Supplemental Information

Protogenin Facilitates Trunk-to-Tail *HOX* Code Transition via Modulating GDF11/SMAD2 Signaling in Mammalian Embryos

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Supplementary Methods

Animals and Genotyping

The *Prtg* conventional knockout allele was generated as previously described (Wang et al., 2013). All procedures involving mice were conducted in strict accordance with university guidelines. Ethical approval for animal experiments was obtained from the Institutional Animal Care and Use Committee of National Yang Ming Chiao Tung University (IACUC No. 1110111nr). For genotyping of postnatal mice, tissue samples (a piece of tail or toe) were lysed by incubation with 300 μL of 50 mM NaOH at 95°C for 30 minutes, followed by neutralization with 50 μL of 1 M Tris (pH 7.9). For embryo genotyping, the yolk sac was lysed in 30 μL of 50 mM NaOH and subsequently neutralized with 5 μL of 1 M Tris (pH 7.9). Extracted DNA was subjected to PCR using Taq DNA polymerase (Geneaid) with primer sequences listed in Supplementary Table 6 VI.

Skeleton and Cartilage Staining

Newborn mice were sacrificed and briefly soaked in 70°C water for 30 seconds before being skinned and eviscerated. Specimens were then fixed in 95% ethanol for at least 3 days. To visualize cartilage, the samples were stained in a solution containing 80% ethanol, 20% acetic acid, and 0.5 mg/mL alcian blue 8GX (Sigma) for 3 days. After staining, the alcian blue-treated specimens were washed twice in 70% ethanol for 2 hours and subsequently immersed in 1% KOH for 1 day to digest soft tissues. The skeletons were stained overnight in a solution containing 1% KOH and 0.3 mg/mL alizarin red S (Sigma) and subsequently destained in 1% KOH for 1 day. The specimens were stored in 20% glycerol for photography.

Synthesis of Digoxigenin-Labeled Riboprobes

DNA templates for probe synthesis were generated by PCR from a mix of E9.5 and E10.5 total cDNA using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Primers used for this experiment were purchased from Integrated DNA Technologies and are listed in Supplementary Table 6. Following PCR, the DNA fragments were purified with the GenepHlow Gel/PCR Kit (Geneaid) and either subcloned into the pCRII-TOPO Vector (Invitrogen) or used directly for probe synthesis. For cloning, digested DNA fragments were gel-purified, inserted into the pCRII-TOPO Vector by TA-cloning or T4 DNA ligase (Thermo Fisher Scientific), and transformed into TOP10 chemically competent *E. coli* cells (Invitrogen). Single colonies were picked, confirmed by colony PCR, and sequence accuracy was verified using Sanger sequencing. Plasmids were purified with the PureLink Quick Plasmid Miniprep Kit (Invitrogen). Digoxigenin-labeled riboprobes were synthesized using T7 RNA polymerase (Roche) or SP6 RNA polymerase (Thermo Fisher Scientific) in the presence of digoxigenin-11-UTP (Roche). Residual DNA templates were removed with DNase I (Geneaid), and the synthesized riboprobes were purified using the Blood/Cell RNA Mini Kit (Geneaid).

Whole-Mount *In Situ* Hybridization

Embryos were obtained from pregnant mice at E9.5, E10.5, or E11.0. Yolk sacs from embryos were preserved for genotyping. The samples were fixed in 4% paraformaldehyde/PBST (PBS [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4] containing 0.1% Tween 20) overnight and then rinsed in PBST. Dehydration was performed through a series of methanol/PBST solutions (20%, 40%, 60%, 80%, then 100% methanol in PBST), followed by storage in absolute methanol at -20°C. After genotyping, samples of the desired genotype were rehydrated, bleached in 6% H_2O_2/PBST, and permeabilized using proteinase K (Roche). Permeabilization was stopped by incubation with 2 mg/mL glycine in PBST. Samples were re-fixed in 4% paraformaldehyde/0.2% glutaraldehyde/PBST, washed twice with PBST, and incubated with hybridization solution (50% formamide [Supelco], 2% blocking

reagent [Roche], 5× SSC pH 7, 5 mM EDTA, 1 mg/mL yeast tRNA [Roche], 0.1 mg/mL heparin, 0.1% Triton X-100, 0.1% Tween-20, 0.5% CHAPS) at 70°C for at least 3 hours. Probe hybridization was performed by incubating samples in hybridization solution containing 200–300 ng/mL digoxigenin-labeled riboprobe at 70°C overnight. After hybridization, samples were washed with wash solution (50% formamide, 4× SSC pH 7, 0.1% Triton X-100, 0.1% Tween-20, 0.5% CHAPS) to remove unbound riboprobes.

Samples were then equilibrated in MABT solution (100 mM maleic acid, pH 7.5, 150 mM NaCl, 0.1% Tween-20) and blocked in blocking solution (MABT containing 10% heat-inactivated sheep serum and 2% blocking reagent) at 4°C for at least 2 hours. The blocking solution was replaced with fresh blocking solution containing anti-digoxigenin-AP Fab fragments (Roche) diluted 1:2000, and samples were incubated at 4°C overnight. Unbound Fab fragments were removed by repeated washing with MABT at room temperature. Samples were then equilibrated in NTMT (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20, 2 mM levamisole [Sigma]), and staining was developed by incubating with NTMT containing 10% polyvinyl alcohol (Sigma), 337.5 µg/mL NBT, and 175 µg/mL BCIP. Staining was terminated by washing with stop solution (100 mM Tris pH 7.4, 1 mM EDTA).

Stained samples were fixed in 4% paraformaldehyde/PBST to prevent color loss. Pooled samples were photographed individually, and heads from each sample were processed for genotyping. For sectioning, samples were soaked in PBST/OCT (Sakura Finetek USA) 1:1 solution overnight, then embedded in OCT solution and immediately stored in a -70°C freezer. Slide sectioning was performed using a Thermo Scientific CryoStar NX70 Cryostat with a thickness of 20 µm. Whole-mount samples were captured using a Leica DFC295 digital color camera with a 0.63× video objective (part #10447367) or an iPhone 11 with a 12.5×, Ø 30 mm eyepiece adaptor. Sections were captured using a QIClick Digital CCD Camera, Mono, 12 -bit instrument on an Olympus BX61 microscope with U-TV1X-2 and U-CMAD3 camera adapters.

Total mRNA Sequencing

Total RNA from the posterior part of E9.5 mouse embryos (Figure 2a) was extracted using the Tissue Total RNA Mini Kit (Geneaid) with on-column DNase I digestion performed using the RNase-Free DNase I Set (Geneaid). Next-generation sequencing library preparation was performed following the manufacturer's protocol. Poly(A) mRNA isolation was conducted using the Poly(A) mRNA Magnetic Isolation Module or the rRNA Removal Kit. mRNA fragmentation and priming were carried out using First Strand Synthesis Reaction Buffer and Random Primers.

First-strand cDNA was synthesized using ProtoScript II Reverse Transcriptase, and second-strand cDNA was synthesized using the Second Strand Synthesis Enzyme Mix. The purified double-stranded cDNA was treated with an End Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by T-A ligation to add adaptors to both ends. Size selection of adaptor-ligated DNA was performed using beads, recovering fragments of ~420 bp (with an approximate insert size of 300 bp). Each sample was then amplified by PCR for 13 cycles using P5 and P7 primers, with both primers carrying sequences for flow cell annealing during bridge PCR, and the P7 primer carrying a six-base index for multiplexing.

The PCR products were cleaned up using beads, validated using a Qsep100 (Bioptic), and quantified with a Qubit 3.0 Fluorometer (Invitrogen). Libraries with different indices were multiplexed and loaded onto an Illumina HiSeq instrument following the manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was performed using a 2×150 bp paired-end (PE) configuration. Image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.6 (Illumina) on the HiSeq instrument. mRNA library preparation and sequencing were carried out by GENEWIZ.

Transcriptome Analysis

Raw sequencing reads were processed using Cutadapt (v1.9.1) to remove adapters, PCR primers, and fragments with base quality scores below 20. Filtered reads were aligned to the reference genome (GRCm38.97) using Hisat2 (v2.0.1). Read counts for each gene were generated using HTSeq (v0.6.1) to calculate FPKM (Fragments Per Kilobase of transcript per Million mapped reads). The FPKM data was analyzed to identify differentially expressed genes (DE genes) using EdgeR (v3.24.1) on the Galaxy platform (https://usegalaxy.org) with a false discovery rate (FDR) threshold of <0.05. Upstream regulator analysis was performed using Ingenuity Pathway Analysis (QIAGEN). Gene Set Enrichment Analysis (GSEA, v4.2.3) with hallmark gene sets was employed to identify altered biological processes in *Prtg*$^{-/-}$ embryos.

Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from embryos using the Tissue Total RNA Mini Kit (Geneaid) and from cells using the Blood/Cell RNA Mini Kit (Geneaid). Genomic DNA was removed by on-column DNase digestion using the RNase-Free DNase I Set (Geneaid). Purified RNA was reverse transcribed into cDNA using SuperScript IV Reverse Transcriptase (Invitrogen) with anchored oligo dT(20)VN primers (Integrated DNA Technologies), following the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed on the ABI StepOnePlus system using either the SYBR Green or TaqMan protocol. For TaqMan assays, each 10 µL reaction included 0.25 µL cDNA, 5 µL PrimeTime Gene Expression Master Mix (Integrated DNA Technologies), 0.5 µM each of forward and reverse primers, and an amplicon -specific probe. For SYBR assays, each 10 µL reaction contained 0.25 µL cDNA, 5 µL PowerTrack SYBR Green Master Mix (Applied Biosystems), 1× yellow sample buffer, and 0.5 µM each of forward and reverse primers. Universal ProbeLibrary probes were obtained from Roche, while other probes and primers were purchased from Integrated DNA Technologies (see Supplementary Table 6). Locked nucleic acids in probes are labeled with +A, +T, +C, or +G.

Western Blotting

Cells were harvested by removing the medium, washing with PBS, and lysing in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, cOmplete EDTA-free protease inhibitor cocktail [Roche], and phosphatase inhibitor cocktail III [TargetMol]). Lysates were incubated on ice for 15 minutes, and cell debris was removed by centrifugation at 13,200 rpm for 15 minutes. The supernatant was transferred to a new tube. Total protein concentration was determined using a Bio-Rad protein assay to ensure equal loading in each lane. Lysates were mixed with 4× sample buffer (Tris-HCl, pH 8.5, 8% SDS, 40% glycerol, 2 mM EDTA, 0.075% SERVA Blue G250, 0.025% phenol red) and 2-ME, denatured by heating at 75°C for 15 minutes, and chilled on ice. Samples were separated by SDS-PAGE at 70 V for 20 minutes, then at 120 V for 1 hour. Proteins were transferred to a PVDF membrane (Millipore) using transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 70 V for 2.5 hours. The membrane was blocked with TBS containing 5% skimmed milk and 0.05% Tween-20 for 30 minutes at room temperature, then incubated with the primary antibody overnight at 4°C. After washing three times with TBST (TBS + 0.05% Tween-20), the membrane was incubated with an HRP-conjugated secondary antibody for 4 hours at 4°C. Signal detection was performed using an ImageQuant LAS 4000 instrument with Immobilon western chemiluminescent HRP substrate (Millipore). For re-probing, the membrane was quenched with 3% H_2O_2/TBST for 20 minutes, washed, and prepared for the next antibody incubation. Antibodies used are listed in Supplementary Table 7.

Whole-Mount Immunofluorescent Staining

Embryos with *Prtg* genotypes (*Prtg*$^{+/+}$, *Prtg*$^{+/-}$, and *Prtg*$^{-/-}$) were collected from the same litter and processed concurrently. E9.5 embryos were harvested using ice-cold DPBS and fixed in 4% paraformaldehyde for 4 hours at 4°C. Fixed embryos were permeabilized in TBS (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% Triton X-100 for 2 hours at room temperature. Embryos were then immersed in a blocking buffer (TBS + 0.2% Triton, 2% BSA, 2% NGS, 0.2% NaN_3) and incubated overnight at 4°C.

Primary antibodies were applied for 3 nights at 4°C. The following day, embryos were washed with blocking buffer for five 1-hour intervals at room temperature before incubating with secondary antibodies overnight at 4°C. After staining, embryos were subjected to another set of five 1-hour washes at room temperature, followed by overnight washes at 4°C. Embryos were mounted in RapiClear 1.52 (SunJin Lab) within a custom-made glass chamber for imaging. Imaging was performed using a Zeiss LSM900 inverted laser scanning confocal microscope with Airyscan 2, employing a Plan-Apochromat 10×/0.45 M27 objective. Laser intensity and photo-multiplier levels were standardized across samples. Antibodies used in whole-mount immunofluorescent staining are listed in Supplementary Table 7. The homemade mouse anti-Prtg [1D5] antibody was biotinylated using the Lightning-Link Biotinylation Kit (Fast, Type A; Abcam).

Plasmids

Mouse *Gdf11*, *Inhba*, *Tgfb1*, and *Pcsk5* sequences were amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and cloned into expression vectors derived from UI4-GFP-SIBR via restriction enzyme digestion. shRNA sequences targeting *Prtg* and scramble controls were cloned into the SIBR cassette to create polycistronic expression vectors. All constructs were verified by sequencing to ensure accuracy. Primers and oligonucleotides used for cloning are listed in Supplementary Table 6. The constructed sequences with the restriction enzyme sites are listed in Supplementary Data 2.

P19 Cell Culture and Transfection

P19 embryonic carcinoma cells were obtained from the Bioresource Collection and Research Center, Taiwan. Cells were maintained in 60-mm tissue culture dishes using a complete growth medium comprising α-MEM, 10% FBS (Gibco), and 1× PSG (Gibco). Cells were passaged every 2–3 days upon reaching 80% confluence. For passaging, the medium was removed, cells were rinsed with DPBS, and detached using TrypLE Express (Gibco). After centrifugation, cells were resuspended in complete growth medium and seeded into new tissue culture dishes.

One day prior to transfection, 5×10^4 cells were seeded per well in 24-well plates, and 10 μM SB431542 was added to inhibit endogenous TGFβ signaling. One hour before transfection, the medium was replaced with Opti-MEM I (Gibco) supplemented with 1% FBS and 1× GlutaMAX. Transfection reagents were prepared by mixing Lipofectamine 3000 (Invitrogen), P3000 reagent (Invitrogen), and purified plasmid DNA according to the manufacturer's instructions. Each well of a 24-well plate received 1.25 μL Lipofectamine 3000, 1 μL P3000, and 500 ng DNA. For the reporter assay, the pRL-US2 vector served as a reference reporter, and the CAGA-driven luciferase reporter (CAGA-Luc) was used to assess TGFβ signaling activity. The ratio of pRL-US2:CAGA-Luc:*Prtg*-related vector:TGFβ ligand-expressing vector was 1:24:9:16. For pSmad2 level measurements, a puromycin-resistant vector under the human ubiquitin C promoter (US2-puro) was included for selection, and a *Pcsk5*-expressing vector was added to enhance *Gdf11* processing. The ratio of US2-puro:*Pcsk5*:*Prtg*-related vector:TGFβ ligand-expressing vector was 2:1:8:9. One day after transfection, the medium was replaced with Opti-MEM I (Gibco) containing 1× GlutaMAX. To select transfected cells, 5 μg/mL puromycin was added. Cells were harvested two days post-transfection. For co-immunoprecipitation experiments, 2.5×10^5 cells were seeded in 30-mm dishes or 6-well plates. Each dish or well received 6.25 μL Lipofectamine 3000, 5 μL P3000, and 2.5 μg

DNA, with a DNA complex comprising US2-puro:*Prtg*-related vector:TGFβ ligand-expressing vector at a ratio of 1:5:4. Cells were treated as described and harvested two days after transfection.

Luciferase Reporter Assay

Cells in 24-well plates were rinsed with PBS and lysed using passive lysis buffer (Promega). Lysates were incubated at room temperature for 15 minutes with periodic shaking to ensure complete lysis. After incubation, lysates were transferred to 96-well white flat-bottom plates. The luciferase activities of rLuc and CAGA-Luc were measured using the Dual-Luciferase Reporter Assay System (Promega), following the manufacturer's instructions.

Co-immunoprecipitation

Cells were washed once with PBS and lysed in IP lysis buffer containing 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% IGEPAL CA-630 (Sigma), and an EDTA-free protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation at 13,200 rpm for 5 minutes at 4°C. A portion of the supernatant was retained as the input sample, while the remaining lysate was transferred to a low-binding tube and incubated overnight at 4°C with rotation, using anti-HA antibody-conjugated agarose resin (Santa Cruz Biotechnology). The agarose resin was pelleted by centrifugation and washed four times with IP lysis buffer to remove unbound proteins. Protein complexes were eluted by incubating the agarose resin in 1× sample buffer (without 2-ME) at 50°C for 20 minutes. After elution, the agarose resin was removed by centrifugation to prevent antibody contamination. Subsequently, 2-ME was added to the eluted proteins, and the samples were denatured by heating at 75°C for 15 minutes before analysis by SDS-PAGE.

Culture of human induced pluripotent stem cells (hiPSCs)

The hiPSC line, NTUH-iPSC-02-02 (abbreviated to N2), was purchased from Bioresource Collection and Research Center of Food Industry Research and Development Institute, Taiwan. The use of these hiPSC lines followed the Policy Instructions of the Ethics of Human Embryo and Embryonic Stem Cell Research guidelines in Taiwan. In addition, approval from the Institutional Review Boards of National Yang Ming Chiao Tung University was obtained (#YM110194W). Human iPSCs were routinely maintained in StemFlex medium (Gibco) on vitronectin (VTN-N, Gibco) coated dishes at 37°C in a 5% CO_2 incubator following the manufacturer's instructions. Cells were subjected to passage when the culture reached 80-90% confluence, typically every 5 days. For passaging, the hiPSCs were rinsed once with Dulbecco's Phosphate-Buffered Saline (DPBS) without calcium and magnesium (Gibco) and then treated with EDTA/DPBS (0.5 mM UltraPure EDTA in DPBS) for 3-4 minutes at 37°C. When the cells started to round up and detach, they were harvested from the dish by gently pipetting. An appropriate number of cells was then transferred to a new vitronectin-coated dish at a ratio of 1:10.

Generation of *PRTG* knockout (*PRTG*^{KO}) hiPSC lines using CRISPR/Cas9 technique

DNA oligonucleotides utilized for gRNA targeting were designed using the TrueGuide™ CRISPR gRNA Design Tool (Thermo Fisher Scientific). The gRNA target sequence is 5'-CGGATCGAGGTTCTTTCTAA-3', located at locus Chr.15: 55740507 - 55740529 on GRCh38. The gRNA was subsequently complexed with the CRISPR-associated protein 9 (Cas9) nuclease (Invitrogen) to create Cas9 protein/gRNA ribonucleoprotein complexes (Cas9-RNPs). These Cas9-RNPs were transfected into hiPSCs via the Neon™ Transfection System (Invitrogen). The efficiency of genomic editing was assessed 48 hours post-transfection using the T7 endonuclease I (T7E1, NEB) assay and primers 5'-TGTTGCGTTGGCCAAACTTC-3' and 5'- TGCCTGTAATTCTGGATGTGC-3'. Surviving cells were allowed to recover and subsequently re-plated at low density for the isolation of individual colonies. Following one week, the Cas9/RNP-transfected hiPSCs were clonally expanded to establish isogenic cell lines. Each *PRTG* knockout clone was verified by Western blotting with an anti-PRTG antibody to assess

protein levels and confirmed via Sanger sequencing. The successfully edited clones were further validated any undesired off-target effect by T7E1 mismatch detection assays. Potential off-target sites in the human genome (hg19) were identified using CCTop - CRISPR/Cas9 target online predictor (https://crispr.cos.uni-heidelberg.de). Unique primers were designed for each identified genomic locus. T7E1 assay was then performed at the predicted off-target site with genomic DNA from CRISPR-edited iPSCs. Primers used in T7E1 assays are listed in Supplementary Table 6V.

Induction of Presomitic and Somitic Mesodermal Cells

For iPSC-PSM differentiation, cells were seeded onto vitronectin-coated 24-well tissue culture plates at a density of 2,500 cells per well in StemFlex medium supplemented with 2 µM Y27632 (Tocris Bioscience). One day after seeding, the medium was replaced with 350 µL of advanced DMEM/F-12 (Gibco) containing 1× N-2 supplement (Gibco), 1× GlutaMAX, 0.5× NEAA (Gibco), 50 ng/mL Activin A (PeproTech), 5 µM CHIR99021 (Tocris Bioscience), and 20 ng/mL FGF2 (PeproTech) to induce PS differentiation. After 24 hours, the medium was replaced with 500 µL advanced DMEM/F-12 containing 1× N-2 supplement, 1× GlutaMAX, 0.5× NEAA, 5 µM CHIR99021, and 20 ng/mL FGF2 to promote PSM differentiation. The culture medium was refreshed daily during the PSM differentiation process. For SM differentiation, the medium was replaced with 500 µL advanced DMEM/F-12 containing 1× N-2 supplement, 1× GlutaMAX, 0.5× NEAA, 10 µM SB431542 (Tocris Bioscience), and 250 nM LDN193189 (Tocris Bioscience) for one day. The medium was then replaced with 500 µL advanced DMEM/F-12 containing 1× N-2 supplement, 1× GlutaMAX, 0.5× NEAA, 1 µM XAV939 (Tocris Bioscience), and 100 nM PD173074 (Tocris Bioscience) for an additional day to induce SM differentiation.

Immunofluorescence

hiPSCs were seeded onto vitronectin-coated coverslips and differentiated into iPS-PSM cells. On differentiation day 5, cells were fixed with 4% PFA/PBS at room temperature for 10 minutes. Fixed cells were rinsed twice with TBST and permeabilized with prechilled 100% methanol at -20°C for 5 minutes. After permeabilization, cells were rinsed twice with TBST and blocked in TBST containing 5% BSA at room temperature for 1 hour. The primary antibody was applied and incubated overnight at 4°C. After primary antibody removal, cells were rinsed twice with TBST and incubated with fluorescent dye-conjugated secondary antibodies and DAPI in TBST containing 5% BSA for 1 hour at room temperature. Cells were then rinsed three times with TBST, mounted on glass slides using Fluoromount-G, and imaged with a fluorescence microscope.

Flow Cytometry

On day 5 of differentiation, iPSC-PSM cells were rinsed with PBS and detached using TrypLE Express. The TrypLE enzyme was inactivated with PBS containing 3% BSA and removed by centrifugation. Cells were resuspended in PBS at a concentration of 1×10^6 cells/mL, with 1 mL used per assay. Fixation was performed with 4% PFA/PBS on ice for 15 minutes, followed by two washes with TBST. For permeabilization, 900 µL of prechilled 100% methanol was added to the cell suspension to achieve 90% methanol, and cells were incubated at -20°C for 10 minutes. Cells were stored at -20°C until immunostaining.

Immunostaining began by pelleting and washing the cells twice with TBST to remove methanol, followed by blocking in TBST containing 10% horse serum for 30 minutes at room temperature. The primary antibody was added directly to the blocking solution and incubated for 45 minutes at room temperature. Afterward, cells were washed twice with TBST and incubated with secondary antibodies in TBST containing 10% horse serum for 45 minutes at room temperature. Following immunostaining, cells were washed twice with TBST, resuspended in PBS, and filtered through a cell strainer into test tubes.

Flow cytometry analysis was conducted using a Beckman CytoFLEX S (model no. B75442), and data were processed with CytExpert v2.4 software.

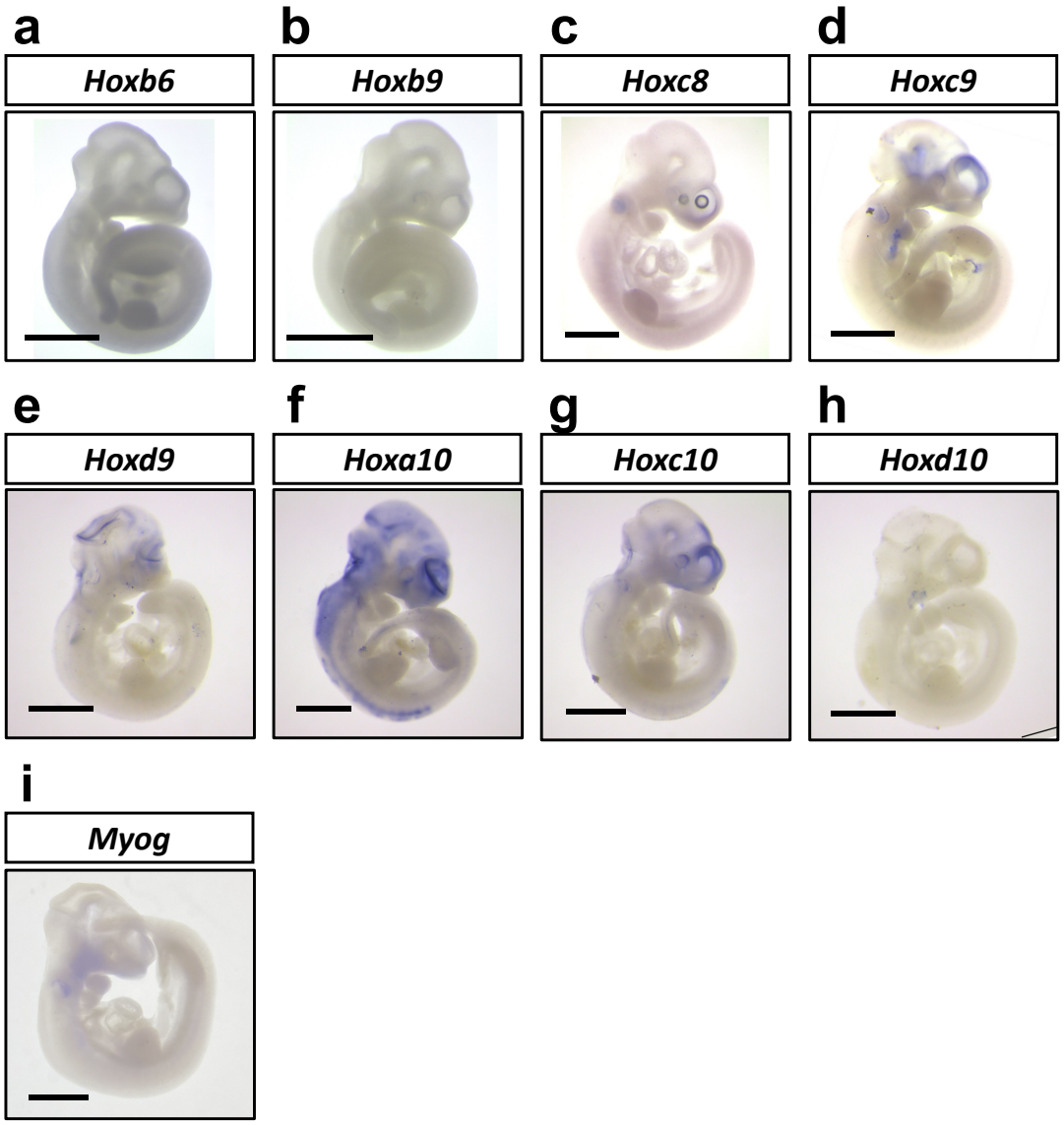
Statistics and Reproducibility

Data are presented as means ± SEM. Statistical significance was assessed using a t-test, one-way ANOVA with Tukey's post hoc test, or two-way ANOVA with the original false discovery rate (FDR) method of Benjamini and Hochberg as the post hoc test. The t-tests were performed in Microsoft Excel, while one-way and two-way ANOVA analyses were conducted using GraphPad Prism software. Statistical significance is indicated in the figures as $p < 0.05$, $p < 0.01$, and $p < 0.001$. Each data point represents a single measurement or the mean of technical replicates from an embryo or an independently treated cell batch. The number of replicates is detailed in the figure legends.

Data and code availability

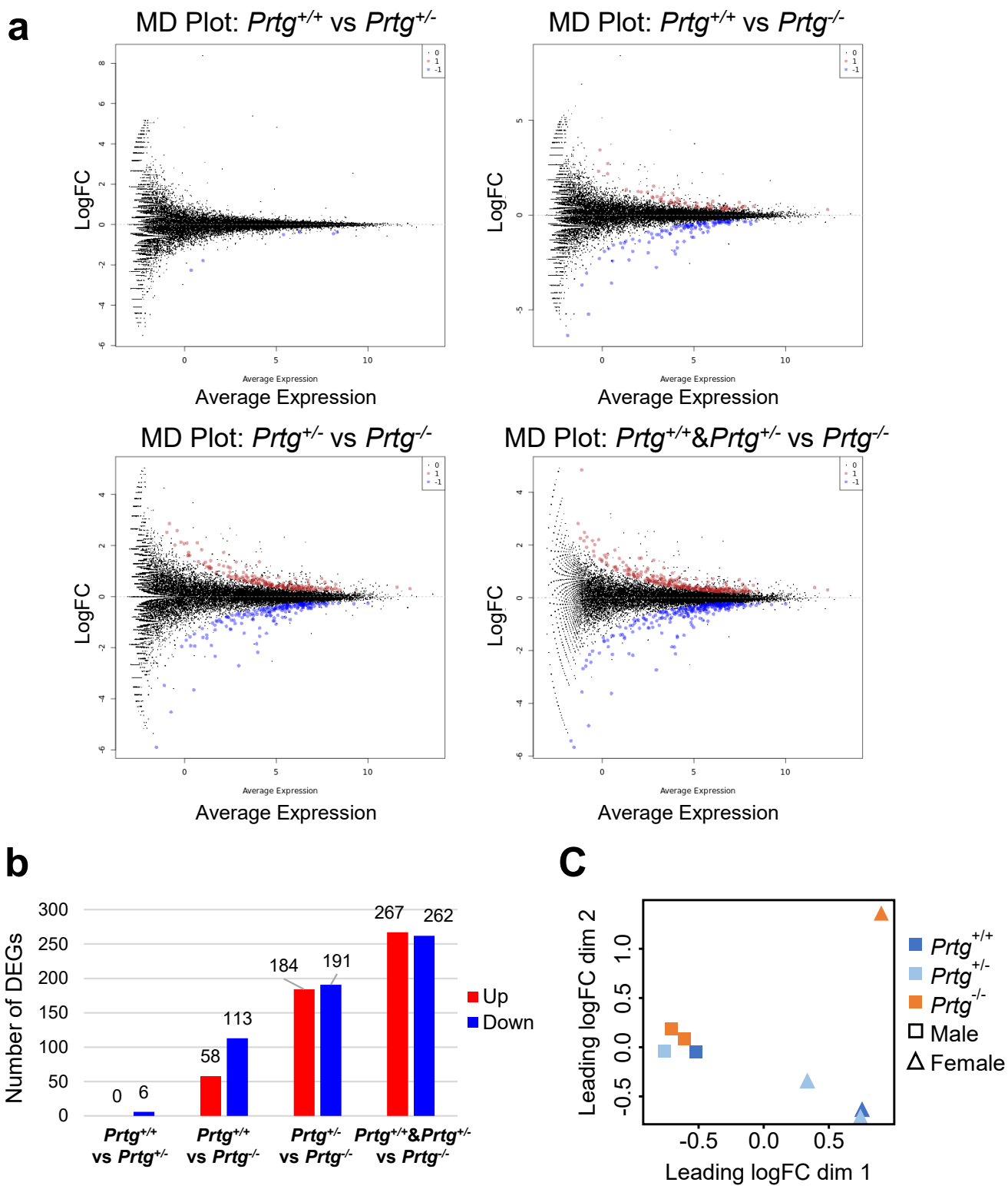
RNA-sequencing data are deposited in the NCBI database under accession number GSE256393. Uncropped blot images are provided in Supplementary Fig. 12. Constructed sequences generated in this study are listed in Supplementary Data 2. Source data underlying the graphs and charts in the main figures are included in Supplementary Data 3.

Supplementary Figures

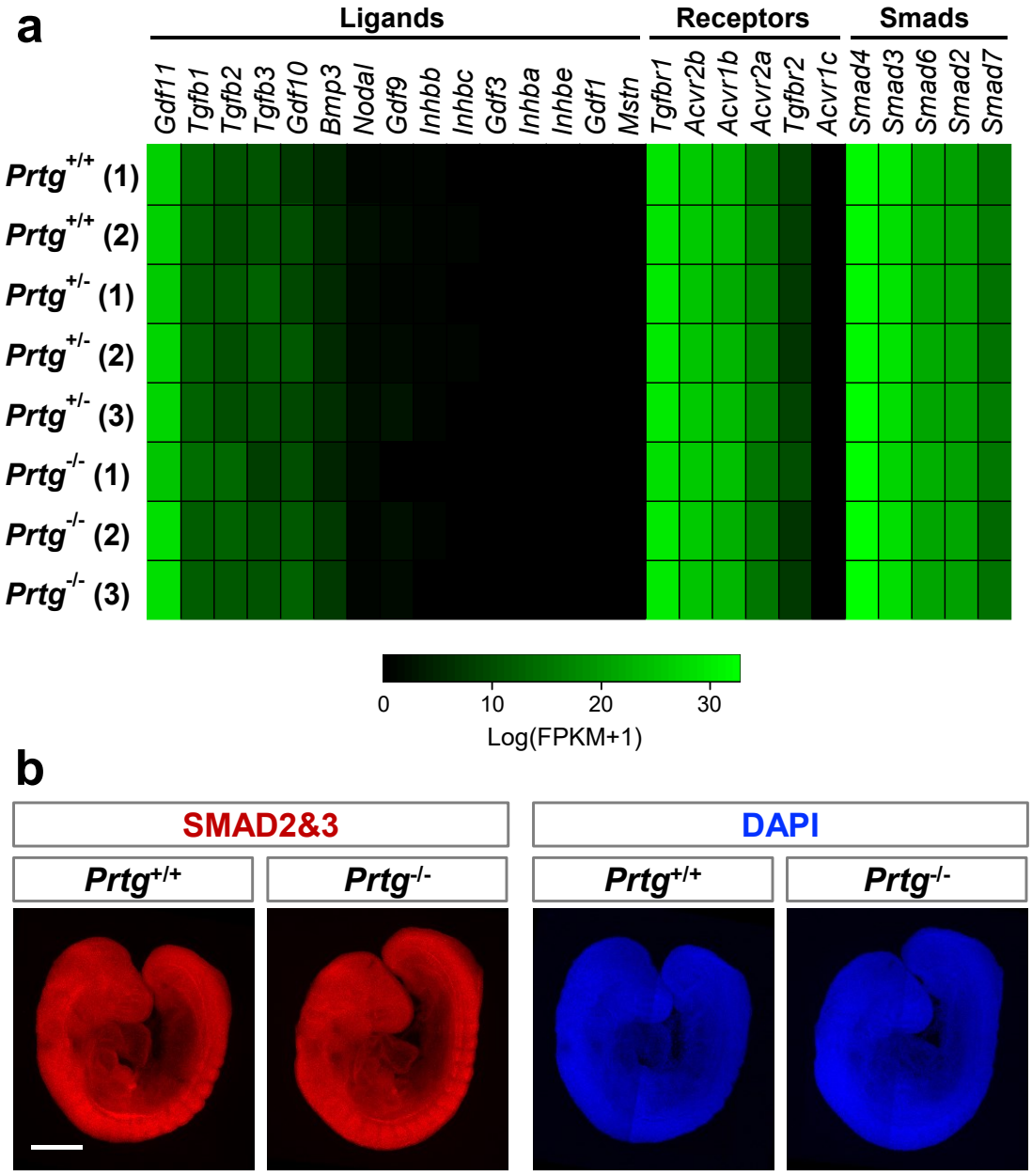


Supplementary Fig. 1: Whole-mount *in situ* hybridization using the sense probes. Related to Fig. 1.

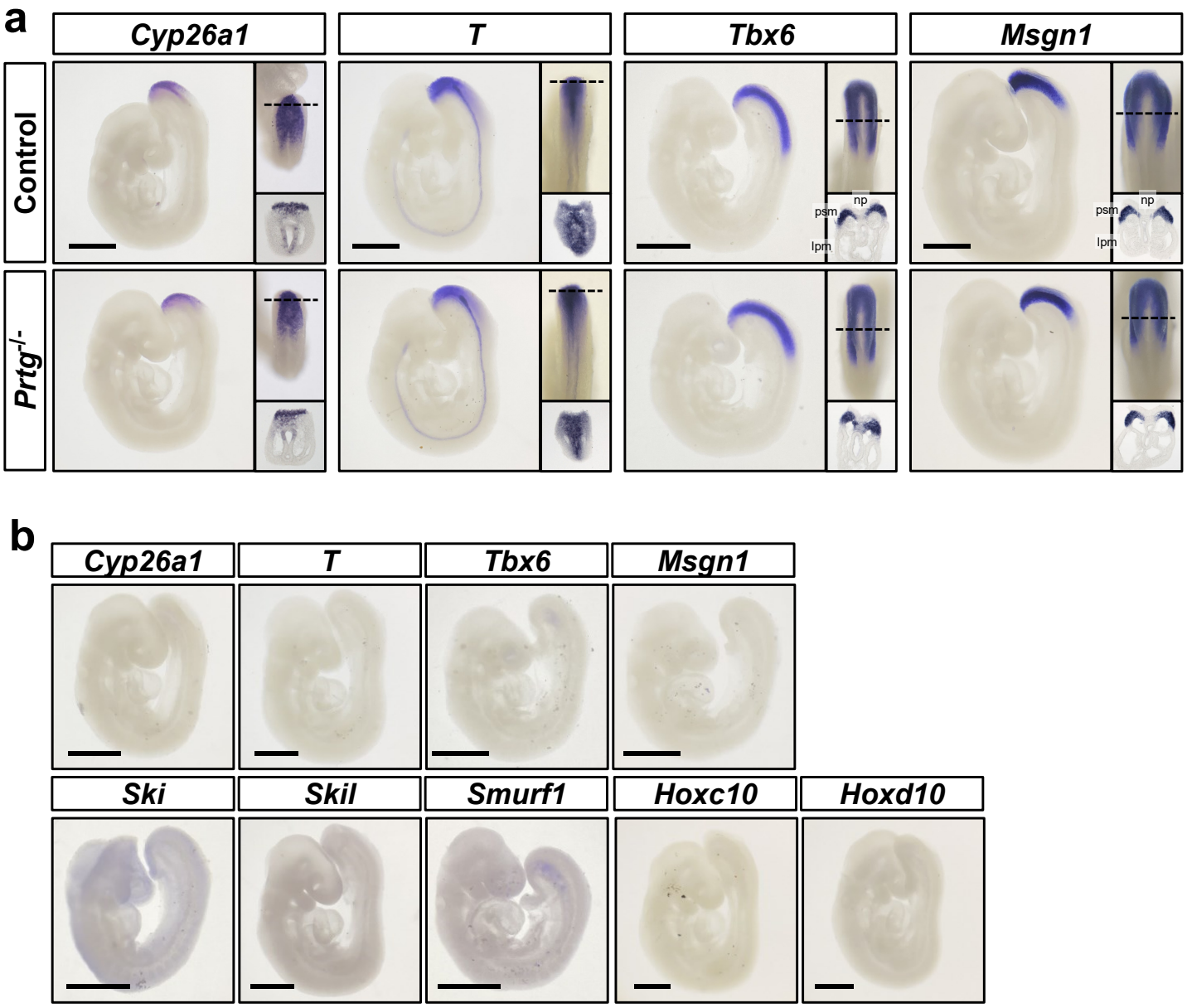
a Whole-mount *in situ* hybridization using the *Hoxb6* sense probe in E10.0 control embryo.
b Whole-mount *in situ* hybridization using the *Hoxb9* sense probe in E10.0 control embryo.
c Whole-mount *in situ* hybridization using the *Hoxc8* sense probe in E10.0 control embryo.
d Whole-mount *in situ* hybridization using the *Hoxc9* sense probe in E10.0 control embryo.
e Whole-mount *in situ* hybridization using the *Hoxd9* sense probe in E10.0 control embryo.
f Whole-mount *in situ* hybridization using the *Hoxa10* sense probe in E11.0 control embryo.
g Whole-mount *in situ* hybridization using the *Hoxc10* sense probe in E10.0 control embryo.
h Whole-mount *in situ* hybridization using the *Hoxd10* sense probe in E10.0 control embryo.
i Whole-mount *in situ* hybridization using the *Myog* sense probe in E10.0 control embryo.
Scale bars, 1 mm.



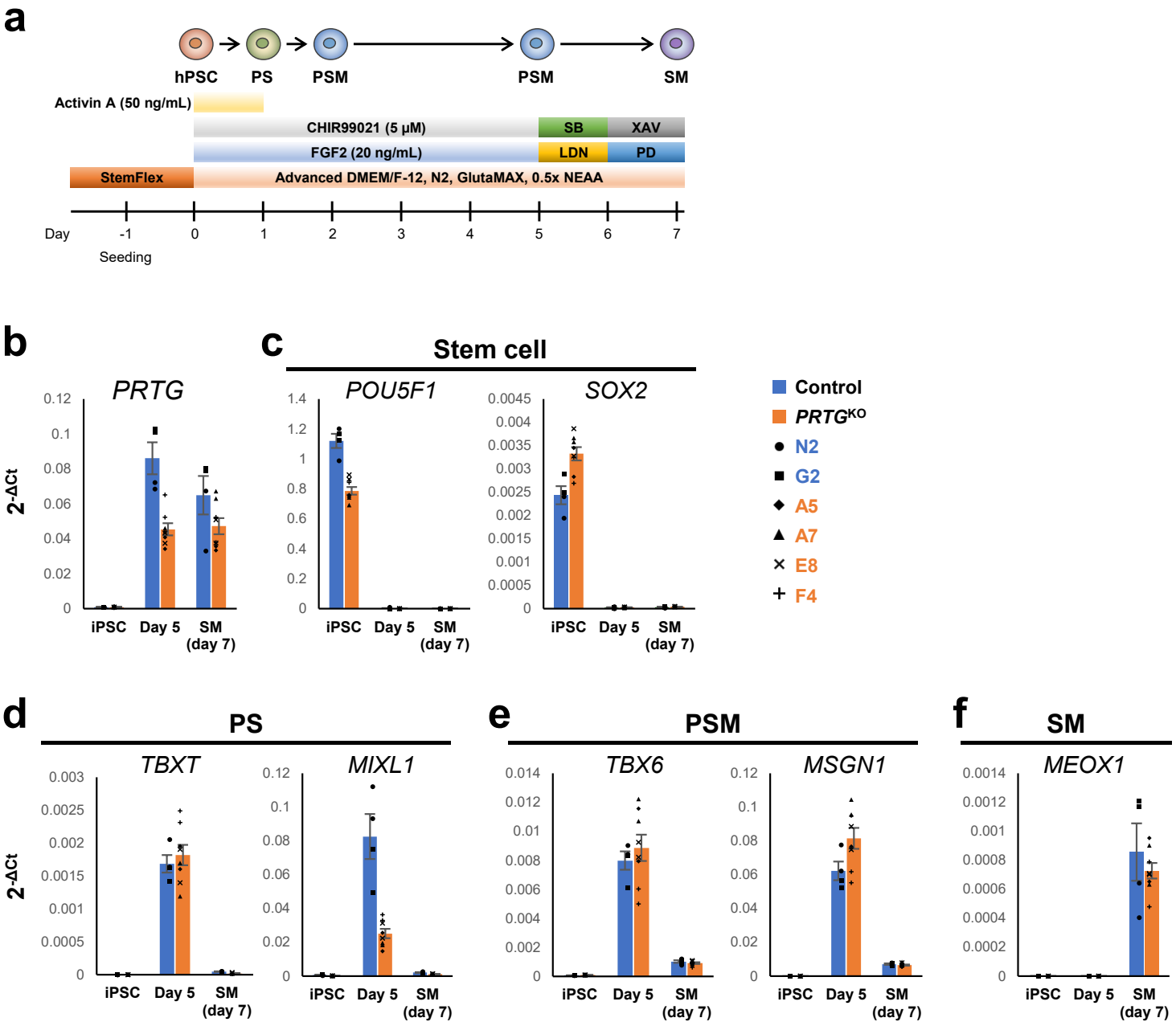
Supplementary Fig. 2: Differential expression gene (DEG) analysis in various sample groups. Related to Fig. 2. a MD plots illustrating gene expression changes in different sample group analyses. Genes with FDR < 0.05 are depicted in red (up-regulated in *Prtg*$^{-/-}$) or blue (down-regulated in *Prtg*$^{-/-}$). MD plots were generated using EdgeR. **b** Summary of the number of DEG resulting from various sample group analyses. **c** Multidimensional scaling (MDS) plots displaying the relationships among the 8 analyzed samples, generated using EdgeR.



Supplementary Fig. 3: Expression of TGFβ ligands, receptors and Smads in E9.5 embryos. Related to Fig. 3 and 4. a The heatmap illustrates the expression levels of TGFβ ligands, receptors, and Smads in the posterior trunk of E9.5 embryos. The data is derived from RNA-seq analysis and is presented on a logarithmic scale (Log(FPKM+1)). **b** Whole-mount immunostaining of total Smad2&3 in E9.5 *Prtg*$^{+/+}$ and *Prtg*$^{-/-}$ embryos. Scale bars, 500 µm.

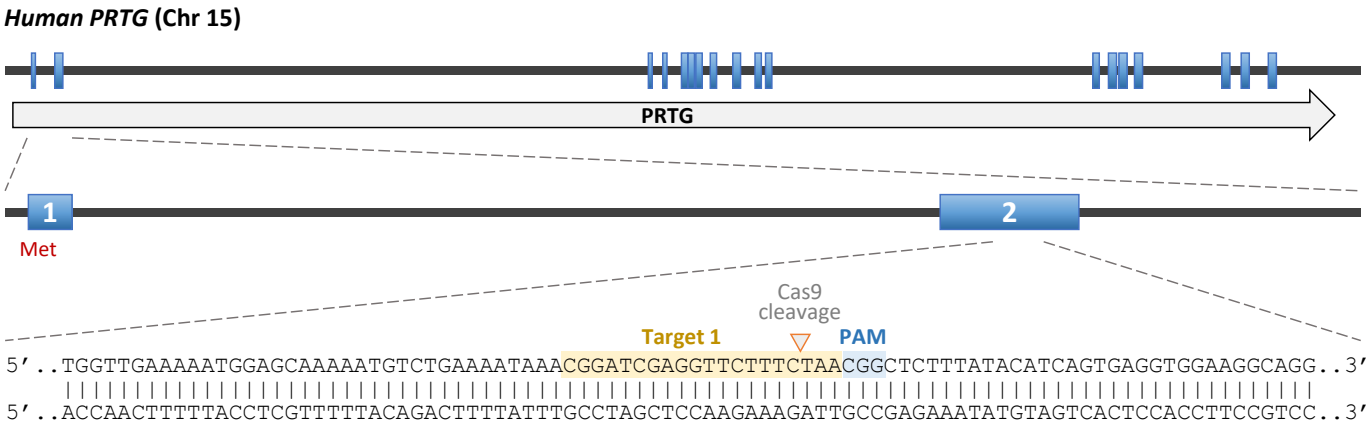


Supplementary Fig. 4: *Prtg*$^{-/-}$ embryos do not exhibit obvious defects in developing mesoderm. Related to Fig. 4. a Whole-mount *in situ* hybridization using markers for posterior progenitor cells (*Cyp26a1* and *T*) and PSM (*Tbx6* and *Msgn1*) in E9.5 control and *Prtg*$^{-/-}$ embryos. Dashed lines indicate the section positions displayed at the bottom. Lpm, lateral plate mesoderm; np, neural plate; psm; presomitic mesoderm. Scale bars, 500 μm. **b** Whole-mount *in situ* hybridization with sense probes for *Cyp26a1*, *T*, *Tbx6*, *Msgn1*, *Ski*, *Skil*, *Smurf1*, *Hoxc10* and *Hoxd10* in E9.5 control embryos. Scale bars, 500 μm.



Supplementary Fig. 5: hiPSC-PSM cells are capable of differentiating into somitic mesoderm-like cells. Related to Fig. 6. a Schematic illustration of somitic mesoderm differentiation protocol. **b-f** Expression levels of *PRTG* (**b**) and markers for stem cells (*POU5F1* and *SOX2*, **c**), primitive streak (PS) (*TBXT* and *MIXL1*, **d**), presomitic mesoderm (PSM) (*TBX6* and *MSGN1*, **e**), and somitic mesoderm (*MEOX1*, **f**) in hiPSCs, hiPSC-PSM Day 5, and hiPSC-derived somitic mesoderm-like cells were quantified by RT-qPCR. Error bars represent ± SEM (n = 4 for control group and 8 for *PRTG*^{KO} group).

a



b

		No. nucleotides deleted or inserted
N2 (parental hiPSC line)	CAAAAATGTCTGAAAATAAACGGATCGAGGTTCTTTC TAACGGCTCTTTATACATCAGTGAGG	
G2 (WT)	CAAAAATGTCTGAAAATAAACGGATCGAGGTTCTTTC TAACGGCTCTTTATACATCAGTGAGG	0
	CAAAAATGTCTGAAAATAAACGGATCGAGGTTCTTTC TAACGGCTCTTTATACATCAGTGAGG	0
A5 (-/-)	CAAAAATGTCTGAAAATAAACGGATCGAGGT------ -------TCTTTATACATCAGTGAGG	-13
	CAAAAATGTCTGAAAATA------------------ -AACGGCTCTTTATACATCAGTGAGG	-20
A7 (-/-)	CAAAAATGTCTGAAAATAAACGGATCGAGGTTCTTTC CTAACGGCTCTTTATACATCAGTGAGG	+1
	CAAAAATGTCTGAAAATA------------------ -AACGGCTCTTTATACATCAGTGAGG	-20
E8 (-/-)	CAAAAATGTCTGAAAATAAACGGATCGAGGTTCTTTC CTAACGGCTCTTTATACATCAGTGAGG	+1
	CAAAAATGTCTGAAAATAAACGGATCGAGGT------ -------TCTTTATACATCAGTGAGG	-13
F4 (-/-)	CAAAAATGTCTGAAAATAAACGGATCGAGGTTCTTTC CTAACGGCTCTTTATACATCAGTGAGG	+1
	CAAAAATGTCTGAAAATAAACGGATCGAGGTTCTTTCTGTA TAACGGCTCTTTATACATCAGTGAGG	+4

Supplementary Fig. 6: Generation of isogenic wild-type and *PRTG*^{KO} hiPSC clones through CRISPR/Cas9 gene editing. Related to Fig. 6. a Schematic representation of the human *PRTG* locus and the guide-RNA (gRNA) targeting site. **b** Confirmation of the edited hiPSC clones, including N2 (parental), G2 (wild-type), A5 (*PRTG*^{KO}), A7 (*PRTG*^{KO}), E8 (*PRTG*^{KO}), and F4 (*PRTG*^{KO}), by Sanger sequencing.

a

T7 out of 32

<Previous Next>

Sequence: CGGATCGAGGTTCTTTCTAACGG

Efficacy score by CRISPRater: 0.67 MEDIUM

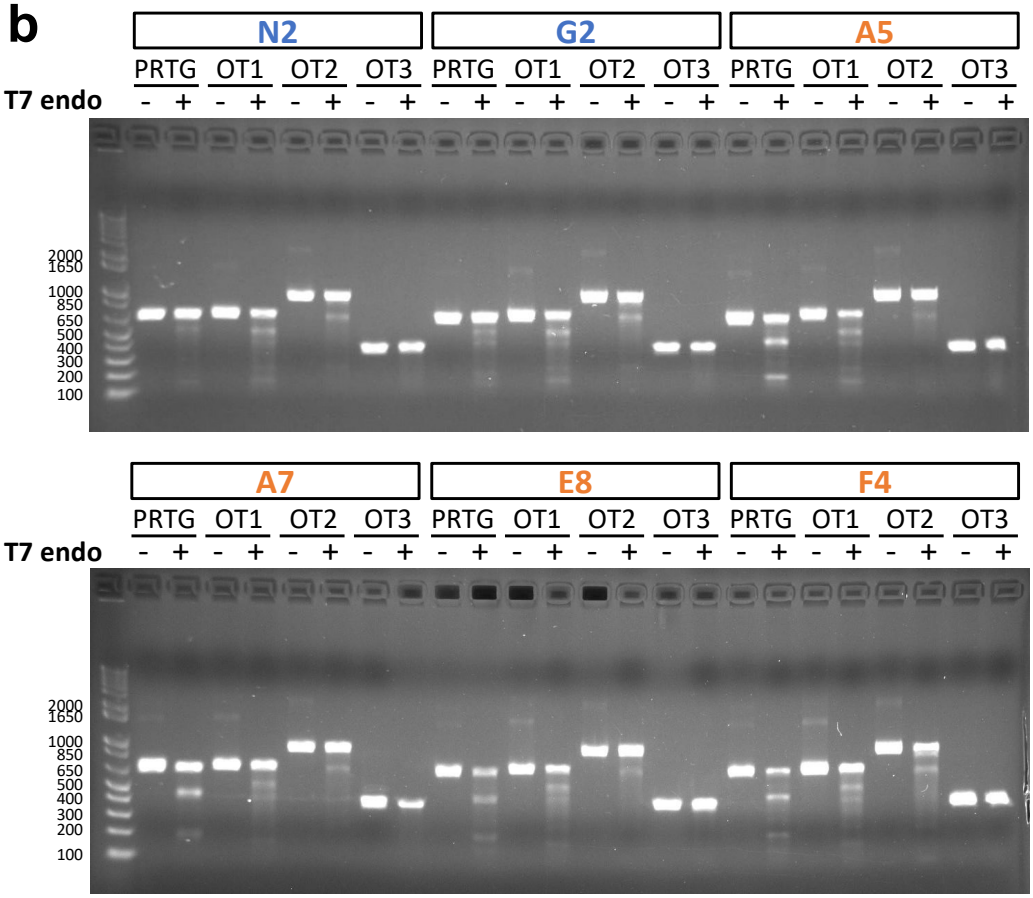
Oligo pair with 5' extension fwd: TAggCGGATCGAGGTTCTTTCTAA rev: AAACTTAGAAAGAACCTCGATCCG

Oligo pair with 5' substitution fwd: TAggGATCGAGGTTCTTTCTAA rev: AAACTTAGAAAGAACCTCGATC

Top 20 offtarget sites out of 43 (including on target; for full list see xls file)

	Coordinates	strand	MM	target_seq	PAM	distance		gene name	gene id
	chr15:55740507-55740529	-	0	CGGATCGA[GGTTCTTTCTAA]	CGG	0	E	PRTG	ENSG00000166450
OT1	chr5:72152131-72152153	+	4	TAGATTGT[GGTTCTTTCTAA]	AGG	17314	I	MIR4803	ENSG00000264099
OT2	chrX:113723441-113723463	-	3	CAGATCGT[AGTTCTTTCTAA]	GGG	NA	I	NA	NA
OT3	chr8:78516959-78516981	-	3	CAGATAGA[GGCTCTTTCTAA]	AGG	491	I	PKIA	ENSG00000171033

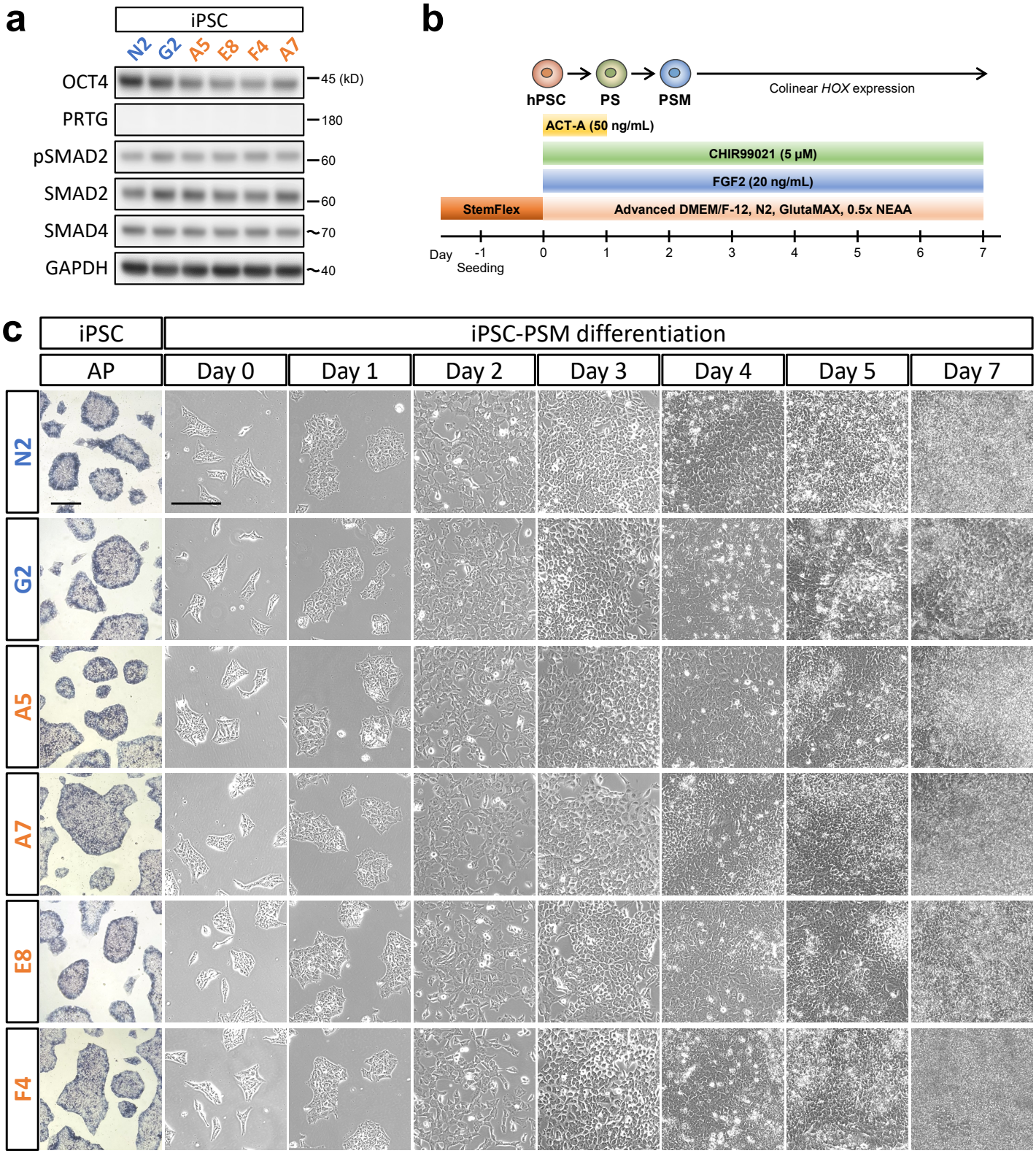
b



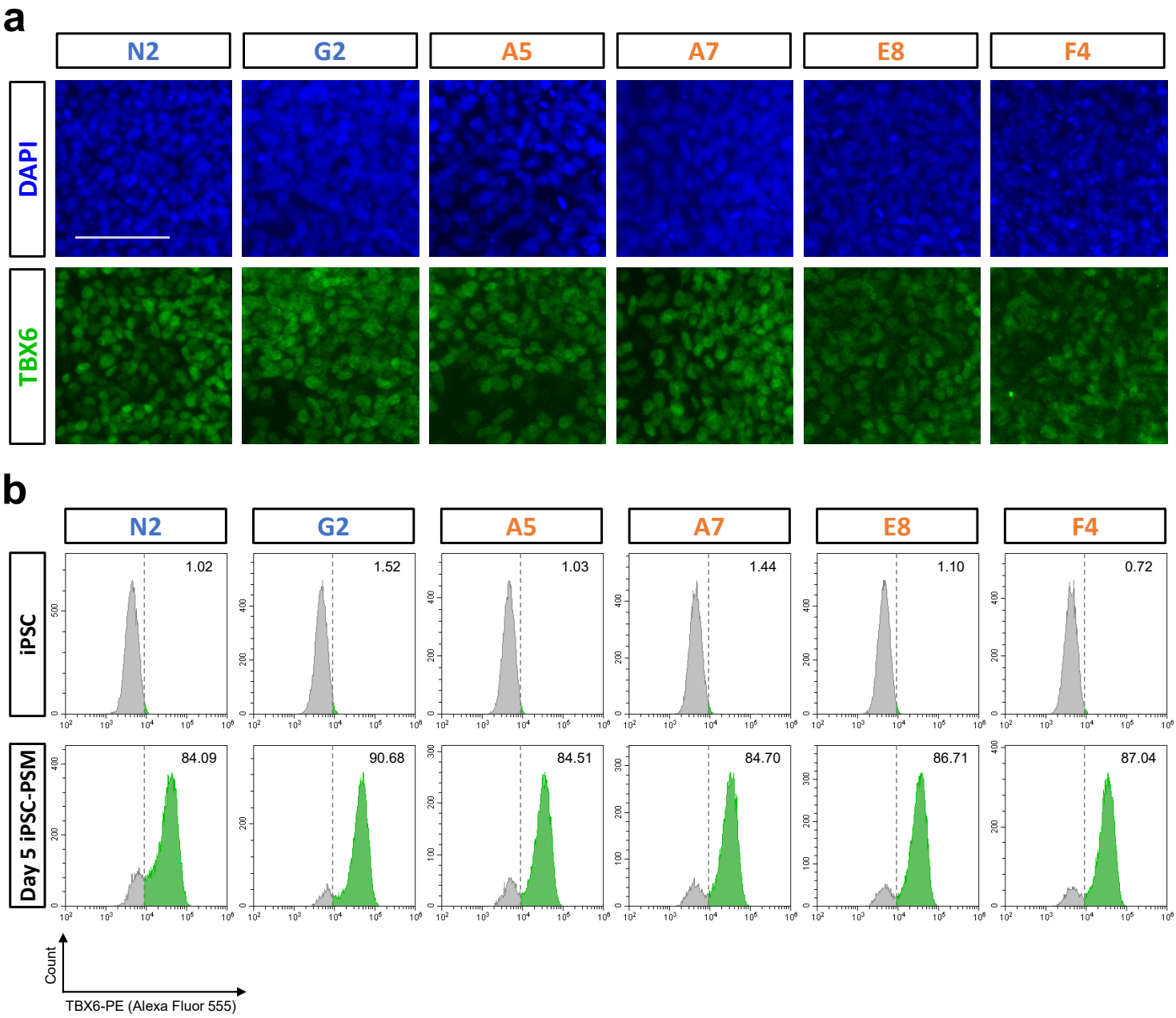
Expected PCR product size after T7E1 digestion (bp)

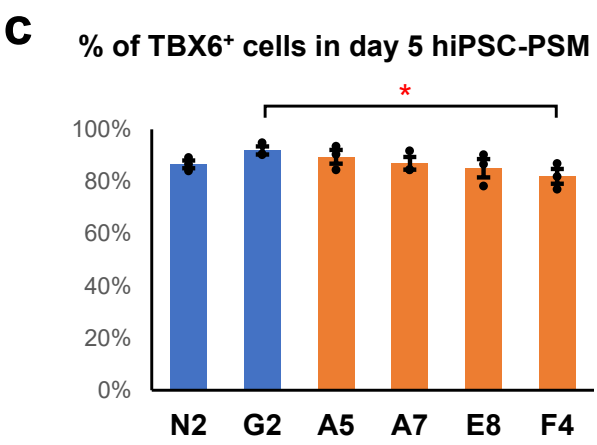
	None	Mismatched
PRTG	662	445 217
OT1	689	443 246
OT2	937	504 433
OT3	404	251 153

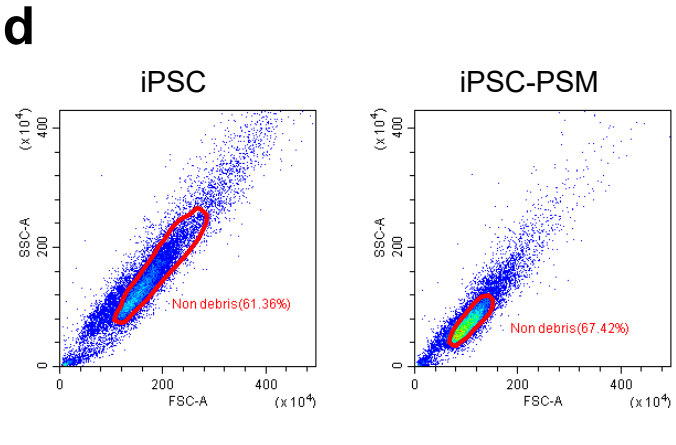
Supplementary Fig. 7: Off-target cleavage by CRISPR/Cas9 editing system was not detected in isogenic hiPSC lines. Related to Fig. 6. a The top 3 possible off-target sites for each target gRNA were predicted using CCTop - CRISPR/Cas9 target online predictor (https://crispr.cos.uni-heidelberg.de). OT1, off-target #1; OT2, off-target #2; OT3, off-target #3. **b** Results of T7E1 mismatch detection assay. T7E1 assay was performed at PRTG and the predicted off-target sites with genomic DNA from CRISPR-edited hiPSCs (N2, G2, A5, A7, E8 and F4 clones). The predicted results are shown in the right table.



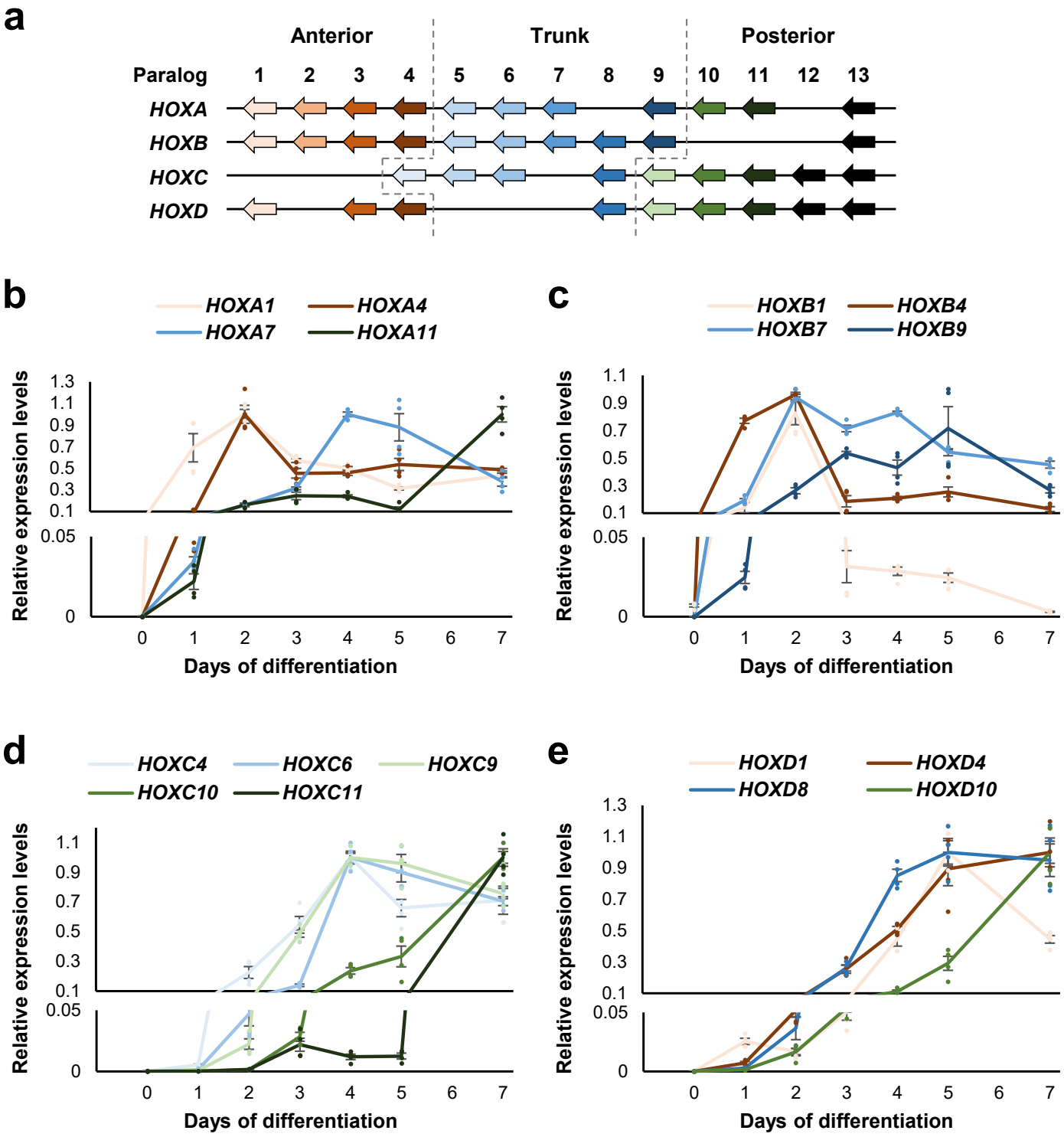
Supplementary Fig. 8: Characterization of CIRSPR/Cas9-edited lines. No obvious difference between control and *PRTG*^{KO} hiPCSs. Related to Fig. 6. a Expression levels of OCT4, PRTG, pSMAD2, SMAD2 and SMAD4 were assessed by western blotting. GAPDH was used as a loading control. **b** Schematic presentation of hiPSC-PSM differentiation protocol. **c** Parental and CRISPR-edited hiPSCs showed an embryonic stem cell-like morphology and expressed pluripotent stem cell marker. Alkaline phosphatase staining (left panel) and phase-contrast images of iPSC-PSM cells during the 7-day differentiation from six hiPSC clones. Scale bars, 200 μm.



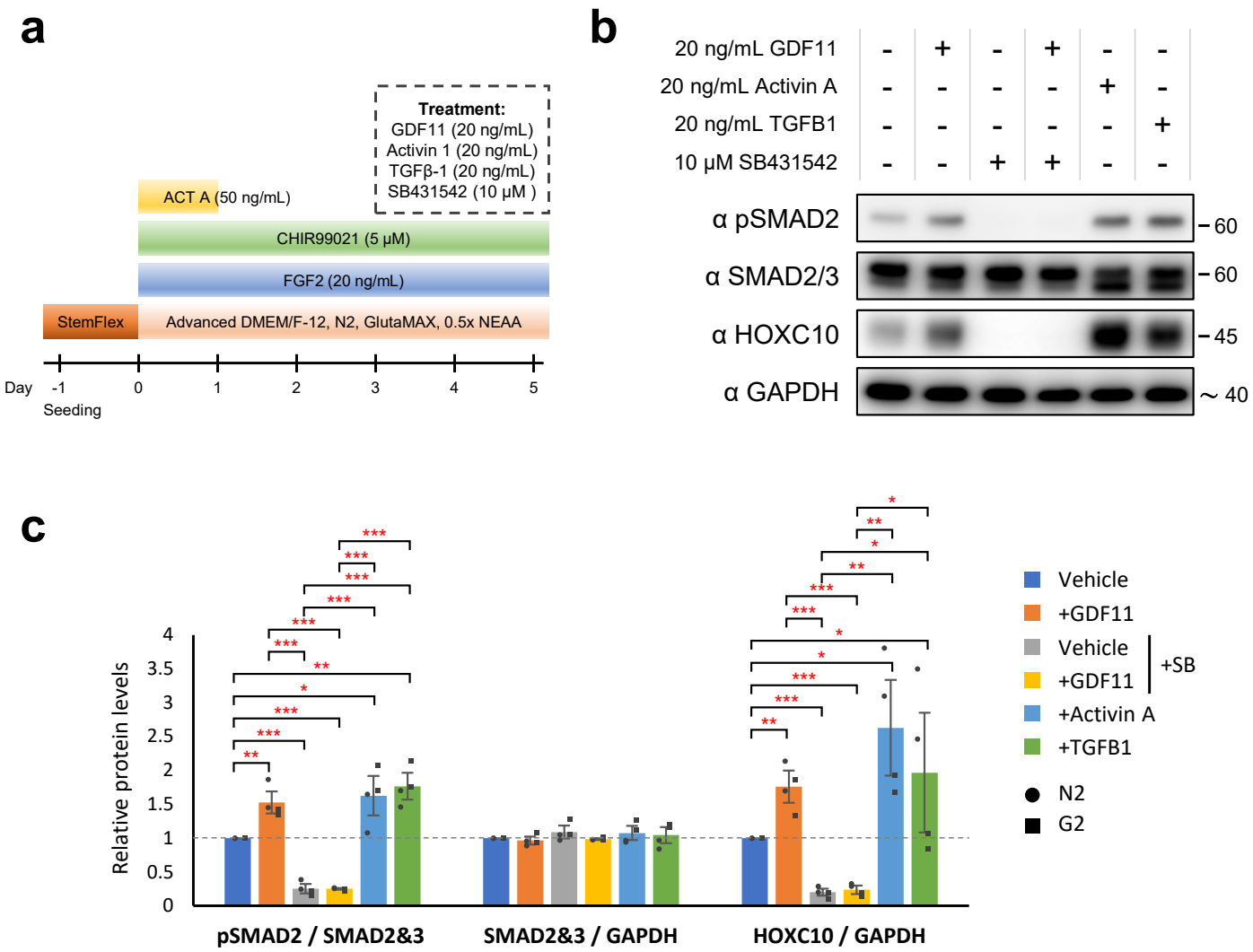




Supplementary Fig. 9: Immunofluorescence and flow cytometry analyses of iPSC-PSM. Related to Fig. 6. a-b Immunostaining (**a**) and flow cytometry (**b**) analyses of iPSC-PSM at day 5 of differentiation using TBX6 antibody to assess the purity of the iPSC-PSM population. Scale bars, 100 µm. **c** Average percentage of TBX6⁺ cells in day 5 iPSC-PSM as determined by flow cytometry (n=3). Data are presented as the mean ± SEM (* $p < 0.05$; by one-way ANOVA). **d** Gating parameters for iPSC and iPSC-PSM experimental cells. Cell debris and clumps of multiple cells were removed by gating with FSC-A and SSC-A.



Supplementary Fig. 10: Collinear expression of *HOX* genes in hiPSC-PSM. Related to Fig. 6. **a** Color codes representing each *HOX* paralog. **b** Expression levels of *HOXA1*, *HOXA4*, *HOXA7* and *HOXA11* in control iPSC-PSM during differentiation were measured by RT-qPCR (n = 4). **c** Expression levels of *HOXB1*, *HOXB4*, *HOXB7* and *HOXB9* in control iPSC-PSM during differentiation were measured by RT-qPCR (n = 4). **d** Expression levels of *HOXC4*, *HOXC6*, *HOXC9*, *HOXC10* and *HOXC11* in control iPSC-PSM during differentiation were measured by RT-qPCR (n = 4). **e** Expression levels of *HOXD1*, *HOXD4*, *HOXD8* and *HOXD10* in control iPSC-PSM during differentiation were measured by RT-qPCR (n = 4).



Supplementary Fig. 11: TGFβ signaling is required for the activation of *HOXC10*. Related to Fig. 7. a Schematic presentation of iPSC-PSM differentiation and treatment protocol. **b** Protein levels of pSMAD2, SMAD2&3 and HOXC10 were measured by western blotting. Gapdh was used as an internal control. **c** Quantification of the results shown in panel **b**. Data are presented as the mean ± SEM (n = 4; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; by one-way ANOVA).

Supplementary Fig. 12: Uncropped Western blot images of indicated Figures in this study

Figure 3e, Prtg

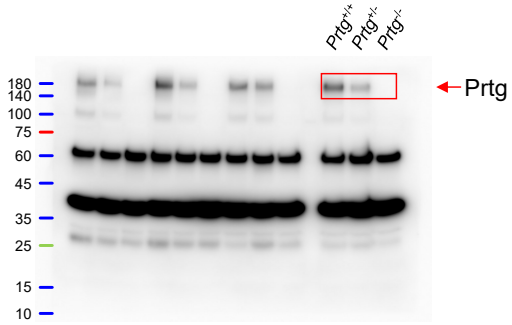


Figure 3e, pSmad2

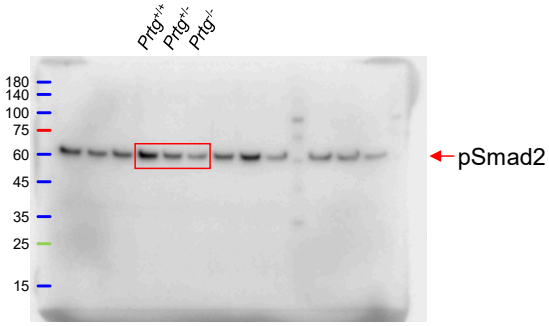


Figure 3e, Smad2

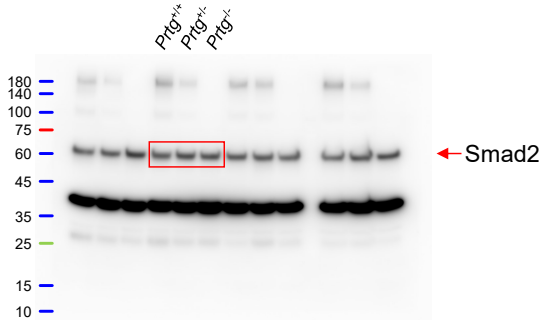


Figure 3e, Gapdh

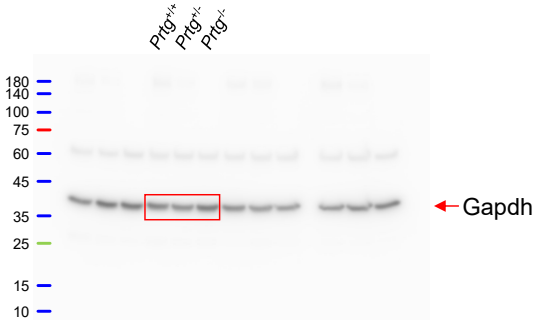


Figure 3e, Smad4

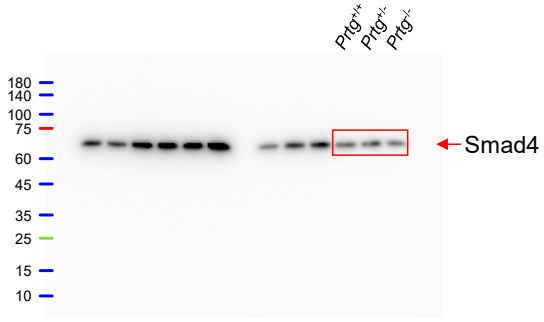


Figure 5b, αPrtg

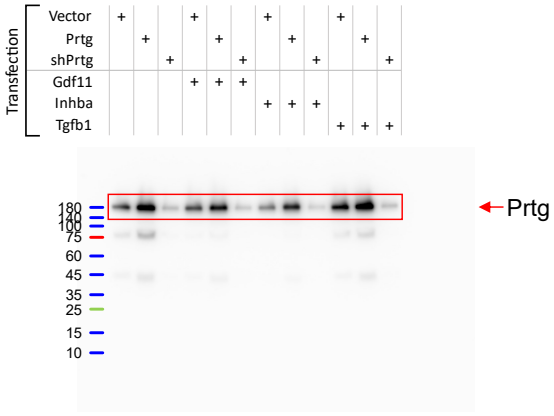


Figure 5b, αpSmad2

Figure 5b, αSmad2&3

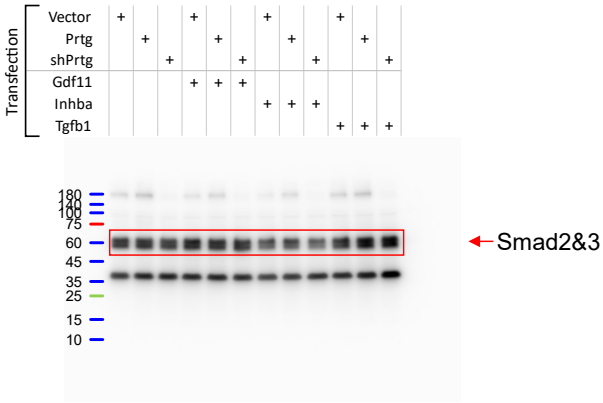


Figure 5b, αGapdh

Figure 5d, Input: αHA

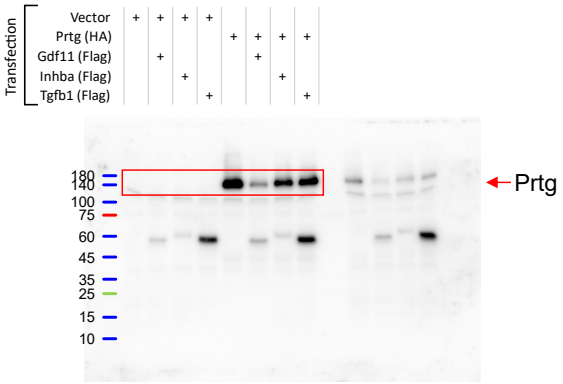


Figure 5d, Input: αFlag

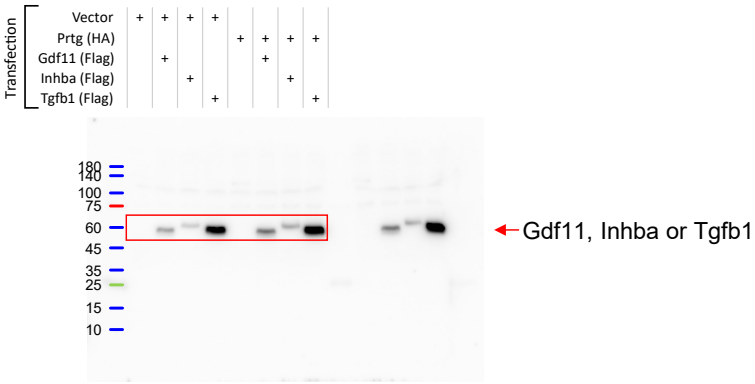


Figure 5d, IP: αHA

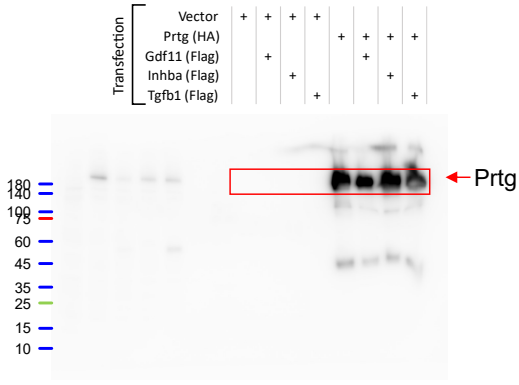


Figure 5d, IP: αFlag

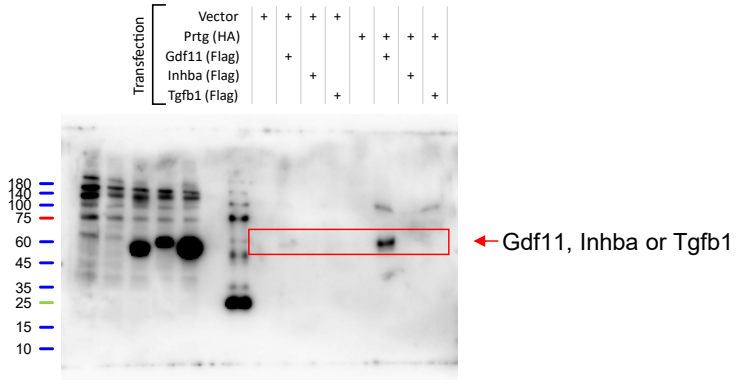
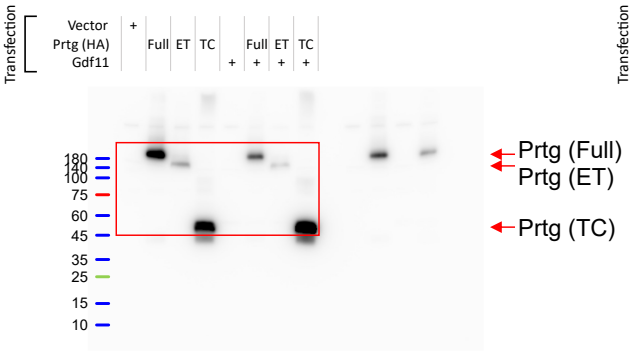


Figure 5f, Input: αHA



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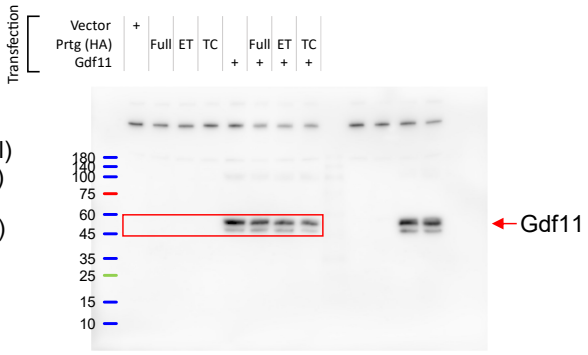

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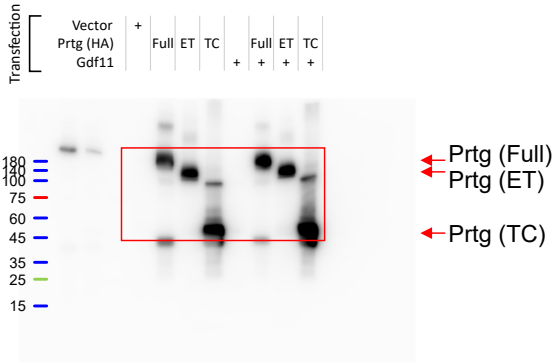

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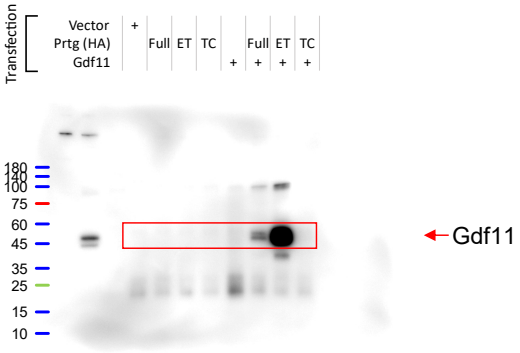


Figure 5h, αPrtg

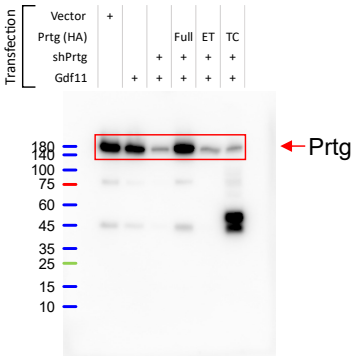


Figure 5h, αHA

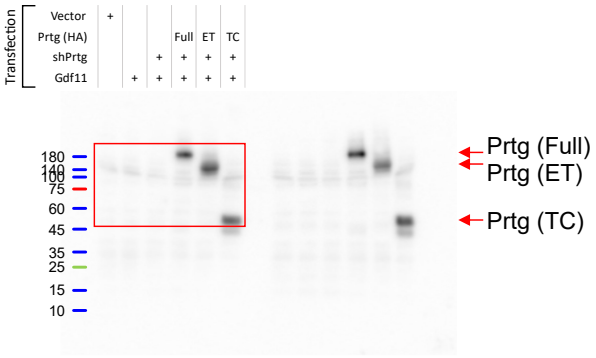


Figure 5h, pSmad2

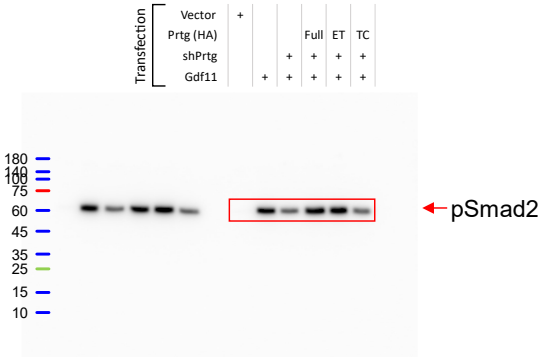


Figure 5h, αSmad2&3

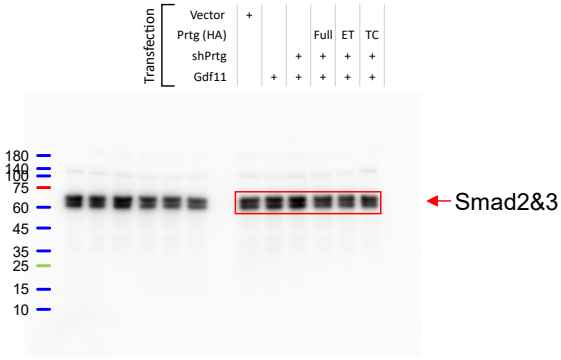


Figure 5h, αGapdh

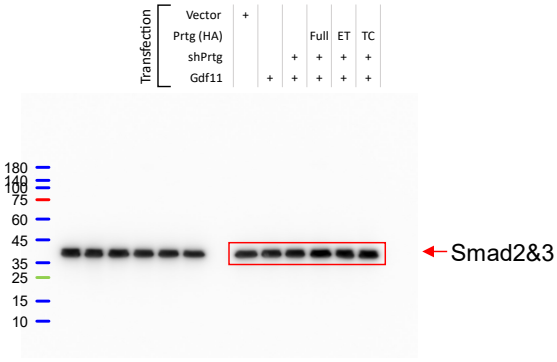


Figure 6b, PRTG

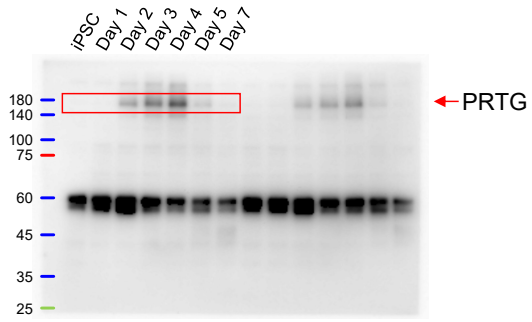


Figure 6b, pSMAD2

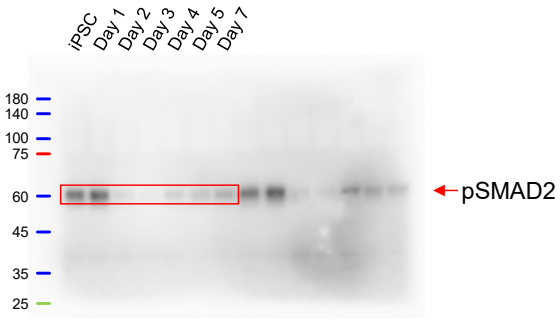


Figure 6b, SMAD2&3

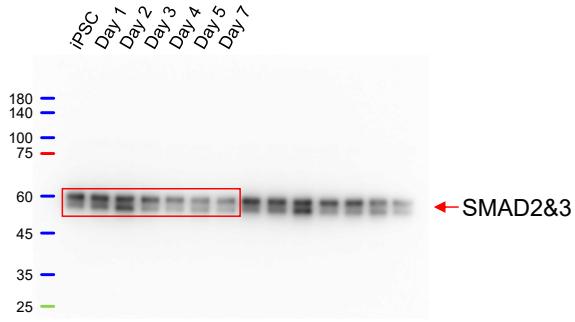


Figure 6b, HOXC10

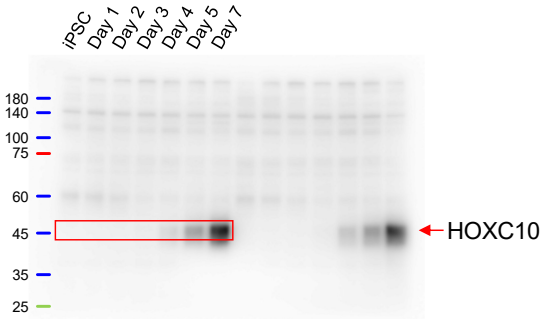


Figure 6b, OCT4

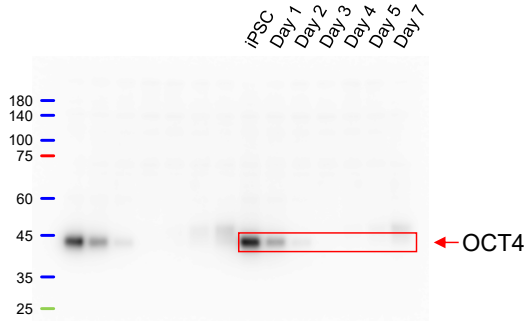


Figure 6b, GAPDH

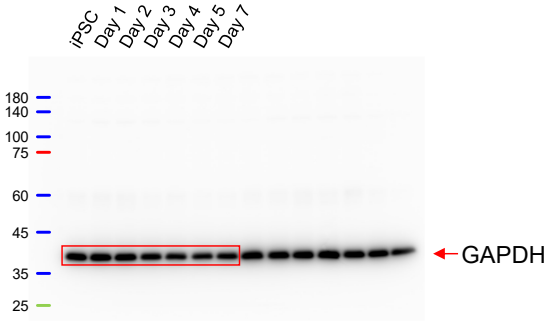


Figure 6e, PRTG

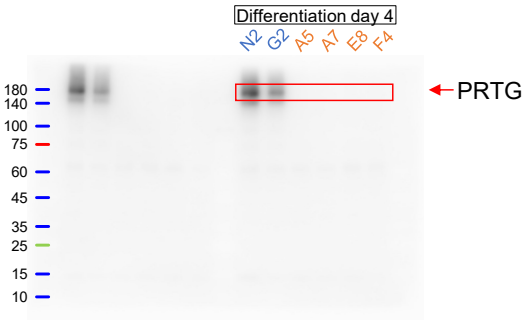


Figure 6e, GAPDH

Figure 6f, pSMAD2

Figure 6f, SMAD2&3

Figure 6f, HOXC10

Figure 6f, GAPDH

Figure 7b, pSMAD2

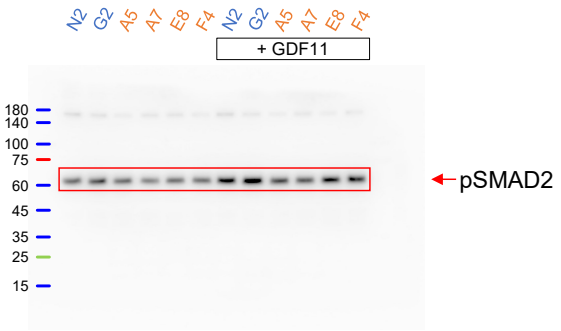


Figure 7b, SMAD2&3 and GAPDH

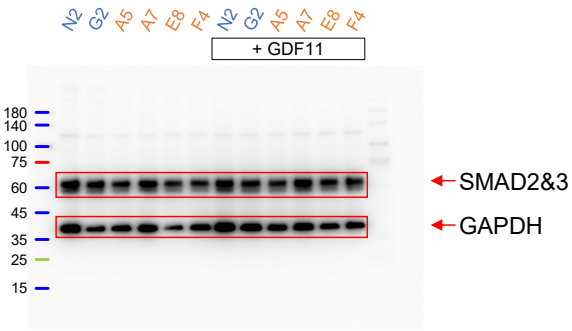
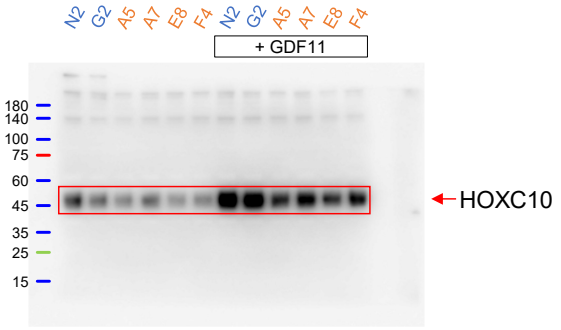


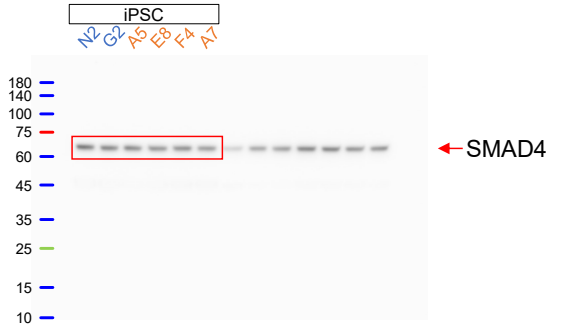
Figure 7b, HOXC10



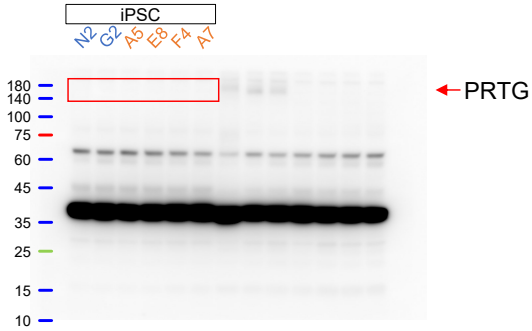
Supplementary Fig. 8, SMAD2 and OCT4

Supplementary Fig. 8, pSMAD2

Supplementary Fig. 8, SMAD4



Supplementary Fig. 8, PRTG



Supplementary Fig. 8, GAPDH

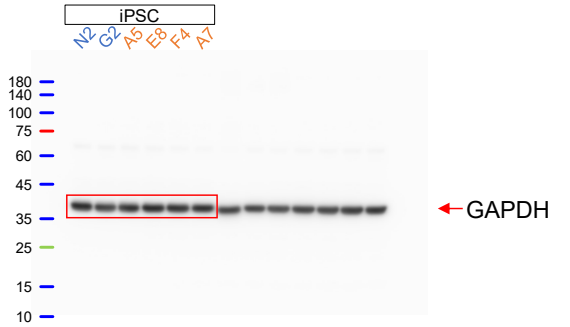
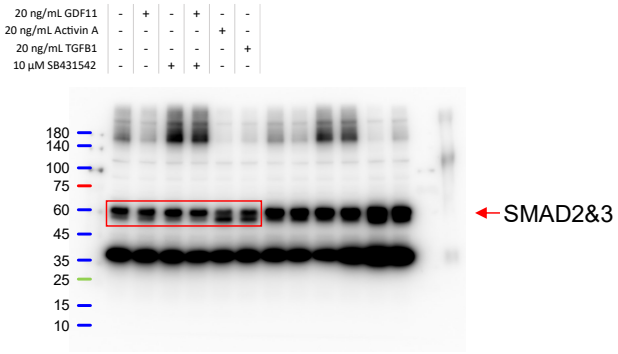


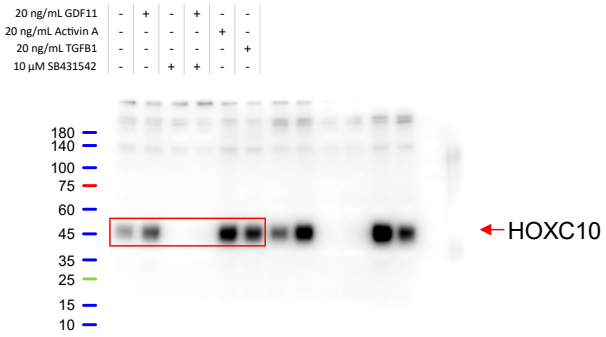
Figure 6e, PRTG

Supplementary Fig. 11, α pSMAD2

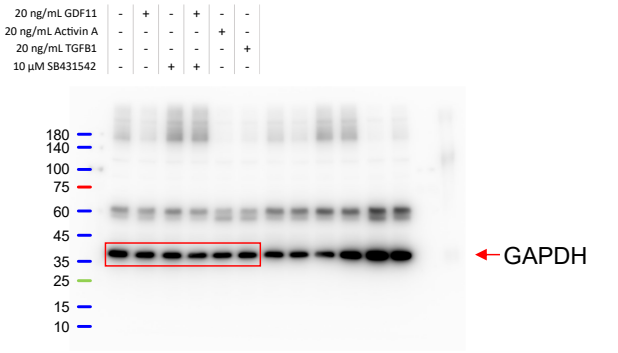
Supplementary Fig. 11, α SMAD2&3



Supplementary Fig. 11, α HOXC10



Supplementary Fig. 11, α GAPDH



Supplementary Tables

Supplementary Table 1: The penetrance of phenotypes in *Prtg*$^{-/-}$ mutants with different genetic background. Related to Fig 1a, b

Mouse strain	C57BL/6			CBA/J	ICR
Generation	N1~N7	N12 (neo+)	N12 (neo-)	N3	N3
Rib cage abnormality	88.2% (15/17)	96.3% (26/27)	100% (23/23)	100% (4/4)	100% (2/2)

The number of *Prtg*$^{-/-}$ mutants assayed is indicated betwenn brackets.

Supplementary Table 2: Number of *Myog*-positive somites in E11 control and *Prtg*$^{-/-}$ embryos. Related to Fig 1c

Prtg genotype	+/+	+/+	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-
Total count of *Myog*-positive somites	30	32	29	30	30	33	30	30	31	31	31	32
The count of somites at the anterior boundary of the hindlimb bud	24	24	24	24	24	24	26	26	26	26	26	25

Supplementary Table 3: Top 20 ranking gene sets of GSEA hallmark analysis. Related to Fig 3b

	Gene Set	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val	RANK AT MAX	LEADING EDGE
1	**TGF_BETA_SIGNALING**	**54**	**0.49**	**1.82**	**0**	**0.014**	**0**	**2748**	**tags=28%, list=15%, signal=33%**
2	WNT_BETA_CATENIN_SIGNALING	41	0.36	1.34	0.055	0.495	0.657	2637	tags=22%, list=15%, signal=26%
3	EPITHELIAL_MESENCHYMAL_TRANSITION	196	0.44	1.25	0.202	0.828	0.909	2219	tags=30%, list=12%, signal=33%
4	MYC_TARGETS_V2	57	0.49	1.24	0.243	0.69	0.951	5870	tags=63%, list=33%, signal=93%
5	MYC_TARGETS_V1	200	0.35	1.21	0.317	0.702	1	6587	tags=60%, list=37%, signal=93%
6	INFLAMMATORY_RESPONSE	198	0.37	1.19	0.08	0.626	1	3124	tags=35%, list=17%, signal=42%
7	ALLOGRAFT_REJECTION	196	0.36	1.14	0.153	0.714	1	3161	tags=30%, list=18%, signal=35%
8	UV_RESPONSE_UP	156	0.28	1.13	0.273	0.668	1	2127	tags=17%, list=12%, signal=19%
9	ANGIOGENESIS	36	0.45	1.07	0.315	0.772	1	1625	tags=25%, list=9%, signal=27%
10	TNFA_SIGNALING_VIA_NFKB	198	0.35	1.03	0.429	0.892	1	2637	tags=28%, list=15%, signal=32%
11	APICAL_SURFACE	44	0.33	0.97	0.592	1	1	2244	tags=25%, list=12%, signal=28%
12	E2F_TARGETS	200	0.29	0.95	0.601	0.989	1	6537	tags=46%, list=36%, signal=71%
13	G2M_CHECKPOINT	196	0.27	0.94	0.529	0.956	1	6540	tags=45%, list=36%, signal=70%
14	COMPLEMENT	193	0.29	0.92	0.605	0.917	1	3091	tags=25%, list=17%, signal=30%
15	KRAS_SIGNALING_UP	199	0.26	0.92	0.678	0.875	1	2144	tags=18%, list=12%, signal=20%
16	APICAL_JUNCTION	198	0.25	0.9	0.608	0.867	1	2574	tags=20%, list=14%, signal=23%
17	IL6_JAK_STAT3_SIGNALING	87	0.26	0.88	0.831	0.863	1	4067	tags=38%, list=23%, signal=49%
18	OXIDATIVE_PHOSPHORYLATION	198	0.2	0.78	0.695	1	1	4779	tags=28%, list=26%, signal=37%
19	MYOGENESIS	200	0.25	0.75	0.911	1	1	2015	tags=17%, list=11%, signal=18%
20	NOTCH_SIGNALING	32	0.19	0.66	0.907	1	1	5481	tags=44%, list=30%, signal=63%

Supplementary Table 4: Fold-change and FDR of GSEA hallmark TGF_BETA_SIGNALING gene set. Related to Fig 3c. Genes with significantly altered expression in *Prtg*$^{-/-}$ embryos are indicated in bold (FDR < 0.05).

Gene Symbol	logFC	logCPM	F test	PValue	FDR
Serpine1	0.24963	1.23186	0.909045	0.34037	1
Ppp1r15a	0.24549	4.655569	6.436026	0.011183	0.419441
Cdh1	0.22905	5.71994	10.37927	0.001278	0.102158
Thbs1	0.205726	4.704567	4.65118	0.031034	0.711791
Id3	0.199467	7.33364	11.45286	0.000714	0.067831
Smad1	0.194857	5.331765	6.204686	0.012742	0.452458
Id2	0.156199	6.801939	6.664859	0.009954	0.39401
Tjp1	0.094495	8.241782	2.493288	0.114334	1
Xiap	0.082541	6.411588	1.824879	0.176735	1
Fnta	0.080725	6.342688	1.693342	0.193162	1
Cdkn1c	0.061862	7.282712	1.090083	0.296453	1
Arid4b	0.057292	5.95149	0.715004	0.397788	1
Nog	0.054546	2.787776	0.111876	0.738019	1
Hipk2	0.047002	6.647589	0.604493	0.436869	1
Wwtr1	0.036068	5.343926	0.212155	0.645084	1
Bmpr1a	0.030386	7.154561	0.260544	0.609747	1
Sptbn1	0.018215	8.115017	0.092626	0.760865	1
Ppm1a	0.015408	6.927521	0.066014	0.797231	1
Hdac1	-0.0104	7.418763	0.030931	0.860394	1
Smad6	-0.01259	5.173936	0.023167	0.879025	1
Id1	-0.01613	7.011764	0.072563	0.787642	1
Bcar3	-0.01898	4.245941	0.029908	0.8627	1
Rhoa	-0.02651	8.180785	0.195189	0.658633	1
Tgfb1	-0.027	4.094806	0.055891	0.813112	1
Ctnnb1	-0.02979	10.5003	0.26941	0.603728	1
Furin	-0.04188	6.018215	0.391202	0.531669	1
Map3k7	-0.04251	7.070727	0.505194	0.477227	1
Smurf2	-0.04273	6.47831	0.49101	0.483478	1
Fkbp1a	-0.04337	8.413367	0.518313	0.471563	1
Trim33	-0.0437	6.766281	0.523696	0.46927	1
Bmp2	-0.04713	3.142879	0.100907	0.750744	1
Ube2d3	-0.0601	8.187859	1.001148	0.317034	1
Ppp1ca	-0.0654	2.359829	0.123552	0.725214	1
Cdk9	-0.06684	7.149628	1.252598	0.263058	1
Tgfbr1	-0.0735	7.544616	1.53396	0.21552	1
Acvr1	-0.11191	4.527621	1.20705	0.271918	1
Apc	-0.11505	7.027645	3.672742	0.055311	0.898379
Tgif1	-0.11614	6.11323	3.124491	0.077125	1
Rab31	-0.13048	5.646884	3.189004	0.074136	1
Slc20a1	-0.16215	6.813033	7.173631	0.007399	0.327397
Smad3	-0.17317	7.170006	8.360061	0.003836	0.213378
Ncor2	-0.18322	7.80871	9.359747	0.002218	0.151254
Ifngr2	-0.1897	4.528332	3.449659	0.063266	0.945379
Smad7	-0.20311	4.41465	3.683037	0.05497	0.893745
Eng	-0.20878	4.797472	4.941995	0.026212	0.64916
Bmpr2	-0.24021	5.582191	10.40554	0.001256	0.101367
Smurf1	**-0.24169**	**5.924032**	**12.30912**	**0.000451**	**0.048021**
Klf10	-0.24479	4.370105	5.206498	0.022503	0.612053
Ski	**-0.294**	**7.826406**	**23.8987**	**1.02E-06**	**0.000351**
Junb	-0.36914	3.189175	6.173124	0.012972	0.45474
Skil	**-0.43607**	**5.558884**	**33.38402**	**7.57E-09**	**5.52E-06**
Ltbp2	-0.65526	-0.18017	2.321266	0.127617	1
Lefty2	-1.83237	-1.05506	7.194748	0.007312	0.324579
Pmepa1	**-2.11773**	**1.463692**	**54.91032**	**1.26E-13**	**2.34E-10**

Supplementary Table 5: Fold-change and FDR of TGFβ ligands, receptors and Smads in E9.5 embryos. Related to Supplementary Fig 3a

Gene Symbol	logFC	logCPM	F test	PValue	FDR
Acvr1b	-0.00297	5.920985	0.001889	0.965333	1
Acvr1c	0.908226	-2.3446	0.612829	0.433725	1
Acvr2a	-0.25515	5.02223	8.487752	0.003576	0.204534
Acvr2b	0.083823	6.143701	1.671587	0.196047	1
Bmp3	0.382346	2.90528	5.988188	0.014403	0.47312
Gdf1	0	-3.09946	0	1	1
Gdf10	0.468842	3.59041	13.09545	0.000296	0.035359
Gdf11	0.098421	6.44025	2.555126	0.110308	1
Gdf3	0.941222	-2.45558	0.594161	0.440815	1
Gdf9	-0.04842	-0.90998	0.007578	0.93063	1
Inhba	-2.47035	-2.24152	3.154121	0.075736	1
Inhbb	-1.02822	-0.81389	3.260558	0.070968	1
Inhbc	-1.28999	-2.34447	0.99416	0.318729	1
Inhbe	-1.7414	-2.51445	1.416692	0.23395	1
Mstn	0	-3.09946	0	1	1
Nodal	-0.48628	-0.85153	0.765318	0.38167	1
Smad2	-0.01617	6.417341	0.069777	0.791662	1
Smad3	-0.17317	7.170006	8.360061	0.003836	0.213378
Smad4	0.089875	7.189903	2.292156	0.13003	1
Smad6	-0.01259	5.173936	0.023167	0.879025	1
Smad7	-0.20311	4.41465	3.683037	0.05497	0.893745
Tgfb1	-0.027	4.094806	0.055891	0.813112	1
Tgfb2	0.469983	3.986899	16.42422	5.06E-05	0.009021
Tgfb3	-0.35236	3.069847	5.287401	0.021481	0.595703
Tgfbr1	-0.0735	7.544616	1.53396	0.21552	1
Tgfbr2	-0.00379	3.689988	0.000883	0.9763	1

Supplementary Table 6: All primers used in this study

I. Primers for generating probes for *in situ* hybridization					
Gene	**NCBI Ref_seq**	**Probe region**	**Length**	**Primers**	
Cyp26a1	NM_007811.2	1079-1518	440	F	CATCAAGCTTCGAGAAGAGATAAAGAGCAAGGGCTTAC
				R	GCCCTCTAGAGGAGGTCCATTTAGAAGCTGCCAATC
Msgn1	NM_019544.1	1-565	565	F	ATCCTCTAGACACACTCTGTGGCCTGGGCT
				R	CCTCGGATCCGAAACGCGTGCCACCATGGACAACCTGGGTGAGACCTTC
Myog	NM_031189.2	524-1334	811	F	CAAGCT***ATTTAGGTGACACTATAGA***CCTGTTCCCGGTATCATCAGC
				R	AATTG***TAATACGACTCACTATAGG***GTGCCCAGTGAATGCAACTC
Ski	NM_011385.2	1800-2243	444	F	ACCATGATTACGCCAAGCT***ATTTAGGTGACACTATAGA***CGCACGCATAGGGGTCCACG
				R	AACGACGGCCAGTGAATTG***TAATACGACTCACTATAGG***GCCAAGAAGGAGAAACTGCGGG
Skil	NM_011386.5	1942-2749	808	F	CATCAAGCTTGGTTGCACCGAATGTGTCGCT
				R	GCCCTCTAGATTGGAGGGCTTGGACGACTTGC
Smurf1	NM_001038627.1	1003-1874	872	F	CAAGCT***ATTTAGGTGACACTATAGA***GTGCTCCACGCAGAAGGTATG
				R	AATTG***TAATACGACTCACTATAGG***GACAACAGTGCAGGGACAAG
T	NM_009309	1049-1768	720	F	GCTCGGATCCGCCCTCTAGATGCAGTCCCATGATAACTGGT
				R	GCTCGGATCCACTAGCTAGCAATTCCAGGATTTCAAAGTCACATA
Tbx6	NM_011538.2	612-1363	752	F	CATCAAGCTTGCTCACCAACAGCACACTGGACC
				R	GCCCTCTAGATGGCATAGGGTTACTACACCGAGG
Hoxa10	NM_008263.3	500-959	460	F	GATTACGCCAAGCT***ATTTAGGTGACACTATAGA***ACTCCCTGGGCAGTTCCAAAGG
				R	ACGTTCACCGAATTG***TAATACGACTCACTATAGG***GCGGCTCCTTGCACCATTGAC
Hoxb6	NM_008269.1	18-791	774	F	AATTG***TAATACGACTCACTATAGG***GTTTCGTGAACTCCACCTTCCCCG
				R	CAAGCT***ATTTAGGTGACACTATAGA***CAGCAAGTCCCCAGACTCCC
Hoxb9	NM_008270.2	692-1328	637	F	TTGCGAAGGAAGCGAGGACAAAGA
				R	AGACTGGAAGAGAGGGGCTGGG
Hoxc8	NM_010466.2	34-838	805	F	ACGCCAAGCT***ATTTAGGTGACACTATAGA***AACTGGAACCGGCCTATTACGACTGC
				R	CAGTGAATTG***TAATACGACTCACTATAGG***GCGTGAGAGACTTCAATCCG
Hoxc9	NM_008272.3	808-1425	618	F	AATTG***TAATACGACTCACTATAGG***GCTGTATTCAGTACGTCGTGGGC
				R	CAAGCT***ATTTAGGTGACACTATAGA***TGGGGCAGGGCTTAGGATTG
Hoxc10	NM_010462.5	349-856	508	F	CATCAAGCTTGTCCTCCTGTTCCTACCCACCTAGT
				R	GCCCTCTAGAGCTGTCAGCCAATTTCCTGTGGTGTT
Hoxd9	NM_013555.4	1091-1817	727	F	CATCAAGCTTGATCCACGCTCGCTCCACCC
				R	GCCCTCTAGAGCAGCTGAGTTGGATTTTAGTTGGGAGA
Hoxd10	NM_013554.5	13-742	730	F	CATCAAGCTTAATTACACCGGGAATGTTTTCCTAGCGATGT
				R	GCCCTCTAGACTCTCCACTTGGGAGACTTTAGTGGGC

Note:

1. Restriction enzyme sites used for cloning are underlined.
2. SP6 or T7 RNA polymerase binding sequences are bolded and italic.
3. TA-cloning was used for amplicons generated by primers lacking restriction enzyme sites, SP6 or T7 RNA polymerase binding sequences.

II. Primers and TaqMan probes for qPCR analysis of mouse samples			
Gene	**NCBI Ref_seq**	**Primers & Probes**	
Tbp	NM_013684.3	F	GGCGGTTTGGCTAGGTTT
		R	GGGTTATCTTCACACACCATGA
		Probe	/5SUN/CACTCCTGC/ZEN/CACACCAGCTTCT/3IABkFQ/
Skil	NM_011386.3	F	TGTGACTCAACCTTGGAGAA
		R	GCTCCTGTCTGAGTTCATCT
		Probe	/56-FAM/AGCTGGCAG/ZEN/AACTGAGGCAAAGACT/3IABkFQ/
Smad7	NM_001042660.1	F	CAACCCCCATCACCTTAGTC
		R	GGAGGAAGGTACAGCATCTG
		Probe	/56-FAM/CCCTCCTCC/ZEN/TTACTCCAGATACCCAATGG/3IABkFQ/
Smurf1	NM_001038627.1	F	TTACAGACCCGAGACAGAATAG
		R	GGGCCTGAGTCTTCATACA
		Probe	/56-FAM/TGTTCCTTC/ZEN/GTTCTCCAGCAGCCC/3IABkFQ/
Snai1	NM_011427.3	F	GGAAGCCCAACTATAGCGA
		R	AGGAGAGAGTCCCAGATGA
		Probe	/56-FAM/AGTTCACCT/ZEN/TCCAGCAGCCCTACG/3IABkFQ/
Prtg	NM_175485.4	F	TCCTCATCTGTGTTCTCATCTTG
		R	CCACTCTGCGCTGTCTTAG
		Probe	/56-FAM/TGACTTCCT/ZEN/GGCTTTGCTTCGGT/3IABkFQ/
Hoxb6	NM_008269.1	F	GCAGCGCATGAATTCGTG
		R	GGGTCAGGTAGCGATTATAGTGA
		Probe	/5SUN/TGGTAGCGT/ZEN/GTGTAGGTCTGGC/3IABkFQ/
Hoxb7	NM_010460.2	F	AAATATCCAGCCGCAAGTTC
		R	AAGCAAAGGCGCAAGAAGT
		Probe	GCTCCAGG (Universal Probe Library #1)
Hoxb8	NM_010461.2	F	CACAGCTCTTTCCCTGGATG
		R	GCGAGTCAGATAGGGATTAAATAGG
		Probe	/56-FAM/CGCCAGACC/ZEN/TACAGTCGCTACCA/3IABkFQ/
Hoxb9	NM_008270.2	F	AAGGAAGCGAGGACAAAGAG
		R	TGGTGAGGTACATATTGAACAGAA
		Probe	/5SUN/AGCGTCTGG/ZEN/TATTTGGTGTAGGGACAG/3IABkFQ/
Hoxc9	NM_008272.3	F	CAGCAAGCACAAAGAGGAGA
		R	CGACGGTCCCTGGTTAAATAC
		Probe	/5SUN/AC+C+A+G+A+CG+CT/3IABkFQ/
Hoxc10	NM_010462.5	F	AGTCCAGACACCTCGGATAA
		R	CGCGTCAAATACATATTGAACAGAA
		Probe	/5SUN/AC+C+A+G+A+CG+CT/3IABkFQ/
Hoxd9	NM_013555.4	F	GCAGCAACTTGACCCAAAC
		R	GGGTGAGGTACATGTTGAAGAG
		Probe	/5SUN/AC+C+A+G+A+CG+CT/3IABkFQ/
Hoxa10	NM_008263.3	F	CCCCTTCAGAAAACAGTAAAGC
		R	TCGAGTAAGGTACATGTTGAATAGA
		Probe	/5SUN/AC+C+A+G+A+CG+CT/3IABkFQ/

III. Primers and TaqMan probes for qPCR analysis of human samples			
Gene	**NCBI Ref_seq**	**Primers & Probes**	
RPL13A	NM_012423.4	F	CAAGCGGATGAACACCAAC
		R	TGTGGGGCAGCATACCTC
		Probe	CCAGCCGC (Universal ProbeLibrary #28)
POU5F1	NM_002701.6	Predesigned qPCR Assays from Integrated DNA Technologies Hs.PT.58.14494169.g	
MEOX1	NM_004527.4	Predesigned qPCR Assays from Integrated DNA Technologies Hs.PT.58.26021003.g	
MIXL1	NM_031944.3	Predesigned qPCR Assays from Integrated DNA Technologies Hs.PT.58.4553924	
MSGN1	NM_001105569.3	Predesigned qPCR Assays from Integrated DNA Technologies Hs.PT.58.24616347.g	
PRTG	NM_173814.6	F	GGAGCCCTGTAGTCTACCATTC
		R	TTAATGTCACTTTTACTCCAACTGGT
		Probe	GCCTGCTG (Universal Probe Library #40)
TBX6	NM_004608.4	F	AGCTCTGTGGGAACAGAAATG
		R	ACCGGAATCACATCCAGAAG
		Probe	CATCACCA (Universal Probe Library #9)
TBXT	NM_003181.4	F	TTCAAGGAGCTCACCAATGA
		R	GAAGGAGTACATGGCGTTGG
		Probe	GGCAGGAG (Universal Probe Library #51)
HOXB1	NM_002144.4	F	CGACGAATGAAGCAGAAGAA
		R	AGTTCAGGAGGTGACAGAG
		Probe	AGCTGGAG (Universal Probe Library #78)
HOXB4	NM_024015.5	F	TTCACTACAACCGCTACCT
		R	GGGCAACTTGTGGTCTTT
		Probe	CTCTGCCT (Universal Probe Library #13)
HOXB6	NM_018952.5	F	CGAGACAGAAGAGCAGAAGT
		R	CGTCAGGTAGCGATTGTAGT
		Probe	/5SUN/AC+C+A+G+A+CG+CT/3IABkFQ/
HOXB9	NM_024017.5	F	AAGGAAGCGAGGACAAAGAG
		R	TGGTGAGGTACATATTGAACAGAA
		Probe	/5SUN/AC+C+A+G+A+CG+CT/3IABkFQ/
HOXC9	NM_006897.3	F	CAGCAAGCACAAAGAGGAGA
		R	CGACGGTCCCTGGTTAAATAC
		Probe	/5SUN/AC+C+A+G+A+CG+CT/3IABkFQ/
HOXC10	NM_017409.4	F	AGACACCTCGGATAACGAA
		R	CGCGTCAAATACATATTGAACAGAA
		Probe	/5SUN/AC+C+A+G+A+CG+CT/3IABkFQ/

III. Primers and TaqMan probes for qPCR analysis of human samples (continued)			
Gene	**NCBI Ref_seq**	**Primers & Probes**	
HOXC11	NM_014212.4	F	GTCATCTCGCCTTCCCAAAT
		R	AACATCGTTCTCCTCTCCTG
		Probe	GGATGGAG (Universal Probe Library #58)
HOXA10	NM_018951.4	Predesigned qPCR Assays from Integrated DNA Technologies Hs.PT.58.39472759.g	
HOXA11	NM_005523.6	F	GGCAGCAGAGGAGAAAGAG
		R	CTCGGATCTGGTACTTGGTATAG
		Probe	CAGCAGCC (Universal Probe Library #66)
HOXD9	NM_014213.4	F	GCAGCAACTTGACCCAAAC
		R	GGGTGAGGTACATGTTGAAGAG
		Probe	/5SUN/AC+C+A+G+A+CG+CT/3IABkFQ/
HOXD10	NM_002148.4	F	GCCTAGAGATCAGTAAGAGCGTTA
		R	TTCTCTCGGCTCATCTTCTTGAG
		Probe	ACAGGCAG (Universal Probe Library #96)
HOXD11	NM_021192.3	Predesigned qPCR Assays from Integrated DNA Technologies Hs.PT.58.585834.g	
HOXB13	NM_006361.6	F	CAAGAAACGCATTCCGTACA
		R	GTCCTTGGTGATGAACTTGTTAG
		Probe	AGCTGGAG (Universal Probe Library #78)
SOX2	NM_003106.4	F	CAAGACGCTCATGAAGAAGGATAAG
		R	TTCATGTGCGCGTAACTGTC
		Probe	CAGCAGCC (Universal Probe Library #66)

Note: Locked nucleic acids are labeled as +N

IV. Primers for SYBR qPCR analysis of human samples			
Gene	**NCBI Ref_seq**	**Primers**	
RPL13A	NM_012423.4	F	CAAGCGGATGAACACCAAC
		R	TGTGGGGCAGCATACCTC
PRTG	NM_173814.6	F	GGAGCCCTGTAGTCTACCATTC
		R	TTAATGTCACTTTTACTCCAACTGGT
TBX6	NM_004608.4	F	AGCTCTGTGGGAACAGAAATG
		R	ACCGGAATCACATCCAGAAG
TBXT	NM_003181.4	F	TTCAAGGAGCTCACCAATGA
		R	GAAGGAGTACATGGCGTTGG
HOXA1	NM_005522.5	F	CTTCTCCAGCGCAGACTTT
		R	GTAGCCGTACTCTCCAACTTTC
HOXA4	NM_002141.5	F	CCTGGATGAAGAAGATCCATGTC
		R	GGGTCAGGTATCGATTGAAGTG
HOXA7	NM_006896.4	F	GAGGCCAATTTCCGCATCTA
		R	CGGTTGAAGTGGAACTCCTT

IV. Primers for SYBR qPCR analysis of human samples (continued)			
HOXA11	NM_005523.6	F	GGCAGCAGAGGAGAAAGAG
		R	CTCGGATCTGGTACTTGGTATAG
HOXB1	NM_002144.4	F	CGACGAATGAAGCAGAAGAA
		R	AGTTCAGGAGGTGACAGAG
HOXB4	NM_024015.5	F	CTGGATGCGCAAAGTTCAC
		R	AATTCCTTCTCCAGCTCCAAG
HOXB7	NM_004502.4	F	CCGAGAGTAACTTCCGGATCTA
		R	CGTCAGGTAGCGATTGTAGTG
HOXB9	NM_024017.5	F	AAGGAAGCGAGGACAAAGAG
		R	TGGTGAGGTACATATTGAACAGAA
HOXC4	NM_153633.3	F	AGCAAGCAACCCATAGTCTAC
		R	GACTTGCTGCCGGGTATAG
HOXC6	NM_004503.4	F	CAGGACCAGAAAGCCAGTATC
		R	TCTGGTACCGCGAGTAGAT
HOXC9	NM_006897.3	F	CAGCAAGCACAAAGAGGAGA
		R	CGACGGTCCCTGGTTAAATAC
HOXC10	NM_017409.4	F	AGACACCTCGGATAACGAA
		R	CGCGTCAAATACATATTGAACAGAA
HOXC11	NM_014212.4	F	GTCATCTCGCCTTCCCAAAT
		R	AACATCGTTCTCCTCTCCTG
HOXD1	NM_024501.3	F	TCGAGTGGATGAAAGTGAAGAG
		R	CAGTTGCTTGGTGCTGAAAT
HOXD4	NM_014621.3	F	GGGCCTACAGTCAGTCCGA
		R	GACTTGCTGCCGGGTGTAG
HOXD8	NM_019558.4	F	GAGGACCCAGACCACTTAAATC
		R	AGCGACTGTAGGTTTGTCTTC
HOXD10	NM_002148.4	F	GCCTAGAGATCAGTAAGAGCGTTA
		R	TTCTCTCGGCTCATCTTCTTGAG

V. Primers for the T7E1 assay			
Name	**NCBI Ref_seq**	**Primers**	
PRTG_seq	NC_000015.10:55740068-55740729	F	TGCCTGTAATTCTGGATGTGC
		R	TGTTGCGTTGGCCAAACTTC
		Amplicon size	662 bp
Off-target_1	NC_000005.10:72151705-72152393	F	ATAGGTGTGGTCCCAAACTGG
		R	CAGACCTGGTGTCAGTCCAC
		Amplicon size	689 bp
Off-target_2	NC_000023.11:113723014-113723950	F	TCCCACATTAAAGGGCCTCC
		R	AGAATAAGGCACAGAGGGGC
		Amplicon size	937 bp
Off-target_3	NC_000008.11:78516714-78517117	F	AGTGCCAGATTCGCGTAGAC
		R	GTCTGTGAATGGGCACAGC
		Amplicon size	404 bp

VI. Primers for mouse genotyping			
Name	**NCBI Ref_seq**	**Primers**	
Prtg_GT	NC_000075.7:72749989-72750313	F	TTGAAGTCCATCCAGTGTCTACT
		R1 (WT)	ACTCTCCAGCCACAGTGAT
		R2 (KO)	CGGAATTCGATAGCTTGGCG
		Amplicon size	WT = 325 bp
			KO = 160 bp

VII. Anchored oligo dT(20)VN			
Sequence	TTTTTTTTTTTTTTTTTTTVN		

VIII. Primers and oligonucleotides for cloning			
Gdf11	F	(HindIII)	AGGT**AAGCTT**GCCACCATGGTGCTCGCGGCCCCGCT
	R	(XbaI)	CTCC**TCTAGA**AGAGCAGCCACATCGATCCACCACCATGCCA
	R(WT)	(XbaI)	CTCC**TCTAGA**TCAAGAGCAGCCACATCGATCCACCA
Inhba	F	(AarI)	AACATTTG**CACCTGC**TCTTAGCTGCCACCATGCCCTTGCTTTGGCTGAGA
	R	(AarI)	GTCAGTCT**CACCTGC**TATTCTAGAGCAGCCACACTCCTCCACAATC
	R(WT)	(XbaI)	ACTC**TCTAGA**TCCATTTTCTCTGGGACCTG
Tgfb1	F	(HindIII)	AGGT**AAGCTT**GCCACCATGCCGCCCTCGGGGCTGCGGCT
	R	(XbaI)	CTCC**TCTAGA**GCTGCACTTGCAGGAGCGCAC
	R(WT)	(XbaI)	CTCC**TCTAGA**TCAGCTGCACTTGCAGGAGCG
Pcsk5	F	(HindIII)	AGGT**AAGCTT**GCCACCATGGACTGGGACTGGGG
	R	(XbaI)	CTCC**TCTAGA**GCCTTGGAATGTACATGTTTTGC
shPrtg	F	GCTGAACAATAACACTAGAATAACTTTTTTGGCCTCTGACTGAAAGTTATTCTAGGGTTAATGTAC	
	R	TCCTGTACATTAACCCTAGAATAACTTTCAGTCAGAGGCCAAAAAAGTTATTCTAGTGTTATTGTT	
shScramble	F	GCTGGACTAAACCAAACAATAATATTTTTTGGCCTCTGACTGAAATATTATTGTTGGGTTAAGTAC	
	R	TCCTGTACTTAACCCAACAATAATATTTCAGTCAGAGGCCAAAAAATATTATTGTTTGGTTTAGTC	

Supplementary Table 7: Antibodies used in this study

Western blotting	Host	Target species	Dilution	Source	Cat. No.	RRID
Prtg [1D5]	Mouse	Mouse	1:2500	Homemade	N/A	N/A
Prtg [2B3]	Mouse	Human	1:5000	GeneTex	GTX83789	AB_10731789
Prtg [OTI2B3]	Mouse	Human	1:5000	OriGene	TA501394	AB_11126435
Phospho-Smad2 (Ser465/Ser467)	Rabbit	Mouse	1:2000	Cell Signaling Technology	18338	AB_2798798
Phospho-Smad2 (phospho Ser465/Ser467)	Rabbit	Human	1:5000	GeneTex	GTX133614	AB_2887051
Smad2/3	Mouse	Human	1:1000	Santa Cruz Biotechnology	sc-133098	AB_2193048
Smad2	Rabbit	Mouse	1:5000	Cell Signaling Technology	5339	AB_10626777
Smad4	Rabbit	Human, Mouse	1:5000	Cell Signaling Technology	38454	AB_2728776
HOXC10	Rabbit	Human	1:5000	GeneTex	GTX118025	AB_11170594
Oct4	Rabbit	Human	1:5000	GeneTex	GTX101497	AB_10618784
GDF11	Rabbit	Mouse	1:2000	Abcam	ab124721	AB_10974143
GAPDH	Mouse	Human, Mouse	1:10000	Thermo Fisher Scientific	AM4300	AB_2536381
Flag tag antibody	Rabbit	FLAG tag	1:10000	Sigma-Aldrich	F7425	AB_439687
HA tag antibody	Rabbit	HA tag	1:10000	Proteintech	51064-2-AP	AB_11042321
Goat Anti-Mouse IgG Polyclonal antibody, HRP Conjugated	Goat	Mouse	1:10000	Millipore	AP124P	AB_90456
Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP	Goat	Rabbit	1:10000	Thermo Fisher Scientific	31460	AB_228341
Whole-mount immunostaining	**Host**	**Target species**	**Dilution**	**Source**	**Cat. No.**	**RRID**
Prtg [1D5]	Mouse	Mouse	1:100	Homemade	N/A	N/A
Mouse Protogenin Antibody	Sheep	Mouse	1:100	R&D Systems	AF4919	AB_2172305
Phospho-Smad2 (phospho S467)	Rabbit	Mouse	1:500	Abcam	ab280888	N/A
HOXC10	Rabbit	Mouse	1:2000	GeneTex	GTX118025	AB_11170594
Smad2/3	Rabbit	Mouse	1:1000	Cell Signaling Technology	8685	AB_10889933
F(ab')2-Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 647	Goat	Rabbit	1:400	Invitrogen	A48285	AB_2896349
Donkey anti-Sheep IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	Donkey	Sheep	1:600	Invitrogen	A21436	AB_2535857
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	Donkey	Rabbit	1:600	Invitrogen	A31573	AB_2536183
Streptavidin, Alexa Fluor 568 conjugate	N/A	Biotin	1:400	Invitrogen	S11226	N/A

Antibodies used in this study (continued)						
Immunofluorescence	**Host**	**Target species**	**Dilution**	**Source**	**Cat. No.**	**RRID**
Human TBX6 Antibody	Goat	Human	1:500	R&D Systems	AF4744	AB_2200834
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 555	Donkey	Goat	1:1000	Invitrogen	A21432	AB_2535853
Flow cytometry	**Host**	**Target species**	**Dilution**	**Source**	**Cat. No.**	**RRID**
Human TBX6 Antibody	Goat	Human	1:300	R&D Systems	AF4744	AB_2200834
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 555	Donkey	Goat	1:300	Invitrogen	A21432	AB_2535853