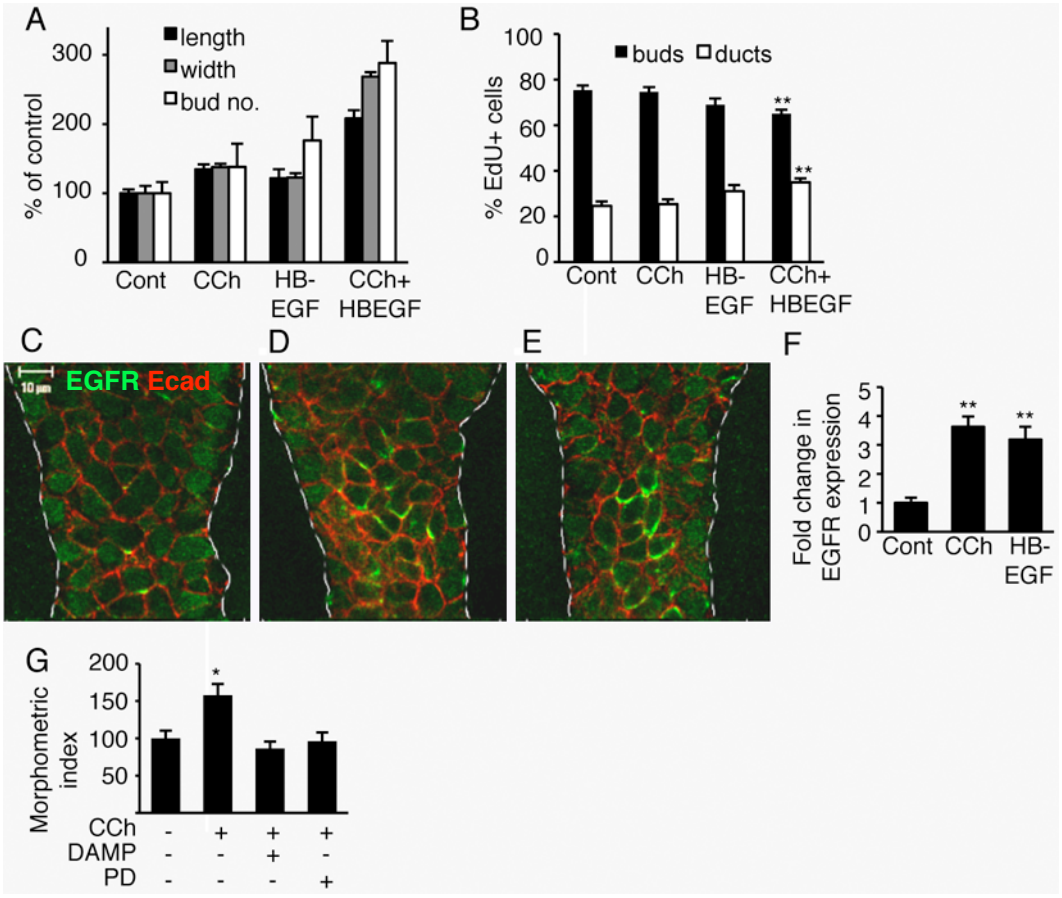

Fig S4. Analysis of CCh- and HBEGF-dependent epithelial morphogenesis. CCh-dependent morphogenesis requires EGFR and CCh upregulates EGFR protein expression in the epithelium. (A-B) CCh and HBEGF both increase epithelial morphogenesis and proliferation. Duct length, bud width and number of buds (**A**) and the number of EdU+ cells in buds and ducts (**B**) was estimated for epithelia treated with CCh, HBEGF or both. There was no significant difference between CCh and HBEGF in their effects on epithelial morphogenesis or cell proliferation. **(C-F)** E13 epithelia cultured for 24 h (**C**) with 10 nM CCh (**D**) or 2 ng/ml HBEGF (**E**) were fixed and stained for EGFR (green) and E-cadherin (red). EGFR expression was quantified as immunofluorescent intensity/epithelial area (**F**). ANOVA with post hoc Dunnetts test; *$P < 0.05$, **$P < 0.01$ (**G**) CCh-dependent morphogenesis requires both M1 and EGFR signaling as it is reduced by both DAMP and PD. E13 epithelia were cultured for 24h with 10nM CCh with 5 μm of either DAMP or PD168393. The morphometric index was determined.

Suppl. Data Fig. 5

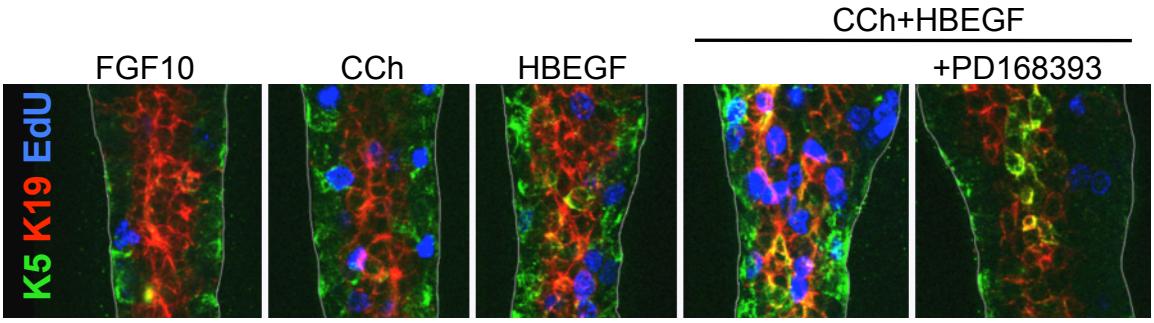

Fig S5. Images were used to count which cells were proliferating in response to CCh and HBEGF in epithelial culture, see Fig 2H for graph. CCh significantly increases the percentage of proliferating cells expressing K5+, whereas HBEGF increases the K5+K19+ and K19+ (red), and both CCh and HBEGF increase proliferation of K5+ and K5+K19+. Addition of PD inhibits proliferation of K5+ and K19+ cells after 44 hrs of culture, although some proliferation still occurs in K5-K19- cells. We used confocal stacks of images to count the number of proliferating cells (EdU+) that were K5+K19-, K5+K19+, or K5-K19+, which were expressed as a percentage of the total EdU+ cells.

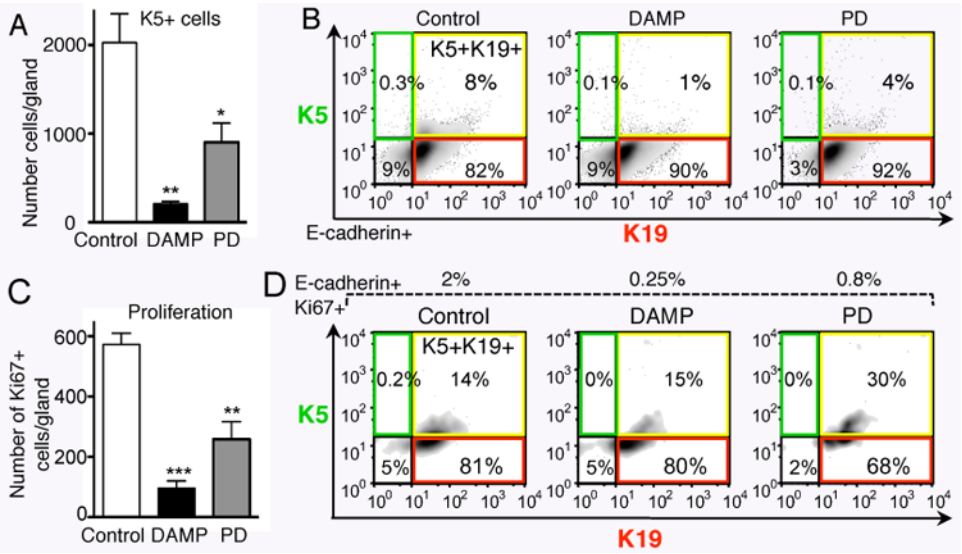

Fig. S6. Inhibition of muscarinic and EGFR signaling in the intact SMG reduces the number of K5+K19- cells and inhibits their proliferation. (**A**) the total K5+ cell population (2,026 K5+ cells out of 27,737 ± 1631 cells/gland, 7.3%), which includes both K5+K19- and K5+K19+ cells, was significantly reduced by both DAMP (207 K5+ cells out of 20,708± 2697 cells/gland, 1.0%) and PD (903 K5+ cells out of 29,448 ± 2288 cells/gland, 3.1%) treatment fro 44 hr. (**B**) FACS analysis of the epithelial cells (E-cadherin+) by their K5 and K19 expression shows that, DAMP and PD reduced the number of both K5+K19- cells (from 0.3% in control to 0.1%) and K5+K19+ cells (from 8% to 1% and 4% with DAMP and PD, respectively. This loss of K5+K19- and K5+K19+ cells was associated with a corresponding increase in the number of K5-K19+ cells (82% control to 90% and 92% with DAMP and PD, respectively), which demonstrates that K19+ cells can differentiate when M1 or EGFR signaling is inhibited. (**C**) The number of proliferating cells (Ki67+) in an intact SMG (573 cells/gland) decreased after DAMP (96 cells/gland) and PD (258 cells/gland) treatment. (**D**) The proliferating epithelial cells (E-cadherin+Ki67+) were further analyzed by FACS for their K5 and K19 expression. DAMP and PD inhibit K5+K19- cell proliferation, confirming that their maintenance by proliferation is both M1- and EGFR-dependent. Proliferation of the K5+K19+ (15 %) and the K5-K19+ (80 %) cells with DAMP treatment was similar to control, suggesting that these cells can proliferate independent of muscarinic activation. PD treatment increased the proliferation of K5+K19+ cells (from 15% to 30%) and decreased proliferation of K5-K19+ cells (from 81% to 68%), which indicates that K5+K19+ and K5-K19+ cell proliferation is not solely dependent on EGFR signaling. All graphs are mean ± SEM from three independent experiments; ANOVA with post hoc Dunnetts test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

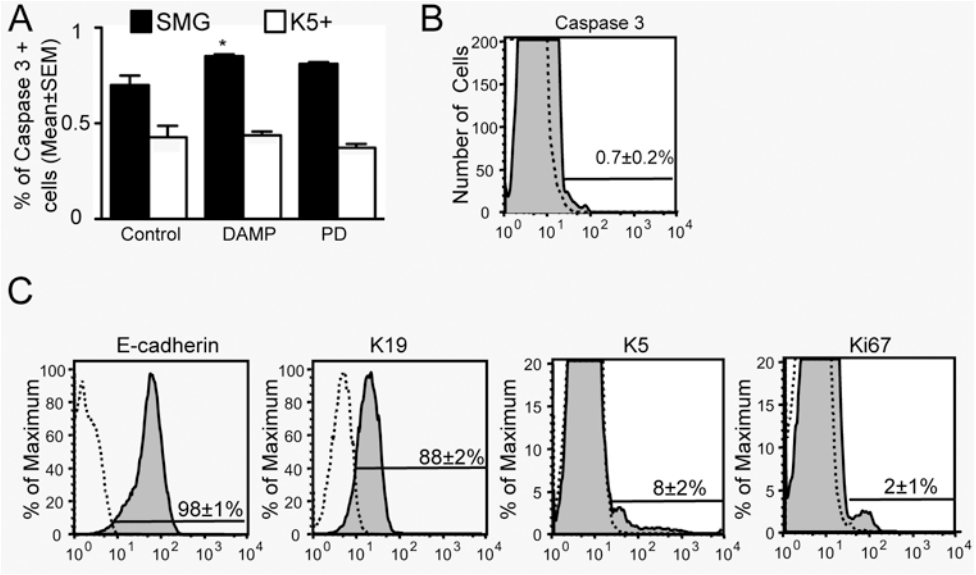

Fig. S7. Inhibition of CCh/M1 and EGFR signaling does not increase K5+ cell apoptosis. (**A**) E13 SMG were treated with either DAMP or PD for 44h and analyzed by FACS for K5 and caspase 3, an early marker of apoptosis. No significant difference was observed between the percentage of caspase 3 positive cells in epithelial K5+ cell types in Control, DAMP, or PD-treated samples. Mean±SEM of three independent experiments (ANOVA; $*P < 0.05$). (**B**) FACS histogram representing cells stained for caspase 3 (grey) or isotype control (dashed line). Caspase 3 positive cells were gated by the horizontal line. (**C**) Histograms generated by FACS analysis of cells immunostained with the epithelial marker E-cadherin, K19, K5, or the proliferation marker Ki67 (gray area) to determine the percentage of cells expressing each protein in SMGs (fig S6A-D). The percentage of cells positive for staining is determined by comparing the histogram of an antibody isotype control (dashed line). X-axis represents the intensity of protein expression. Y-axis shows the number of cells with a particular protein intensity, which is normalized when comparing different sized populations.

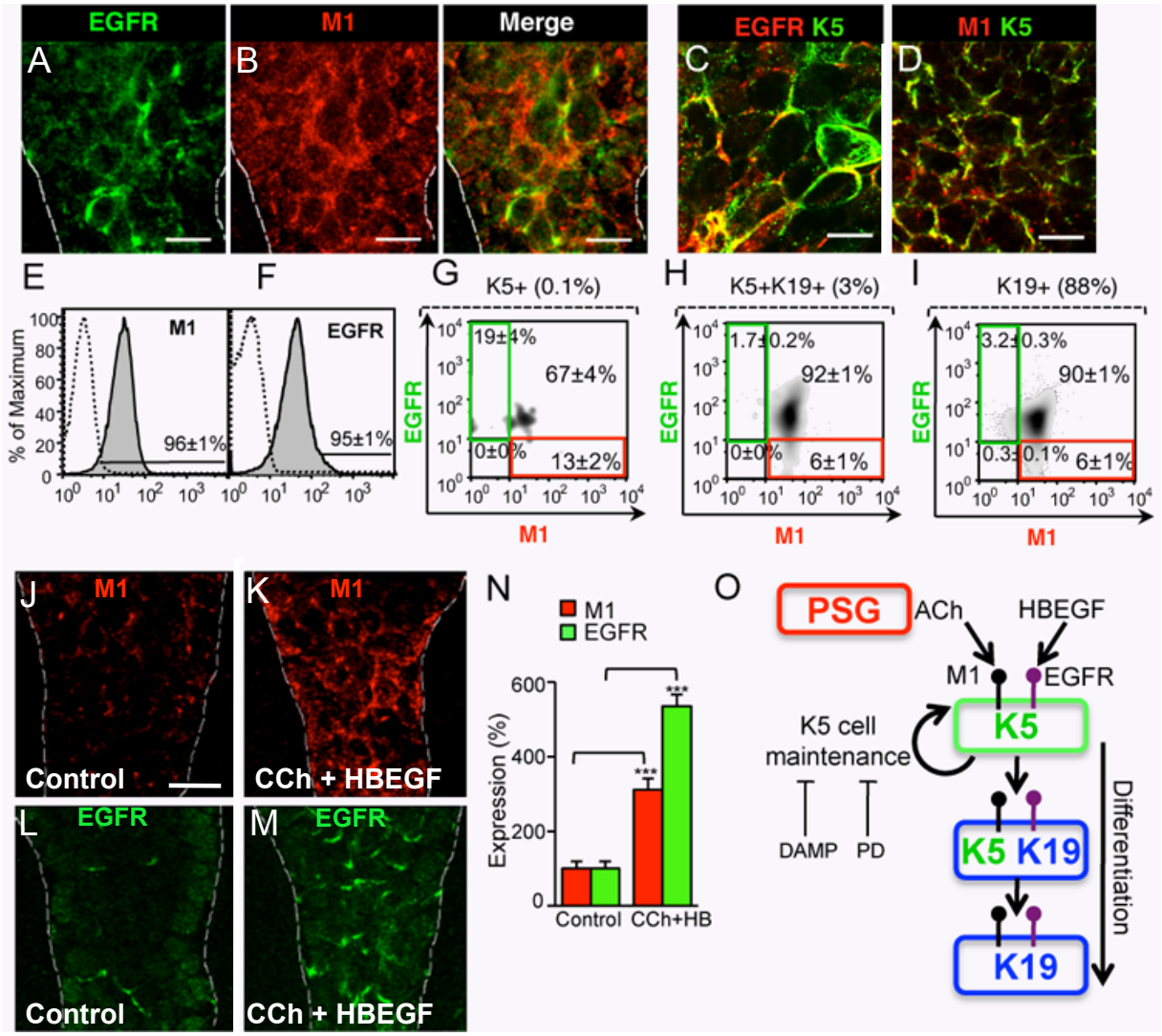

Fig S8. EGFR and M1 receptors are both present in K5+ cells and the addition of CCh + HBEGF upregulates M1 and EGFR. (**A-D**) EGFR and M1 are co-expressed in epithelial cells and with K5. E13 epithelia (**A-C**) in FGF10 supplemented media treated with CCh (10nM) and HBEGF (2ug/ml) or E13 SMG (**D**) were immunostained for M1 or EGFR. Scale bar = 10µm. Ct=control. (**E-F**) The majority of cells in the SMG expressed M1 (96 %) and EGFR (95 %) suggesting that the effects of CCh and HBEGF are cell-autonomous. (**G-I**) The majority of K5+ (67%), K5+K19+ (92%) and K19+ (90%) cells express both M1 and EGFR. Interestingly, 19 % of the K5+ cells expressed only EGFR, and 13 % expressed only M1. We speculate that these subpopulations represent quiescent K5+ cells, and that the co-expression of both receptors results in proliferation. E13.5 SMGs were analyzed by FACS. Average mean of three independent experiments (ANOVA; ***$P < 0.001$). (**J-N**) CCh and HBEGF upregulate M1 and EGFR at the cell surface. These data imply that positive feedback increases receptor expression to enhance cell proliferation. The quantification of fluorescence per unit area was expressed as a percentage of control. Mean ± SEM; ***$P < 0.001$; Student's t test. Scale bar = 20µm (**O**) Model of K5+ progenitor cell differentiation. All images of immunostaining are single confocal sections (1 µM).

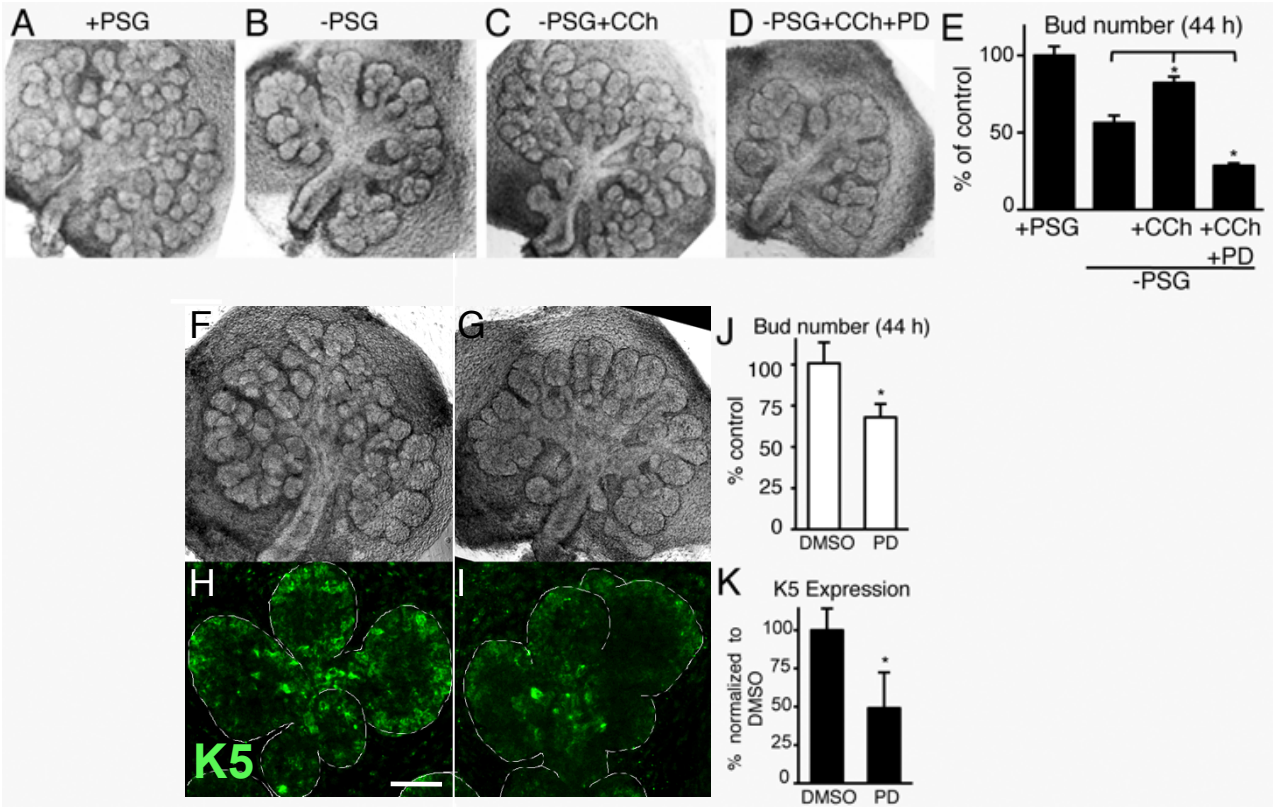

Fig S9. CCh rescues branching morphogenesis in SMG explant culture without the PSG, in an EGFR-dependent manner. SMG recombination explants with (**A**) or without (**B**) the PSG cultured for 48 h with 50 nM CCh (**C**) or 50 nM CCh+10 μM PD (**D**). Images of the K5 staining are in Figure 3A-D. (**E**) End bud number at 48 h.

The inhibition of EGFR alone, also decreases epithelial morphogenesis and K5 expression in the presence of the PSG. (F-K) Recombined SMG explants were treated with DMSO (**F** and **H**) or 10μM PD168393 (**G** and **I**). End buds were counted (**J**) and the glands fixed and stained for K5 (C and D). Scale bar = 50 μm. K5 expression was determined per epithelial area and presented as percentage of DMSO control (**K**). Mean ± SEM of three independent experiments (**P* <0.05*, Students t-test*).

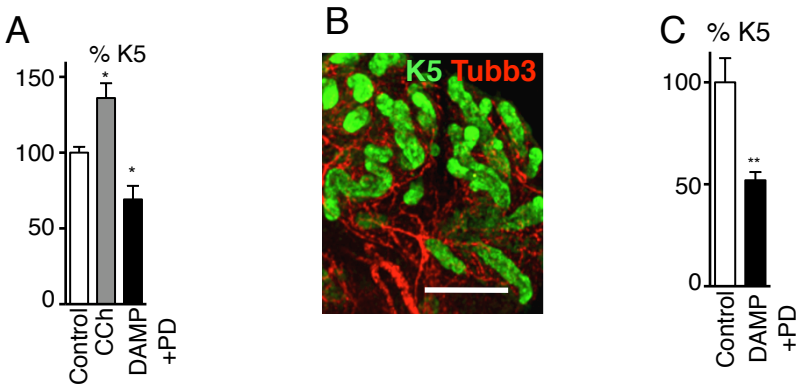

Fig S10. Muscarinic receptor/EGFR signaling also controls K5+ protein expression in the adult SMG and in the developing prostate. (**A**) Quantitation of K5 staining from Figure 4 A-C. Denervated lobules of adult SMGs were cultured for 44 h with CCh or DAMP+PD and immunostained for K5. CCh increases K5 whereas DAMP+PD reduce K5 compared to control. (**B**) Ventral prostates from P6 mice were stained for K5 (green) and beta3 tubulin (Tubb3; red), scale bar = 200 μm. (**C**) Quantitation of K5 staining from Figure 4 E-F. Prostates cultured for 48 h ± DAMP+PD were stained for K5. (*$P < 0.05$, **$P < 0.01$, *Students t-test)*