Supplemental Information Inventory

Supplementary Figures and Legends and Tables

Supplementary Experimental Procedures

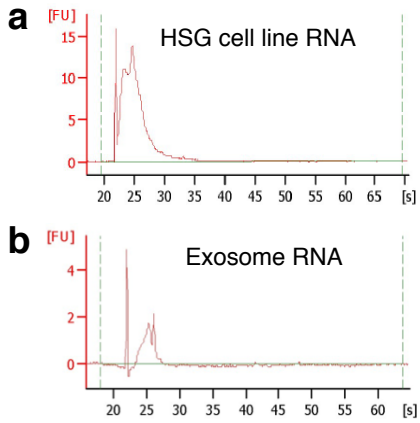


Figure S1, related to Figure 1. Bioanalyzer analysis of control samples during exosome purification. (**a**) Human Submandibular Gland (HSG) cell-line conditioned medium as a positive control for miRNA. (**b**) The exosome fraction contained small RNAs after washing twice with PBS. N = 3, graph is a representative experiment.

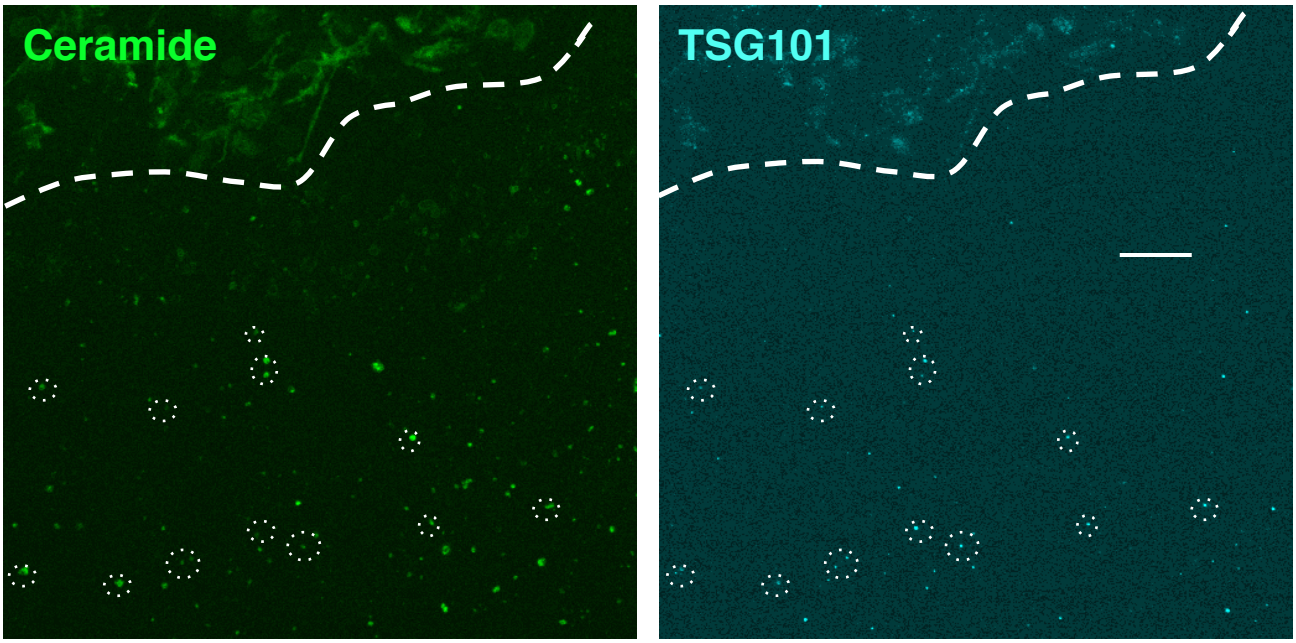
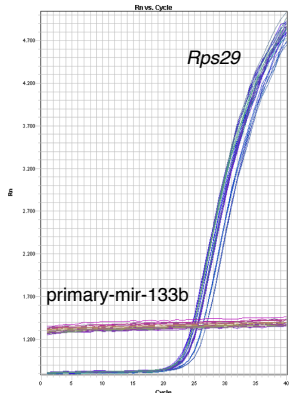


Figure S2, related to Figure 2. Ceramide-labeled mesenchyme was cultured on ECM that contained isolated epithelium (similar to Fig 2b) and stained with the exosome marker TSG101. Mesenchyme cells, above the white dotted line, are releasing ceramide-labelled vesicles that co-stain with TSG101. Some examples of ceramide-labelled vesicles in the ECM that co-stain with antibodies to TSG101 are outlined in small circles. N = 3. Scale bar = 25 μm.



Ct average (STDEV)

	E12	E13	late E13
Rps29	21.6 (0.40)	22.8 (0.69)	21.5 (0.31)
Primary mir-133b	ND	ND	ND

Figure S3, related to Figure 3. Amplification curve in the TaqMan qPCR of the Rps29 and primary mir-133b in the SMG epithelium at E12, E13, and late E13. The primary mir-133b transcript was not detected in the SMG epithelium while *Rps29* as endogenous control showed ~Ct values of 22. N = 3.

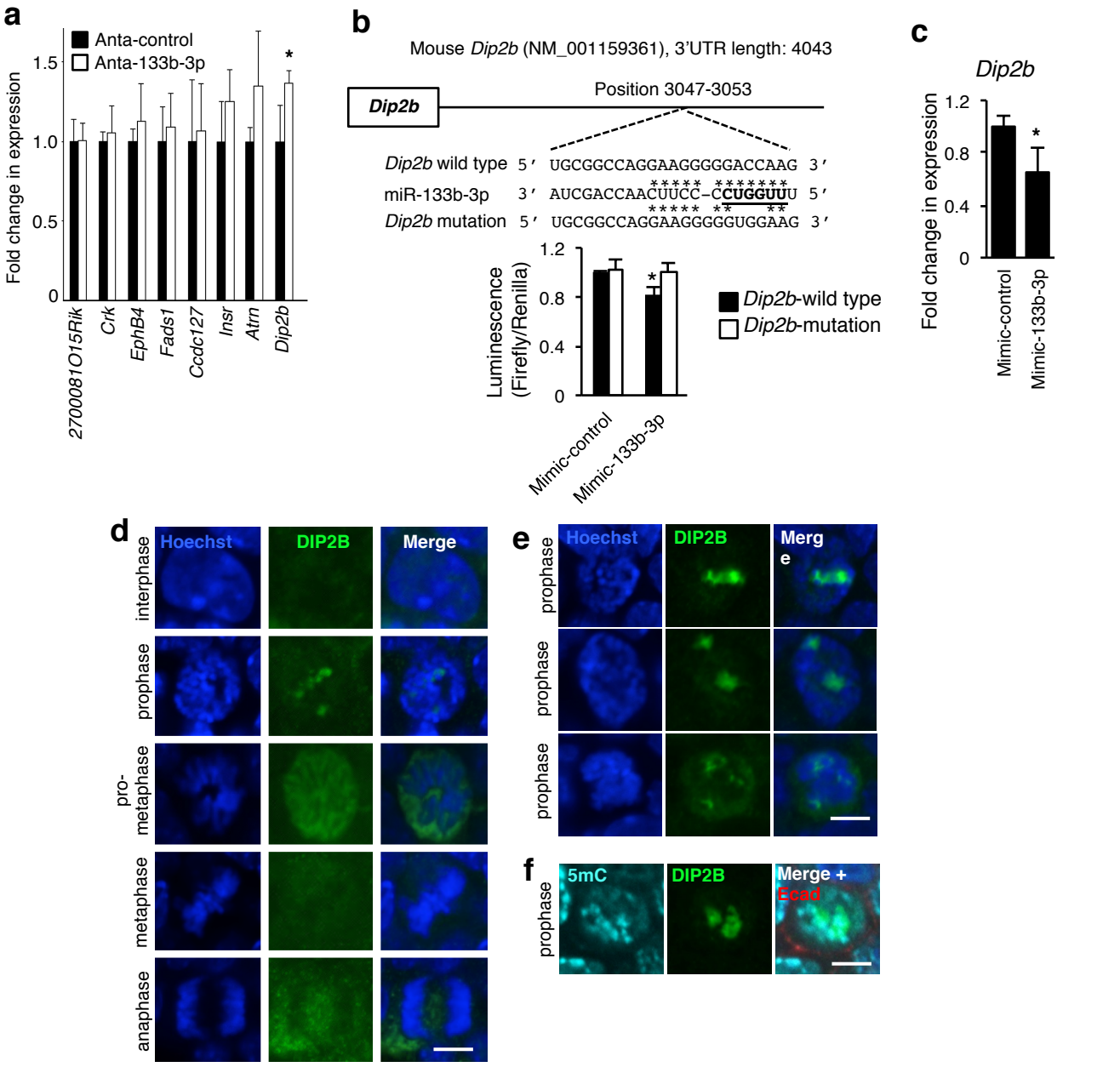


Figure S4, related to Figure 4. (**a**) miR-133b-3p loss-of-function increases disco-interacting protein 2 homolog B (*Dip2b*) expression. Isolated-epithelia were treated with an antagomir (Anta-133b-3p) at 200 nM and predicted targets of mir-133b-3p were analyzed by qPCR after knock-down. *Dip2b* was significantly increased after 20 h compared to a control antagomir (Anta-control). Student's *t*-test, *P< 0.05. N = 3. (**b**) The seed region of the miR-133b-3p target site of the *Dip2b* 3'UTR,shown in **bold**, was mutated (ACC to UGG) and tested in an NIH3T3 cell luciferase reporter assay, comparing the wild-type miR-133b-3p binding site (*Dip2b*-wild type) to a construct with 3-nt substitutions. Disrupting base-pairing of the seed region of miR-133b-3p (*Dip2b*-mutant) reduces luminescence. N = 3. Student's *t*-test, *P< 0.05. (**c**) miR-133b-3p gain-of-function decreases *Dip2b* expression. Isolated-epithelia were treated with mimic at 200 nM for 20 h. There was no change in morphogenesis 20 h after mimic treatment. *Dip2b* expression was normalized to *Rps29* and compared with epithelia treated with negative control mimic (Black bar, dashed line). N = 3. Student's *t*-test, *P< 0.05. (**d**) Additional examples of DIP2B expression patterns in SMGs epithelial cells during mitosis. Scale bar 5µm. DIP2B aggregated in the prophase nuclei of epithelial cells and was disassociated from mitotic chromatin in other phases. (**e**) Additional images of DIP2B in prophase nuclei. Scale bar 5µm. (**f**) Additional images showing that DIP2B was partially associated with methylated-cytosine (5mc) in heterochromatin in SMG E-cadherin+ epithelial cells (Ecad). Images are 1µm optical sections. Scale bar 5µm.

Supplemental Table 1, related to Fig. 3a.
List of exosomal microRNAs (81) identified from the media of intact E13 SMGs cultured for 48 h.

mmu-let-7b-5p, mmu-let-7c-5p, mmu-miR-100-5p, mmu-miR-106a-5p, mmu-miR-106b-5p, mmu-miR-125b-5p, mmu-miR-126-3p, mmu-miR-126-5p, mmu-miR-127-3p, mmu-miR-128-3p, mmu-miR-130a-3p, mmu-miR-130b-3p, mmu-miR-132-3p, mmu-miR-133a-3p, mmu-miR-133b-3p, mmu-miR-134-5p, mmu-miR-138-5p, mmu-miR-139-5p, mmu-miR-145a-5p, mmu-miR-152-3p, mmu-miR-16-5p, mmu-miR-17-5p, mmu-miR-181a-5p, mmu-miR-182-5p, mmu-miR-183-5p, mmu-miR-188-5p, mmu-miR-191-5p, mmu-miR-193b-3p, mmu-miR-199a-3p, mmu-miR-19a-3p, mmu-miR-19b-3p, mmu-miR-200b-3p, mmu-miR-200c-3p, mmu-miR-204-5p, mmu-miR-205-5p, mmu-miR-20a-5p, mmu-miR-211-5p, mmu-miR-214-3p, mmu-miR-218-5p, mmu-miR-224-5p, mmu-miR-24-3p, mmu-miR-25-3p, mmu-miR-28a-5p, mmu-miR-296-5p,, mmu-miR-301a-3p, mmu-miR-301b-3p, mmu-miR-30a-5p, mmu-miR-30b-5p, mmu-miR-30c-5p, mmu-miR-30e-5p, mmu-miR-320-3p, mmu-miR-323-3p, mmu-miR-324-3p, mmu-miR-328-3p, mmu-miR-331-3p, mmu-miR-335-3p, mmu-miR-335-5p, mmu-miR-337-5p, mmu-miR-34b-3p, mmu-miR-351-5p, mmu-miR-365-3p, mmu-miR-370-3p, mmu-miR-376b-3p, mmu-miR-376c-3p, mmu-miR-382-5p, mmu-miR-409-3p, mmu-miR-410-3p, mmu-miR-425-5p, mmu-miR-433-3p, mmu-miR-434-3p, mmu-miR-484, mmu-miR-494-3p, mmu-miR-495-3p, mmu-miR-501-3p, mmu-miR-543-3p, mmu-miR-574-3p, mmu-miR-667-3p, mmu-miR-676-3p, mmu-miR-687, mmu-miR-92a-3p, mmu-miR-99a-5p

Supplemental Table 2, related to Fig. 4 and Fig. S4a.
List of predicted targets of miR-133b-3p that increased in expression (2x) in isolated SMG epithelium treated for 20 h with an antagomir to miR-133b-3p (from 2 independent microarray experiments). Predicted targets were identified using TargetScan (www.targetscan.org), DIANA-microT (www.microrna.gr/microT-CDS), and miRDB (www.mirdb.org/miRDB).

Gene ID	Log_2 [Data 1]	Log_2 [Data 2]	Prediction	Species with binding site (conserved or not) Seed region match in TargetScan	DIANA score	Description
Fads1	1.338227	3.234816	TargetScan	Mmu 7mer-m8		Mus musculus fatty acid desaturase 1 (Fads1), mRNA [NM_146094]
Dip2b	1.327165	2.655222	TargetScan	Mmu (2 biding sites) 7mer-m8		Mus musculus DIP2 disco-interacting protein 2 homolog B (Drosophila) (Dip2b), transcript variant 1, mRNA [NM_001159361]
Ephb4	1.292360	3.649177	TargetScan	Broadly conserved among vertebrates 7mer-1A		Mus musculus Eph receptor B4 (Ephb4), transcript variant 1, mRNA [NM_001159571]
Ccdc127	1.243082	1.913761	DIANA		0.71	Mus musculus coiled-coil domain containing 127 (Ccdc127), transcript variant 2, mRNA [NM_024201]
Insr	1.224900	1.141031	TargetScan DIANA	Broadly conserved among vertebrates 7mer-1A	0.72	Mus musculus insulin receptor (Insr), mRNA [NM_010568]
Atrn	1.197171	1.383234	TargetScan	Mmu, Rno, Oga, Eca 8mer		Mus musculus attractin (Atrn), mRNA [NM_009730]
2700081 O15Rik	1.134024	2.039974	TargetScan	Mmu 7mer-m8		Mus musculus RIKEN cDNA 2700081O15 gene (2700081O15Rik), mRNA [NM_175381]
Crk	1.089519	1.095187	TargetScan	Broadly conserved among vertebrates 7mer-m8		Mus musculus v-crk sarcoma virus CT10 oncogene homolog (avian) (Crk), mRNA [NM_133656]

Supplementary Experimental Procedures

SMG organ and epithelial culture

Fetal submandibular gland (SMG) explants and isolated SMG epithelium from timed-pregnant ICR mice (Harlan, IN) were cultured as previously described (Rebustini, et al., 2009). SMGs were cultured on Track-etch filters (13 mm, 0.1 μm pore size; VWR) floated on 200 μl of DMEM/F12 in 50-mm glass-bottom microwell dishes (MatTek). The medium was supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 150 μg/ml vitamin C and 50 μg/ml transferrin. Five rudiments were cultured on each filter at 37°C in a humidified 5% CO2/95% air atmosphere. Isolated epithelia were cultured with FGF10 400 ng/ml. In some cases, media was also supplemented with FGF10 800 ng/ml (R&D Systems), heparan sulfate 500 ng/ml (Sigma-Aldrich), and recombinant mouse stem cell factor 200 ng/ml (R&D Systems). To evaluate isolated-epithelial morphogenesis (Morphogenic Index), the number of buds, width of buds, and length of ducts were measured and expressed in arbitrary units as an indicator of overall morphogenesis, as previously described(Patel, et al., 2008).

Mouse lines

Wild-type embryos were obtained from ICR timed pregnant females (Harlan). K5-*Venus* mice were produced as previously described using the bovine-K5 promoter driving Venus expression (Knox, et al., 2010). All experiments were approved by the Animal Care and Use Committee at NIDCR, NIH. For timed matings the morning of plug detection was considered day 0.5 postcoitum. Genotyping of mice was performed on tail biopsies by PCR and agarose gel electrophoresis (primers available on request).

Exosome preparation and visualization

The exosome fraction was prepared from conditioned medium of E13 SMG after 48 h of culture and processed for negative staining by transmission electron microscopy (TEM) as previously described (Thery, et al., 2006). For western blotting only, SMG were stimulated with monensin (10 μM) for 3 h after culture to promote exosome secretion (Pegtel, et al., 2010). Conditioned medium was centrifuged at 1,500 X g for 10 min at 4°C to remove cells. The supernatant was centrifuged at 12,000 X g for 15 min at 4°C to remove cellular debris. The supernatant was then ultracentrifuged at 100,000 X for 70 min at 4°C to obtain an exosome pellet. The pellets were washed with 10 ml of PBS twice, and after ultracentrifugation, the pellets were resuspended in PBS. For TEM, the exosome fraction was fixed with an equal volume of 4% (w/v) paraformaldehyde (PFA) and applied on Formvar-carbon coated EM grids. Images were obtained by TEM (Tecnai T12, FEI).

Western blotting

Exosome pellets, cultured SMGs, and NIH3T3 cells were lysed with 300 μl lysis buffer (20 mM HEPES buffer (pH 7.4) containing 1% Triton X-100, 0.5% Nonidet P-40, 150 mM sodium chloride, 12.5 mM β-glycerophosphate, 1.5 mM magnesium chloride, 10 mM sodium fluoride, 2 mM DTT, 1 mM sodium orthovanadate, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixtures, and phosphatase inhibitor mixtures) at 4°C for 15 min. The amount of protein was measured using the micro BCA protein assay reagent kit (Pierce). Lysates were boiled with SDS sample buffer and separated on 10-20% gradient SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore). The membranes were blocked in 5% skim milk in PBS. Immunoreactive bands were detected using the Alix (Cell Signaling, #2171), TSG101 (Abcam, ab30871) and actin (Santa Cruz, sc-8432) antibodies. Signal detection was carried out using the ECL Plus Western Blotting Detection System (Amersham Bioscience).

Ceramide labeling
Fluorescent-conjugated ceramide was used to label cell membranes in SMGs. SMG epithelium and mesenchyme were separated and incubated in medium with BODIPY-TR-ceramide (Molecular Probes) 5 μM for 30 min at 37°C. The free ceramide was removed from the tissue by washing 3 times with medium. Finally, the tissues were incubated in fresh medium for 1 h at 37°C. and washed twice with medium to remove unbound ceramide. The labeled tissue was then recombined with unlabeled tissue in culture or placed on top of laminin extracellular matrix to observe ceramide transfer to unlabeled epithelium.

Whole-mount immunofluorescence
Whole-mount immunofluorescence was performed as previously described (Rebustini, et al., 2009). Briefly, the whole SMG, epithelia and mesenchyme were fixed in 4% PFA in PBS, and washed with PBS. The samples fixed with PFA were permeabilized with 0.1% triton X-100 for 10 min. For antigen retrieval to stain 5mC, the samples were treated with DNase I (1 U/μl) for 10 min, followed by incubation in 2N HCl for 10 min. The samples were blocked for 1 h in 10% heat-inactivated donkey serum, 1% BSA, MOM IgG blocking reagent (Vector Laboratories), prior to overnight incubation at 4°C with primary anti-antibodies. The tissues were washed in PBS-T (PBS containing 0.1% Tween 20) and the antibody was detected using Alexa-conjugated secondary Fab fragment antibodies (Jackson Laboratories). Nuclei were detected with Hoechst. Fluorescence was analyzed using a Zeiss 710 laser-scanning microscope (Zeiss). Antibodies used included rabbit anti-Ecadherin (1/100, Cell Signaling), rat anti-E-cadherin (1/125, TaKaRa), rabbit anti-DIP2B (1/200, Abcam), goat anti-KIT (1/100, Santa Cruz), mouse anti-5mC (1/500, Eurogentec).

RNA isolation
Total RNA was prepared using mirVana miRNA isolation kits. For pri-miRNA analysis the total RNA was treated with DNase twice for 20 min at 37°C (DNA-free Kit, Ambion) to remove DNA. To analyze RNA in the exosome fraction, electrophoresis was carried out using the Agilent Bioanalyzer 2100 (Agilent RNA 6000 Pico kit, Agilent Technologies). The exosome fraction and SMG total RNA were treated with RNase A (300 μg/ml, Sigma-Aldrich) for 30 min at 37°C. After incubation, RNA was extracted with mirVana miRNA isolation kits (Ambion).

Analysis of microRNA in the exosome fraction using TaqMan low density arrays
Total RNA (2 ng) was analyzed using the Megaplex Pools protocol and reagents (Applied Biosystems) to detect miRNAs in both the exosome fraction and the cultured gland as previously described (Rebustini, et al., 2012). The relative fold change in miRNA expression in the exosomes was calculated relative to cultured SMGs using the global mean normalization method (D'Haene, et al., 2012). A PCR cycle threshold of 33 cycles was used as a cutoff to exclude low abundance miRNAs.

Quantitative real time PCR (qPCR) for mature and primary microRNAs
For mature miRNA analysis, miR-133a-3p, miR-133b-3p, miR-409-3p, and miR-200c-3p TaqMan microRNA assays were performed (Applied Biosystems). Total RNA (4 ng) was used as a template for each specific microRNA reverse transcription reaction. Expression levels were normalized to the housekeeping gene, *snRNA-U6*. For primary miRNA analysis, TaqMan Pri-miRNA Assays were used for mir-133a-1, mir-133a-2, mir-133b, mir-409, and mir-200c (Applied Biosystems). Complementary DNA (cDNA) 4 ng was used as template for qPCR reaction. Expression was normalized to the housekeeping gene, *Rps29*. TaqMan Universal PCR Master Mix (Applied Biosystems) was used for qPCR reactions, and comparative Ct method was used to quantify expression levels.

qPCR for messenger RNAs (mRNA)
qPCR for mRNAs was performed as previously described (Rebustini, et al., 2012). Total RNAs were treated with DNase (DNA-free Kit, Ambion). Reverse transcription was carried out using SuperScript reagents (BioRad). SYBR-green qPCR was performed using 2 ng of cDNA, with primers designed by Beacon Designer Software (sequences available on request). Gene expression was normalized to *Rps29* and to the corresponding experimental control, and reactions were run in triplicate and repeated three times.

Transfection assays
E13 isolated epithelia were transfected with 400 nM of an miRNA antagomir (Exiqon) or with 200 nM of an miRNA mimic (Ambion) in 200 µl of culture medium using RNAiFect (Qiagen) reagents as previously described (Rebustini, et al., 2012). Total RNA was isolated after 20 h or 45 h of culture, and gene expression levels were analyzed by qPCR. For analysis of the miR-133 family, TaqMan microRNA assays (Applied Biosystems) were used for miR-133a-3p and miR-133b-3p. Expression was normalized to the housekeeping gene snRNA-U6.

Microarray analysis
Antagomir-control (Anta-control) or Antagomir-133b-3p (Anta-133b-3p) was transfected into isolated epithelia, and epithelia were lysed at 20 h culture. Total RNA was isolated, converted to cDNA probe, and hybridized to SurePrint G3 Mouse GE Microarray (8 x 60K) (Agilent). The signals for specific probes and fold change (Anta-133b-3p/Anta-control) were determined using Genespring Software, identifying genes with 2-fold up-regulation in epithelia treated with Anta-133b-3p. Experiments were repeated using two independent samples.

MicroRNA target prediction
Candidate target genes of miR-133b-3p were selected from genes that were up-regulated 2-fold Anta-133b-3p treatment of isolated-epithelia, combined with three different prediction algorithms: TargetScan Release 6.2, DIANA-microT version 5.0, and miRDB. An RNAHybrid algorithm predicted the miR-133b-3p matching target sites on the *Dip2b* 3'UTR.

Luciferase reporter assay
Firefly/Renilla Duo-Luciferase reporter vector (pEZX-MT01) with a *Dip2b* 3'UTR was used (GeneCopoiea). Three nucleotides in the 3'UTR corresponding to seed region of miR-133b-3p were mutated with QuickChange Site-Directed Mutagenesis Kit (Agilent). Plasmids were transformed into competent cells optimized for large plasmids (*E.coli* HST08 Premium competent cells, TaKaRa). Mutation of nucleotides was confirmed by Sanger sequencing. NIH3T3 cells were grown to 90% confluence and co-transfected with *Dip2b* (400 ng/well) plasmid constructs and miRNA mimics at 20 nM (Ambion) using Lipofectamine 2000 (Invitrogen). After 24 h transfection, luciferase activity was determined by Luc-Pair miR Luciferase Assay Kit (GeneCopoeia). Luminescence was measured using an Infinite 200 PRO multimode reader (TECAN).

Fluorescence-activated cell sorting
SMGs were prepared for FACS analysis as previously described (Knox, et al., 2010). Anti-E-cadherin APC (FAB7481A, R&D Systems) and anti-Kit PE (BD Biosciences) were also used. Fluorescence levels were analyzed using FlowJo (Tree Star).

Next generation sequencing analysis
Total RNA was extracted from epithelial cells sorted by FACS analysis (N=3 separate experiments) and RNAseq performed by Cofactor Genomics (www.cofactorgenomics.com).

Single-ended reads (50 nt in length) were produced using Illumina sequencer (Illumina). Reads were mapped to the GRCm38/mm10 using the STAR 2.4.2a aligner and gene counts generated with Subread's feature Counts tool. The RNA-seq data are available in the GEO database. GEO accession is GSE89896.

Statistics

Experiments were performed with at least three biological replicates. To determine the significance between two groups, a Student's t test was used. For statistical tests, $p < 0.05$ was considered significant.

References for Supplementary Experimental Procedures

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