Protein Arginine Methyltransferase PRMT1 Is Essential for Palatogenesis

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Appendix

SUPPLEMENTAL MATERIALS AND METHODS

Skeletal staining

Skeletal staining of the heads of *Wnt1-Cre;Prmt1*$^{fl/fl}$ and control mice was performed using a modified Alcian Blue-Alizarin Red S staining protocol. Briefly, newborns or E17.5 mice were sacrificed then fixed in 4% PFA, followed by a 1 h wash in double-distilled (dd) H_2O and post-fixation in 70% ethanol. The skin and internal organs were carefully removed. Next, the skeletons were stained with 0.02% Alcian Blue 8GX for 2 days, followed by washing with ethanol/glacial acetic acid (7:3) for 1 h. Then, they were soaked in 100% ethanol overnight followed by ddH_2O for 1 day. Once the cartilage was obviously detectable, Alizarin Red staining was performed overnight. Finally, the samples were treated with a KOH/glycerol series and stored in glycerol (Parada, Li et al. 2013, Parada, Han et al. 2015).

MicroCT scan and 3D reconstruction

Mouse heads were dissected from euthanized control and *Wnt1-Cre;Prmt1*$^{fl/fl}$ newborn mice, and fixed in 4% paraformaldehyde (PFA) on a shaker at 4°C overnight. The heads were scanned by a micro-computed tomography (micro-CT) system (Scanco Medical; V1.2a) as previously described (Parada et al. 2013). Visualization and 3D micro-CT reconstruction of the heads were performed using Isosurface parameters in Avizo 7 (Visualization Sciences Group).

Analysis of cell proliferation and apoptosis

To evaluate cell proliferation within the palate, intraperitoneal 5-bromo-2′-deoxyuridine (BrdU, Sigma) injection (100 mg/kg body weight) was given at E13.5 or E14.5. Two hours after injection, mice were sacrificed and embryos were fixed in 4% PFA and processed for immunohistochemistry (Parada et al. 2015). Detection of BrdU-labeled cells was carried out in

paraffin section using a BrdU labeling and detection kit (ab125306) according to the manufacturer's protocol. The numbers of BrdU-positive cells and total number of cells within the palatal mesenchyme, Meckel's cartilage, and mandible were counted in three continuous sections per sample. Apoptosis assays were performed using TUNEL staining (Invitrogen, cat# C10617) of frozen sections according to manufacturer's protocol.

Histological analysis

For histological analysis, mouse heads were fixed in 4% PFA overnight, embedded in paraffin and processed for serial sectioning using standard procedures. For morphology examination, deparaffinized sections were stained with hematoxylin and eosin (H&E) using standard protocols (Li et al. 2011).

Immunohistochemistry

TSA Plus Cyanine 3 kit (PerkinElmer, NEL744001KT) was used to amplify signals of phospho-Smad2, phospho-Smad3, phospho-Smad1/5/9. Staining proceeded according to routine protocol (Parada et al. 2015). Antibodies used for immunohistochemistry included Myod1 (Abcam, cat# ab203383; 1:50), Myogenin (DSHB, cat# F5D; 1:20), MHC (DSHB, cat# MF20; 1:10), Sp7/osterix (Abcam, cat# ab22552; 1:100), Cyclin D1 (Abcam, cat# ab16663; 1:100), phospho-Smad2 (Cell signaling, cat# 3108; 1:100), phospho-Smad3 (Cell signaling, cat# 9520; 1:100), phospho-Smad1/5/8 (Cell signaling, cat# 13820; 1:100) and active β-Catenin (Cell signaling, cat# 19807; 1:100).Alexa Fluor 488 and 594 fluorescent secondary antibodies (Invitrogen Life Technologies; 1:400) were used. Sections were counterstained with DAPI and imaged by fluorescence microscopy.

Western blotting

The total protein concentration in the palates was determined by comparison with BSA standards. Twenty micrograms of total protein from each sample were loaded in each well of a 10% polyacrylamide gel. Western analysis was carried out as previously described (Iwata, Tung et al. 2012). Antibodies used for western blot were phospho-Smad1/5/9 (Cell signaling, cat# 13820; 1:1000), phospho-Smad2 (Cell signaling, cat# 3108; 1:1000), phospho-Smad3 (Cell signaling, cat# 9520; 1:1000), Smad1 (Cell signaling, cat# 6944; 1:1000), Smad2 (Cell signaling, cat# 5339;

1:1000), Smad3 (Cell signaling, cat# 9523; 1:1000), active β-Catenin (Cell signaling, cat# 19807; 1:1000), Axin 2 (Abcam, cat# ab32197; 1:1000), Histone H4 (Active Motif, cat# 61299; 1:1000), Histone H4R3me2a (Abcam, cat# ab129231; 1:1000) and α-Tubulin (Sigma, cat# T9026; 1:1000).

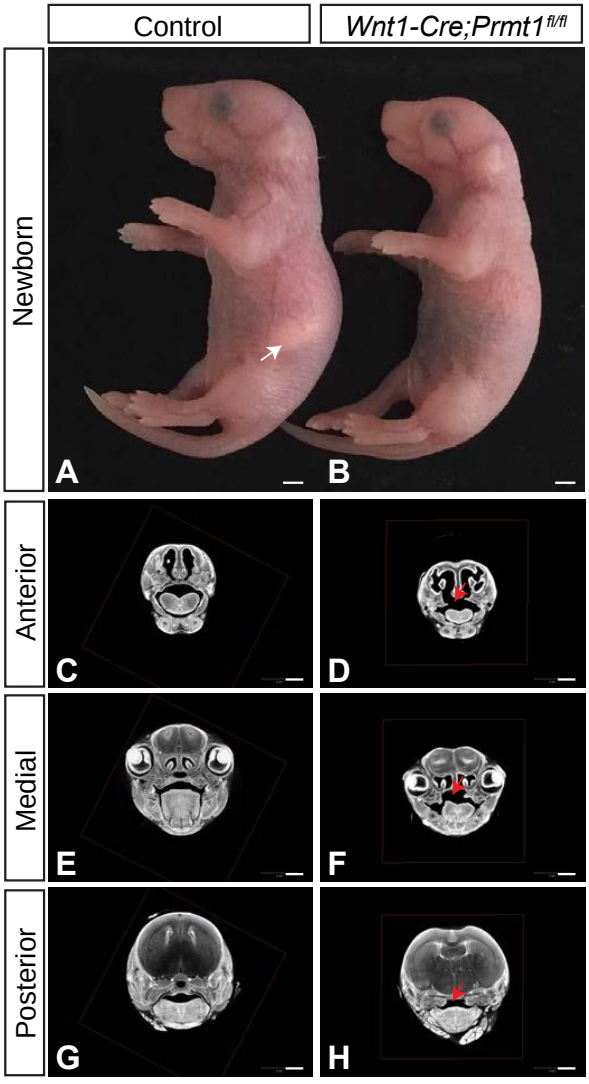
RNA-sequencing analysis

Total RNA isolated from palatal shelves at E14.5 was extracted using TRIzol (Thermo Fisher Scientific). Total RNA was enriched for poly(A) RNA and fragmented for library construction. RNA fragments were reverse transcribed followed by A-tailing and index adapter ligation, then denatured and amplified on cBot. Library analysis and quality checks were performed on a LabChip GX. Sequencing was performed on a HiSeq 4000 (Illumina) with paired end reads of 100 bp. Analysis was performed using Partek Flow software (Partek Inc.). Reads were first trimmed to remove adaptor sequences and then mapped to the mouse genome (mm10) using STAR (Version 2.4.1d). Aligned reads were then quantified using Partek E/M. Differential expressed genes in each condition were identified using gene set analysis (GSA), and further analyzed using Partek Flow and Ingenuity Pathway Analysis (IPA). The raw data has been deposited with NCBI under accession number GSE114894.

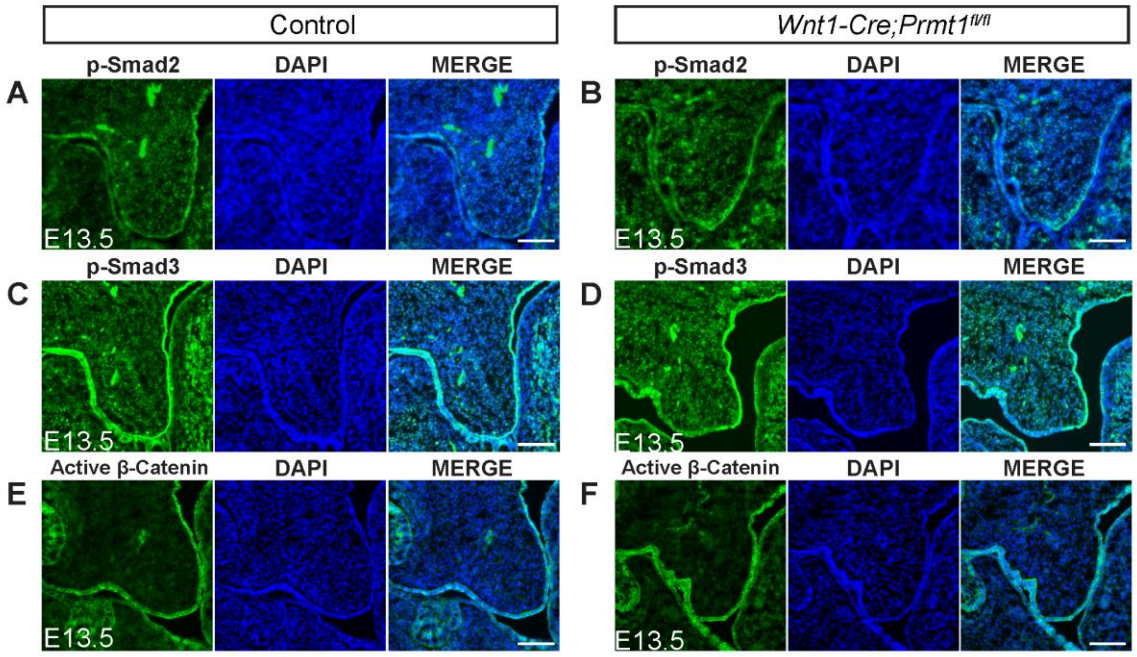
Statistical analysis

Two-tailed Student's t-tests were applied for statistical analysis. For all graphs, data are represented as mean ± s.d. $P<0.05$ was considered statistically significant.

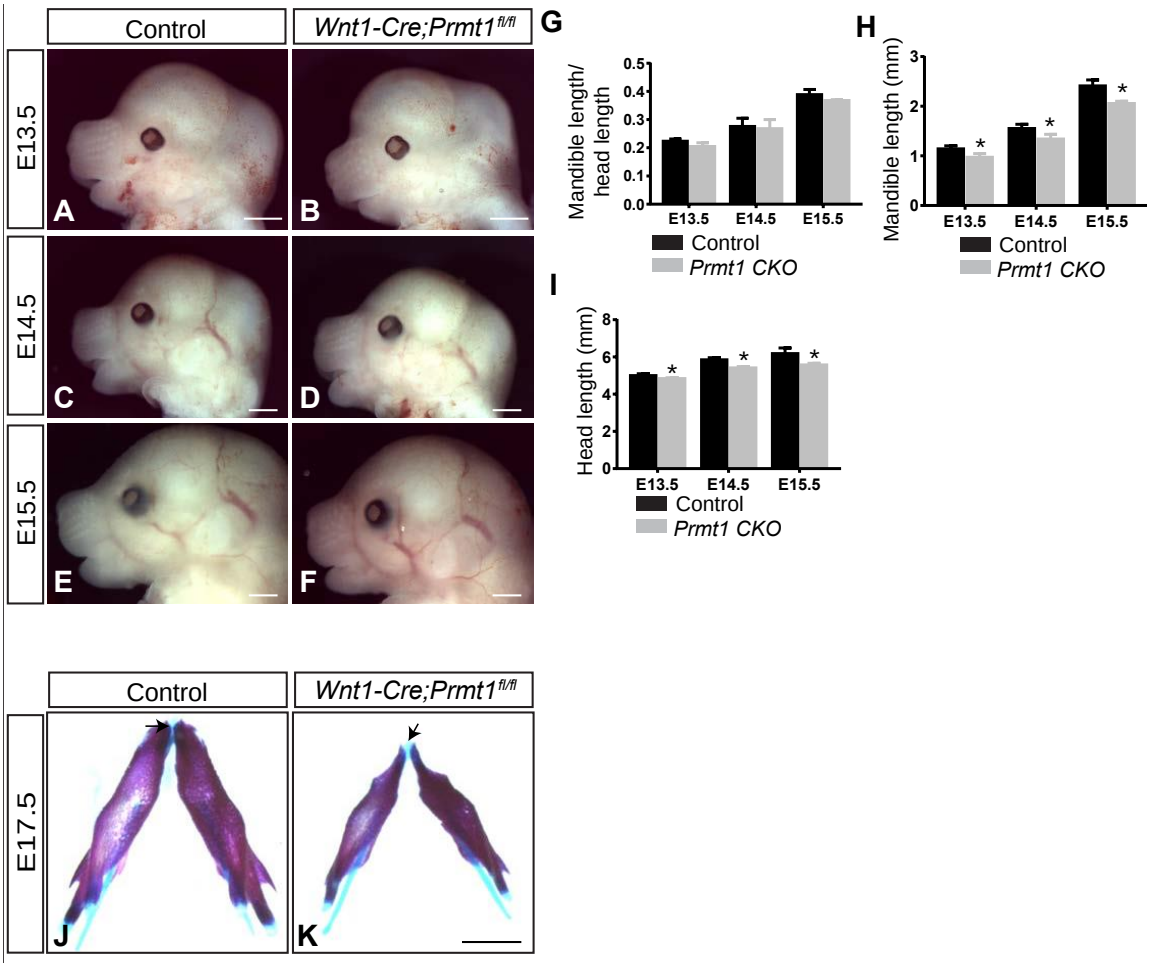
Appendix Figure 1. ***Wnt1-Cre;Prmt1*$^{fl/fl}$ mice die at postnatal day 0 and display cleft palate.** (A,B) Macroscopic views of control and *Wnt1-Cre;Prmt1*$^{fl/fl}$ newborns. Arrow indicates milk in the stomach of the control pup, which is absent in the mutant pup. (C-H). Sections from microCT scan of anterior, medial, and posterior regions of control and *Wnt1-Cre;Prmt1*$^{fl/fl}$ mouse heads at newborn stage. Red arrows indicate cleft palate. n=4.



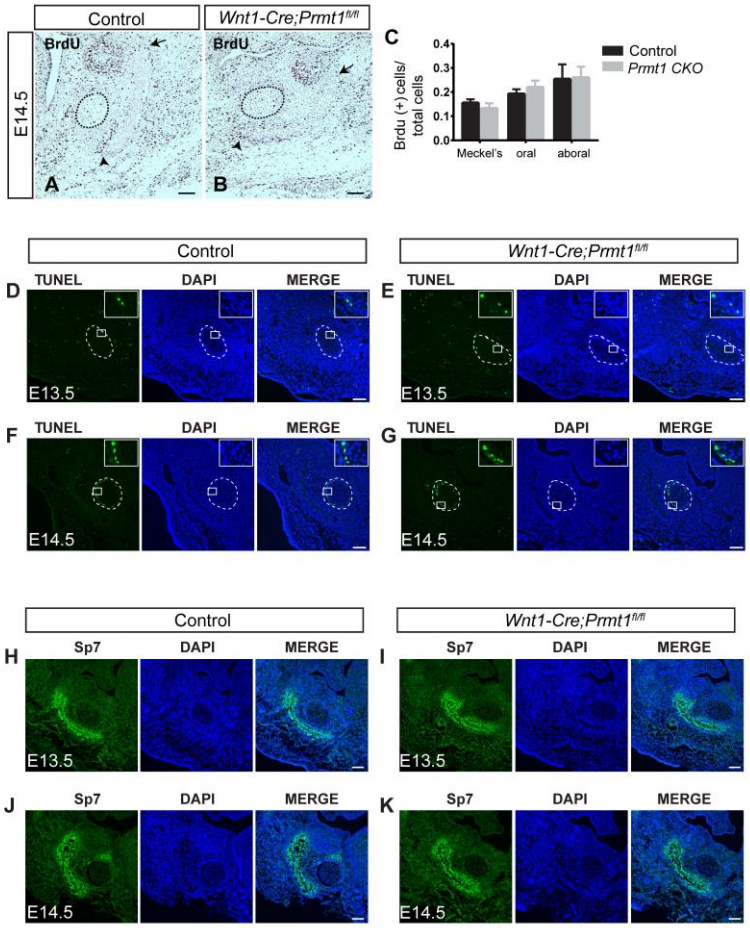
Appendix Figure 2. TGFβ and WNT signaling activation are not altered in *Wnt1-Cre;Prmt1$^{fl/fl}$* mice. (A-F) Immunostaining (green) of control and *Wnt1-Cre;Prmt1$^{fl/fl}$* palates at E13.5 for: p-Smad2 (A,B), p-Smad3 (C,D), and active β-catenin (E,F). Data shown are representative of three independent samples for each staining and Western. Scale bar = 100 μm.



Appendix Figure 3. **Mandibular size decreases proportionally to the head size in *Wnt1-Cre;Prmt1$^{fl/fl}$* mice.** (A-F) Heads of control and *Wnt1-Cre;Prmt1$^{fl/fl}$* embryos at (A, B) E13.5, (C, D) E14.5, and (E, F) E15.5. (G) Mandible length to head length ratio at E13.5, E14.5 and E15.5. n=4. *P<0.05. (H) The length of control and *Wnt1-Cre;Prmt1$^{fl/fl}$* mandibles at E13.5, E14.5 and E15.5. n=4. *P<0.05. (I) The length of control and *Wnt1-Cre;Prmt1$^{fl/fl}$* heads at E13.5, E14.5 and E15.5. n=4. *P<0.05. (J, K) The *Wnt1-Cre;Prmt1$^{fl/fl}$* mandibles exhibit a normal symphysis (indicated by black arrows) of Meckel's cartilage. n=3.



Appendix Figure 4. **Osteogenic progenitor formation in the mandible is unaffected in *Wnt1-Cre;Prmt1$^{fl/fl}$* mice.** (A-B) BrdU staining (dark brown) of control and *Wnt1-Cre;Prmt1$^{fl/fl}$* mandibles at E14.5. Dashed line indicates Meckel's cartilage. Arrowheads and arrows indicate oral side and aboral side of osteogenic front of mandibles, respectively. (C) Quantification of cell proliferation in A & B. n=4. *P<0.05. (D-G) TUNEL staining (green) of E13.5 (D, E) and E14.5 (F, G) control and *Wnt1-Cre;Prmt1$^{fl/fl}$* mandibles. Arrowheads indicate positive staining in Meckel's cartilage. n=4. High magnification of TUNEL-positive cells in the white square was shown in right upper corner of D-G. (H-K) Sp7 immunostaining (green) of control and *Wnt1-Cre;Prmt1$^{fl/fl}$* mandibles at E13.5 (H, I) and E14.5 (J, K). n=4. Scale bar = 100μm in A-B, D-K.



Appendix Figure 5. **Muscle differentiation of the tongue is unaffected in *Wnt1-Cre;Prmt1$^{fl/fl}$* mice.** (A, B) MyoD and (C, D) Myogenin immunostaining (green) of control and *Wnt1-Cre;Prmt1$^{fl/fl}$* tongues at E13.5. n=4. (E, F) MHC immunostaining (green) of control and *Wnt1-Cre;Prmt1$^{fl/fl}$* tongues at E15.5. n=4. Scale bar =100 μm.