Supplemental Information

Submandibular parasympathetic gangliogenesis requires Sprouty-dependent Wnt signals from epithelial progenitors

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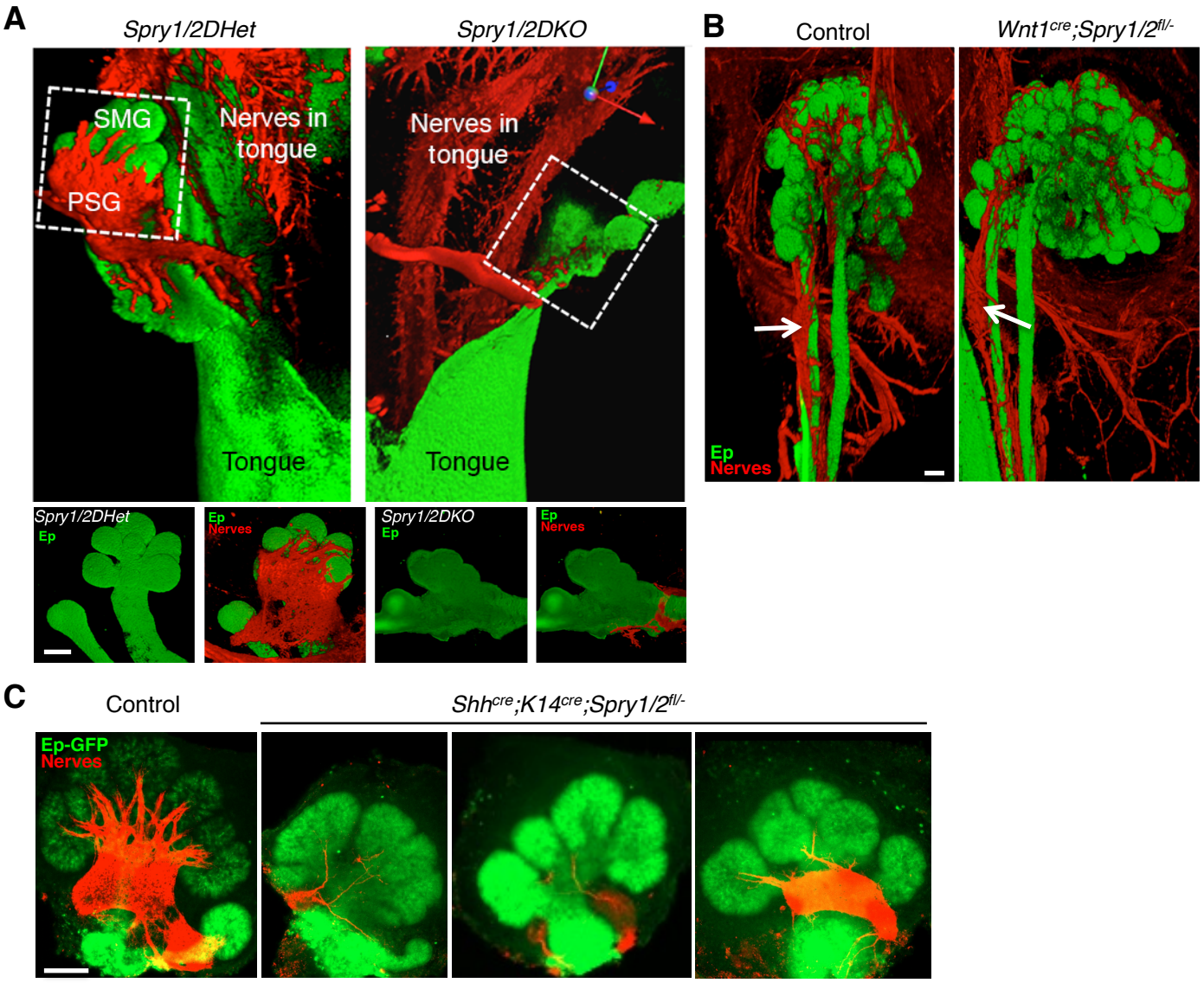

Figure S1, related to Figure 1.

(A) The tongue is still innervated in the *Spry1/2DKO* mouse. Whole mount immunostaining for the epithelium (Ep, E-cadherin) and nerves (Tubb3) in *Spry1/2DHet* and *Spry1/2DKO* tongues with SMGs attached. Stained tissues were cleared in BABB and 3D reconstructions generated with Volocity software. The SMGs (in white box) are in the lower panels. Scale bar, 100 µm.

(B) *Wnt1*^{cre} neural crest conditional knockout of *Spry1* and *Spry2* does not affect PSG formation and innervation of the SMG. *Wnt1*^{cre} was used to delete *Spry1* and *Spry2* in the neural crest by crossing *Wnt1*^{cre}*;Spry1/2*$^{+/-}$ males with *Spry1/2*$^{flox/flox}$ females to generate *Wnt1*^{cre}*;Spry1/2*$^{flox/-}$ embryos that are *Spry1/2DKO* in the neural crest, which gives rise the SMG mesenchyme and PSG. E14 tongue and associated SMGs were dissected, fixed, immunostained for epithelium, (Ep, E-cadherin) and nerves (Tubb3), cleared in BABB and 3D reconstructions generated with Volocity software. The nerves around the SMG are within the tongue. Scale bar, 100 µm.

(C) Deletion of *Spry1/2* in a subset of epithelial cells disrupts the PSG and innervation. Both *Shh*^{cre} and *K14*^{cre} were used to delete *Spry1* and *Spry2* in 80 +/- 4 percent of epithelial cells. E13 SMGs were immunostained for Tubb3 (nerves), green staining is from an endogenous membrane-bound GFP expressed in the Cre-recombined cells. The phenotype of the *Shh*^{cre}*;K14*^{cre}*;Spry1/2*$^{fl/-}$ SMGs varies as shown, the PSG is typically smaller and innervation is reduced. Scale bar, 100 µm, n= 4.

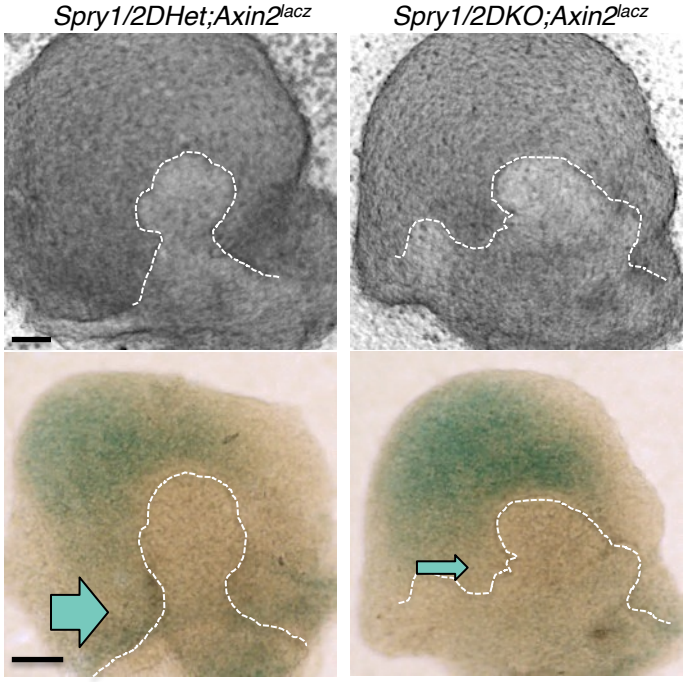

Figure S2, related to Figure 2.
Endogenous Wnt signaling is reduced around the duct in *Spry1/2DKO* SMGs. Whole mount brightfield and XGAL staining images of E13 *Spry1/2DHet;Axin2^{lacZ}* control and *Spry1/2DKO;Axin2^{lacZ}* mutant SMGs. Blue arrows indicate XGAL staining around the epithelial duct. *Spry1/2DKO* SMGs carrying the *Axin2^{lacZ}* allele have reduced XGAL staining adjacent to the epithelial duct when compared to *Spry1/2DHet;Axin2^{lacZ}* controls. This confirms a reduction in Wnt signaling in *Spry1/2DKO* SMGs, which correlates with a loss of PSG formation in these mutants. $n = 4$. Scale bars, 50 μm.

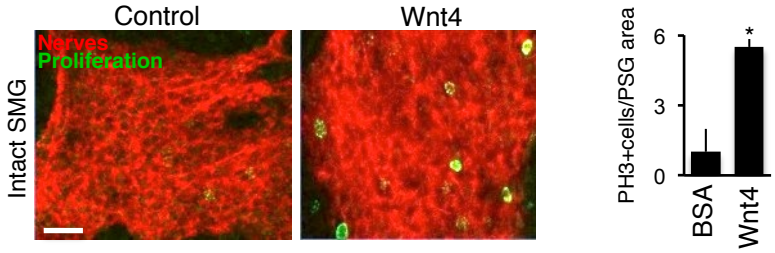

Figure S3, related to Figure 3.
Wnt signaling increases neuronal proliferation in intact SMGs. E12 SMGs cultured for 24 h with BSA (control) or Wnt4 protein and immunostained for nerves (Tubb3) and phospho-histone H3 (Proliferation). The graph shows quantitative analyses of proliferation. *n* = 3. Student's t test, **P* < 0.05; Error bars are s.d. Scale bar, 25 μm.

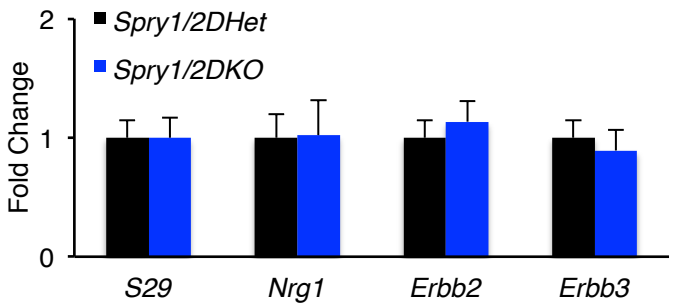

Figure S4, related to Figure 4.
Gene expression of *Nrg1*, *Erbb3*, and *Erbb4* does not change in the *Spry1/2DKO*. qPCR analysis of E13 *Spry1/2Dhet* and *Spry1/2DKO* SMGs. $n = 4$.

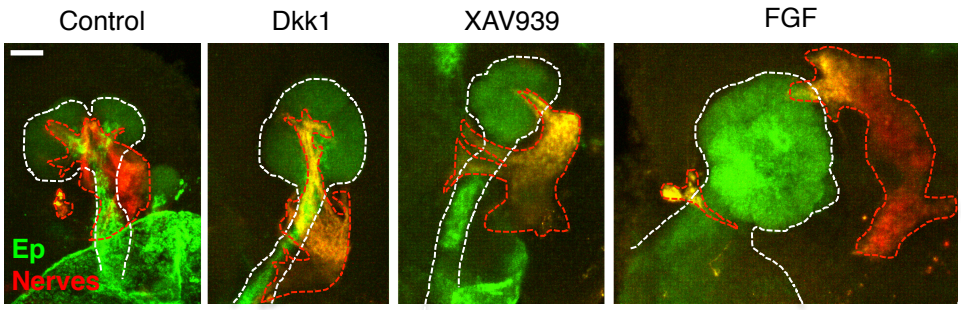

Figure S5, related to Figure 5.
Inhibition of Wnt signaling disrupts SMG development. Additional images of immunostained control, Dkk1-, XAV939-, FGF-treated mandible cultures after 48 h. White dashed lines outline the SMG epithelium, red dashed lines outline the ganglia. Scale bar, 100 µm.

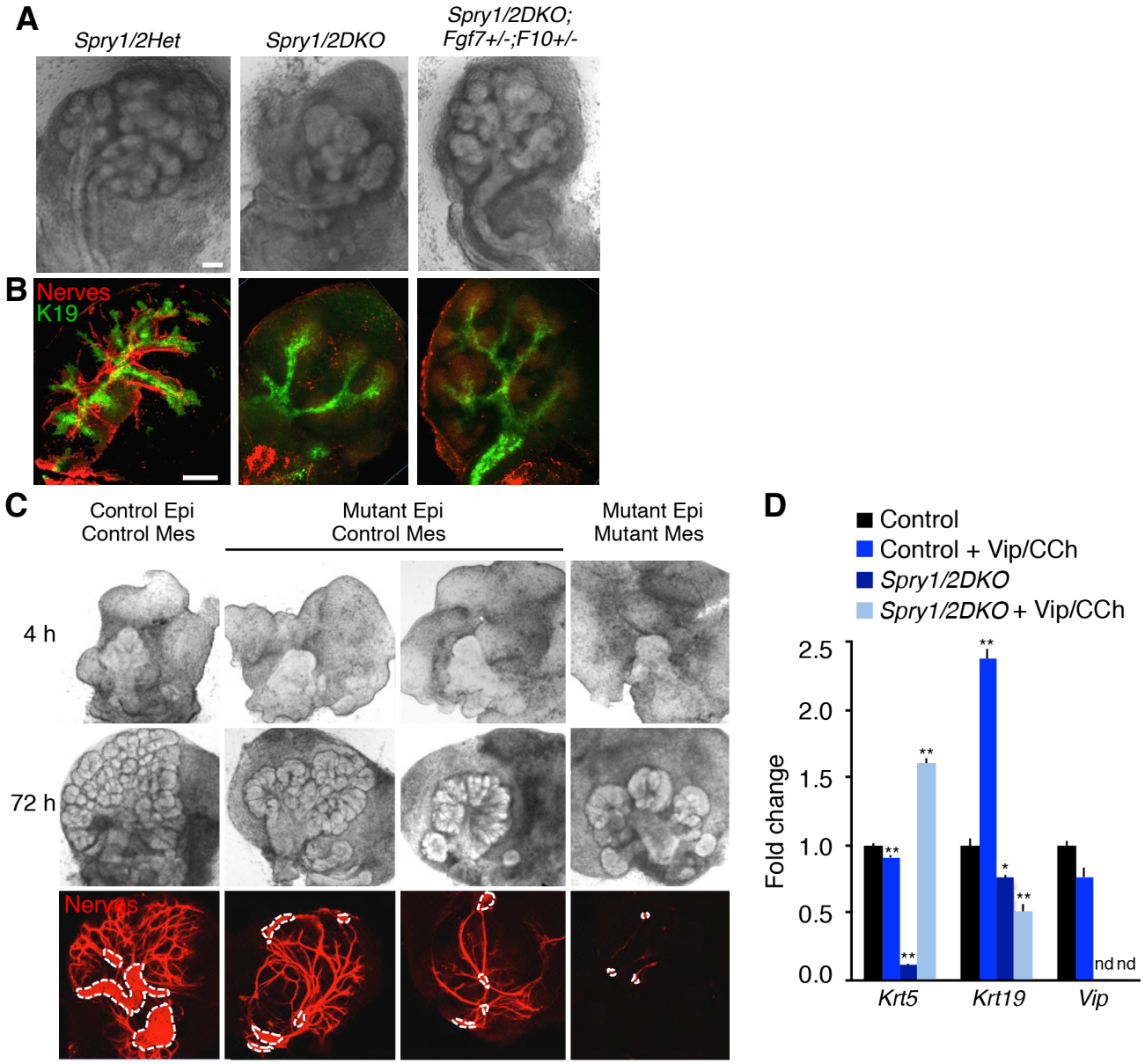

Figure S6, related to Figure 6.

(A) Genetic deletion of *Fgf7* and *Fgf10* alleles rescues primary duct formation in *Spry1/2DKO* SMGs. Brightfield images of E14 SMGs from (left-right) *Spry1/2DHet*, *Spry1/2DKO* and *Spry1/2DKO;Fgf7*$^{+/-}$*;Fgf10*$^{+/-}$ embryos. $n = 3$

(B) Genetic deletion of *Fgf7* and *Fgf10* alleles does not rescue the PSG in *Spry1/2DKO* SMGs. Immunostaining for nerves (Tubb3) and Keratin 19. Scale bars, 200 μm.

(C) Recombining *Spry1/2DKO* epithelia with control PSGs does not rescue innervation. E13 epithelia were dissected from control and *Spry1/2DKO* SMGs and an epithelial rudiment was recombined with 3 mesenchymes from either control E13 SMGs (Control Mes) or *Spry1/2DKO* SMGs (Mutant Mes). The recombined tissues were cultured for 72 h and immunostained for nerves (Tubb3). The wild-type PSGs do not associate with duct when cultured with mutant epithelia, rather the PSG becomes fragmented and fewer axons extend towards the endbuds compared to controls. Dashed white lines outline the PSG cell bodies. $n = 4$.

(D) Treatment of *Spry1/2DKO* SMGs with Vip and Carbachol increases *Krt5* expression. E14 SMGs were cultured for 48 h with 500 nM Vip and 50nM Carbachol (CCh) and gene expression was analyzed by qPCR. *Krt5* expression is increased in *Spry1/2DKO* after Vip + CCh treatment. ANOVA compared to control, error bars show SEM, * $p < 0.05$, ** $p < 0.01$. $n = 2$.

ProbeName	Spry1/2 Het/Het	SD	Spry1/2 DKO	SD	Fold Decrease	Gene	GeneName
A_51_P368313	1004	222	5	2	191	**Vip**	Vasoactive intestinal polypeptide
A_55_P2038787	938	206	5	1	186	**Olfr78**	olfactory receptor 78
A_55_P2000284	13104	4076	91	27	144	**Phox2a**	paired-like homeobox 2a
A_55_P2027421	676	117	8	3	82	**Fam163a**	family with sequence similarity 163A
A_55_P2160676	507	16	7	2	73	**Ret**	ret proto-oncogene
A_52_P1130990	465	72	7	2	66	**Trim67**	tripartite motif-containing 67
A_55_P2167486	321	43	5	1	64	**Resp18**	regulated endocrine-specific protein 18
A_55_P2041828	12141	1528	202	103	60	**Tubb3**	tubulin, beta 3
A_51_P191669	2466	507	42	16	58	**Chgb**	chromogranin B
A_55_P2068673	6353	830	112	89	56	**Stmn2**	stathmin-like 2
A_51_P140690	3005	239	54	27	56	**Stmn3**	stathmin-like 3
A_55_P2040549	2229	239	40	41	55	**Phox2b**	paired-like homeobox 2b
A_55_P2086240	607	151	11	9	53	**Kcnq2**	potassium voltage-gated channel, subfamily Q2
A_51_P379552	393	90	8	3	52	**Nwd2**	NACHT and WD repeat domain containing 2
A_55_P2055127	228	51	5	1	48	**Slc7a14**	solute carrier family 7, member 14
A_55_P1968895	6264	794	131	18	48	**Prph**	peripherin
A_55_P2164659	2233	509	47	49	48	**Tlx2**	T-cell leukemia, homeobox 2
A_55_P1990309	468	162	10	8	47	**Elavl4**	Embryonic lethal, abnormal vision-like 4
A_55_P2081323	2320	640	56	16	41	**Gnas**	Guanine nucleotide binding protein complex locus
A_51_P448478	229	59	6	1	37	**Slc10a4**	solute carrier family 10, member 4

Supplementary Table 1 related to Figure 1b.
Top 20 genes with reduced expression in the *Spry1/2DKO* SMG compared to *Spry1/2DHet* by Agilent Microarray Analysis (n=3 for each group)

Supplementary Experimental Procedures

Immunostaining and Antibodies

For wholemount staining, SMGs and mandibles were fixed in ice-cold acetone:methanol (1:1) for 10 minutes. PSG cultures were fixed in 4% PFA for 20 minutes followed by permeabilization in ice-cold acetone:methanol (1:1) for 5 minutes. Pancreas samples were fixed in 4% PFA overnight followed by fixation in ice-cold acetone:methanol (1:1) for 15 minutes. The following primary antibodies were used: rabbit anti-E-cadherin (1:200, #3195, Cell Signaling), mouse anti-β-III-tubulin (1:200, #BAM1195, clone TUJ1, R&D Systems), rabbit anti-cytokeratin 5 (1:2,000, #ab24647, Abcam), rat anti-cytokeratin 19 (1:200, troma-III cytokeratin, Developmental Studies Hybridoma Bank), rabbit anti-caspase3 (1:200, #9664L, Cell Signaling), rat anti-PECAM-1 (1:100, #550274, Clone MEC13.3, BD Pharmingen), rabbit anti-phospho-Histone H3 (Ser10) (1:500, #06-570, EMD Millipore), rabbit anti-β-galactosidase (1:2,000, #08559761, MP Biomedicals), goat anti-GFRα2 (1:100, #AF429, R&D Systems). Secondary antibodies were all donkey DyLight F(ab)2 fragments labeled with Cy2, Cy3 or Cy5 (Jackson Immunoresearch Laboratories).

Ex Vivo Culture Treatments

FGF treated cultures were incubated with both rhFGF7 (1 μg/ml, R&D Systems) and rhFGF10 (1.5 μg/ml, R&D Systems). The following proteins and chemicals were also used in organ culture: rmWnt4 (125 ng/ml, R&D Systems), rmDkk1 (2.5 μg/ml, R&D Systems) and XAV939 (10 μM, Tocris). SMG epithelial rudiments were dissected and cultured in laminin-111 gel (Trevigen) as previously described (Lombaert et al., 2013).

XGAL staining and Cryosections

SMGs were fixed in 0.2% glutaraldehyde, 2% PFA solution for 20 minutes at 4°C, rinsed once and incubated for 30 minutes in Tissue Rinse Solution A (Millipore, #BG-6-B) at room temperature. SMGs were then briefly rinsed and incubated for 5 minutes in Tissue Rinse Solution B (Millipore, #BG-7-B). XGAL (20 mg/ml) was diluted 1:25 in fresh staining buffer (0.1 M phosphate buffer (pH7.5), 2 mM $MgCl_2$, 0.02% NP-40, 20 mM Tris-HCl (pH7.5), 8 mM potassium hexacyanoferrate(II) trihydrate, 13 mM potassium

hexacyanoferrate(III)). Samples were incubated in the XGAL staining solution at 37°C, then washed in PBS and refixed in 4% PFA for 10 minutes. For cryosections, tissues were fixed in 4% PFA for 20 minutes, washed in PBS and then incubated for 1 h in 15% sucrose followed by 2 h in 30% sucrose. Tissues were then placed in OCT, positioned and snap frozen on dry ice. 15 μm cryosections were cut using a Leica CM3050S cryostat and immunostained.

Microarray analysis

E13 SMG epithelia were isolated from mesenchyme and then the endbud was mechanically separated from the duct. Four independent samples of RNA were made from pooled endbuds and ducts. Agilent whole mouse genome arrays were analyzed with Genespring Software, identifying duct genes with a >5x increase in expression and a p-value < 0.05. The 332 genes were analyzed using the Molecular Signatures Database and KEGG Pathways analysis (MSigDB at www.broadinstitute.org).

Recombination experiments

For recombination experiments, E13 SMG were dispase-treated, then separated into mesenchyme (including PSG) and epithelia, and collected in 5 % BSA. After washing in DMEM/F12, epithelia were recombined with 3-4 mesenchymes on a filter and floated on culture medium. Recombined epithelia + mesenchymes were cultured for at least 72 h at 37°C (Knox et al., 2010).