

Figure S1. The local landscape of *Pitx1* interactions. The TAD that encompasses *Pitx1* is shown in black as is the ChIA-PET interaction between the *Pitx1* promoter and the PDE identified by DeMare *et al.* (2013). Hindlimb 4C signal tracks in wildtype and *PDE*^{-/-} mice are shown in blue; FourCSeq peak calls are shown below each track. H3K27ac (green) profiles in E11.5 hindlimb from wild type and *PDE*^{-/-} mice are also shown. Enriched regions identified for each mark in each tissue are shown below each signal track. Distal *Pitx1* promoter interactions identified by Capture-C (Andrey *et al.* 2017) are shown in blue below the hindlimb 4C signal track. Regions enriched for H3K27ac in mouse E11.5 forelimb and hindlimb identified by Cotney *et al.* (2012) are shown in dark and light teal, respectively. Enhancers predicted to be active in embryonic mouse limb are shown in orange (Monti *et al.* 2017). The enhancer located in a deletion associated with feathered feet in pigeons is boxed (Domyan *et al.* 2016).

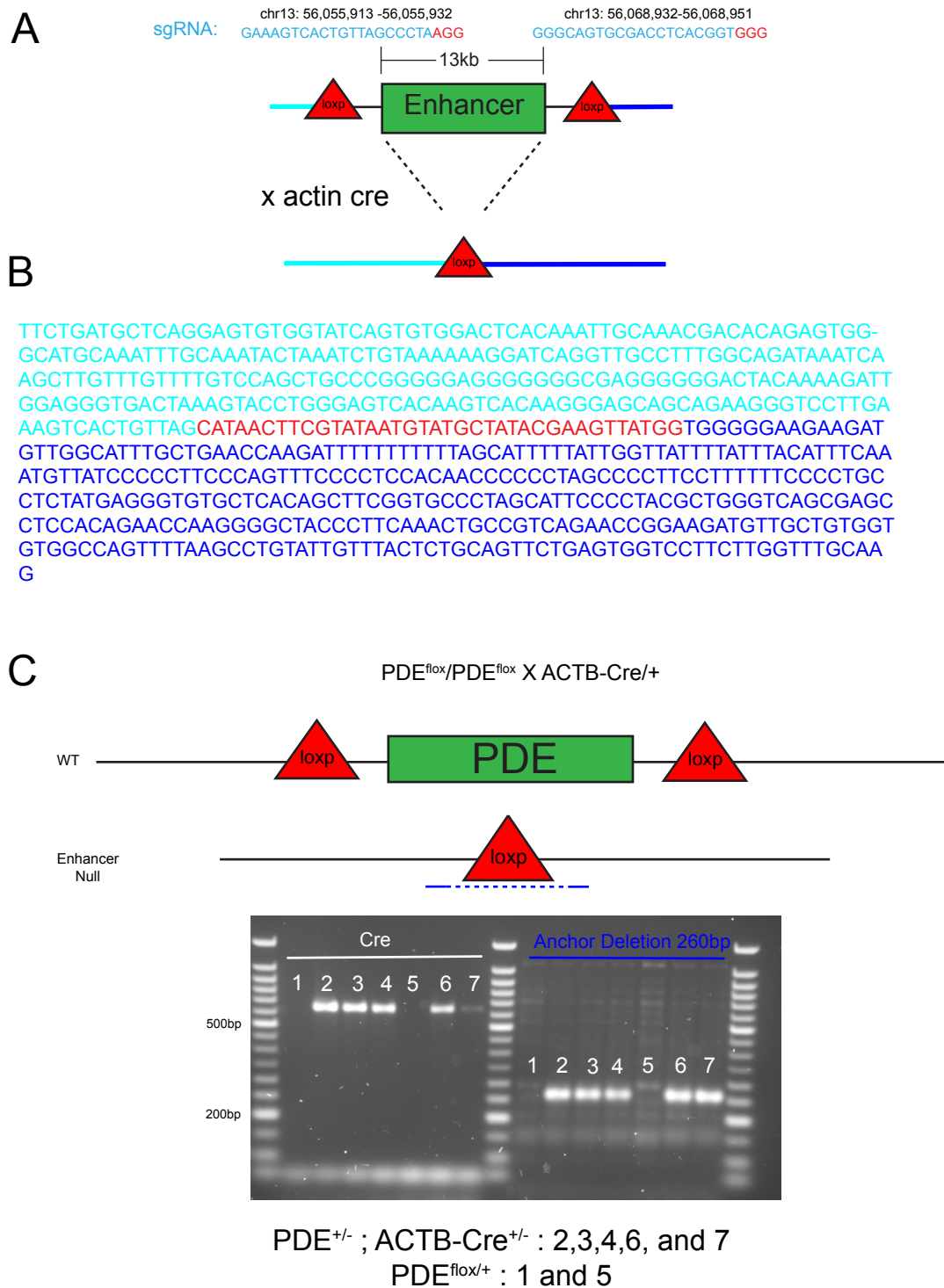


Figure S2. Generation and validation of the PDE deletion allele.

A. Top. Schematic illustrating the location of the PDE (green) and flanking loxP sites (red). The sgRNA sequences used for genome editing are shown at the top of the figure with the PAM sites highlighted in red (chr13: 56,055,913 -56,055,932 ; chr13: 56,068,932-56,068,951; mm9). **Bottom.** Mice with a floxed PDE allele were crossed with Actin-Cre mice to generate the constitutive PDE knockout line (also see panel C). **B.** Validation of the PDE deletion allele using Sanger sequencing. After Cre recombination, a single loxP sequence (in red) remains. Genomic sequence immediately 5' of the PDE is shown in light blue (chr13: 56,055,668-56,055,927), and sequence immediately 3' of the PDE is shown in dark blue (chr13: 56,068,951-56,069,282). **C.** PCR genotyping to identify PDE^{+/-} offspring resulting from the PDE^{flox/flox} x ACTb-cre^{+/+} cross used to generate the PDE constitutive knockout line. A 260 bp PCR amplicon (in blue) spanning the remaining loxP site was used to detect PDE deletion alleles. A 680 bp PCR amplicon was used to detect the presence of the ACTb-cre allele. PDE deletion alleles were only detected in mice carrying ACTb-cre (lanes 2, 3, 4, 6 and 7).

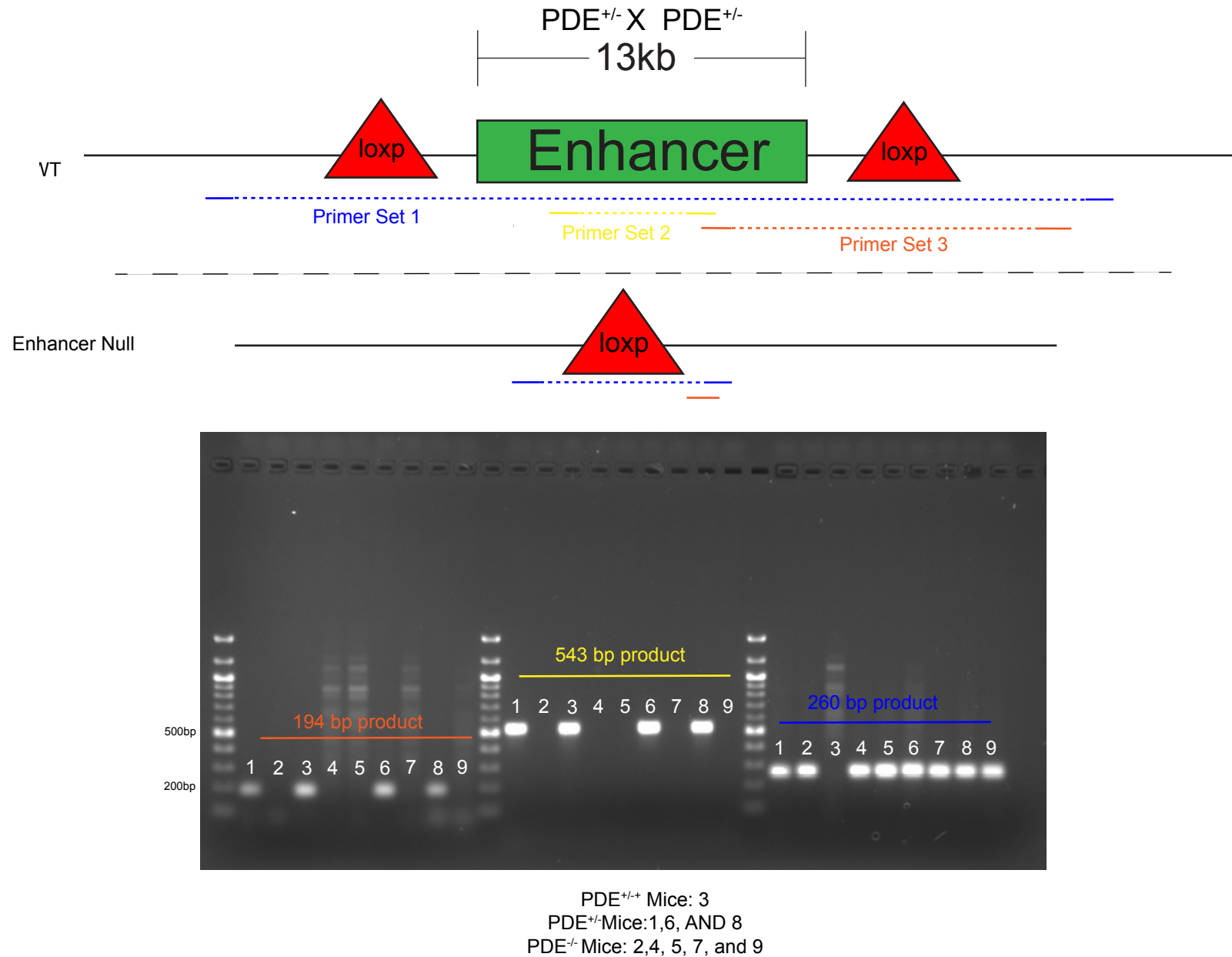


Figure S3. Genotyping strategy.

A. Schematic representation of the three PCR primer sets used to distinguish wild type, heterozygous, and homozygous PDE deletion mice. Primer set 1 (blue) spans the 13 kb PDE and produces a 260 bp product in PDE^{+/-} and PDE^{-/-} mice, but not wild type mice. Primer set 2 (yellow) is located within the PDE and produces a 543 bp product in wild type and PDE^{+/-} mice, but not PDE^{-/-} mice. Primer set 3 (orange) spans the junction between the PDE and flanking genomic sequence and produces a 194 bp product in wild type and PDE^{+/-} mice, but not PDE^{-/-} mice. B. Representative genotyping assay; lanes corresponding to mice of each genotype are indicated below the gel image.

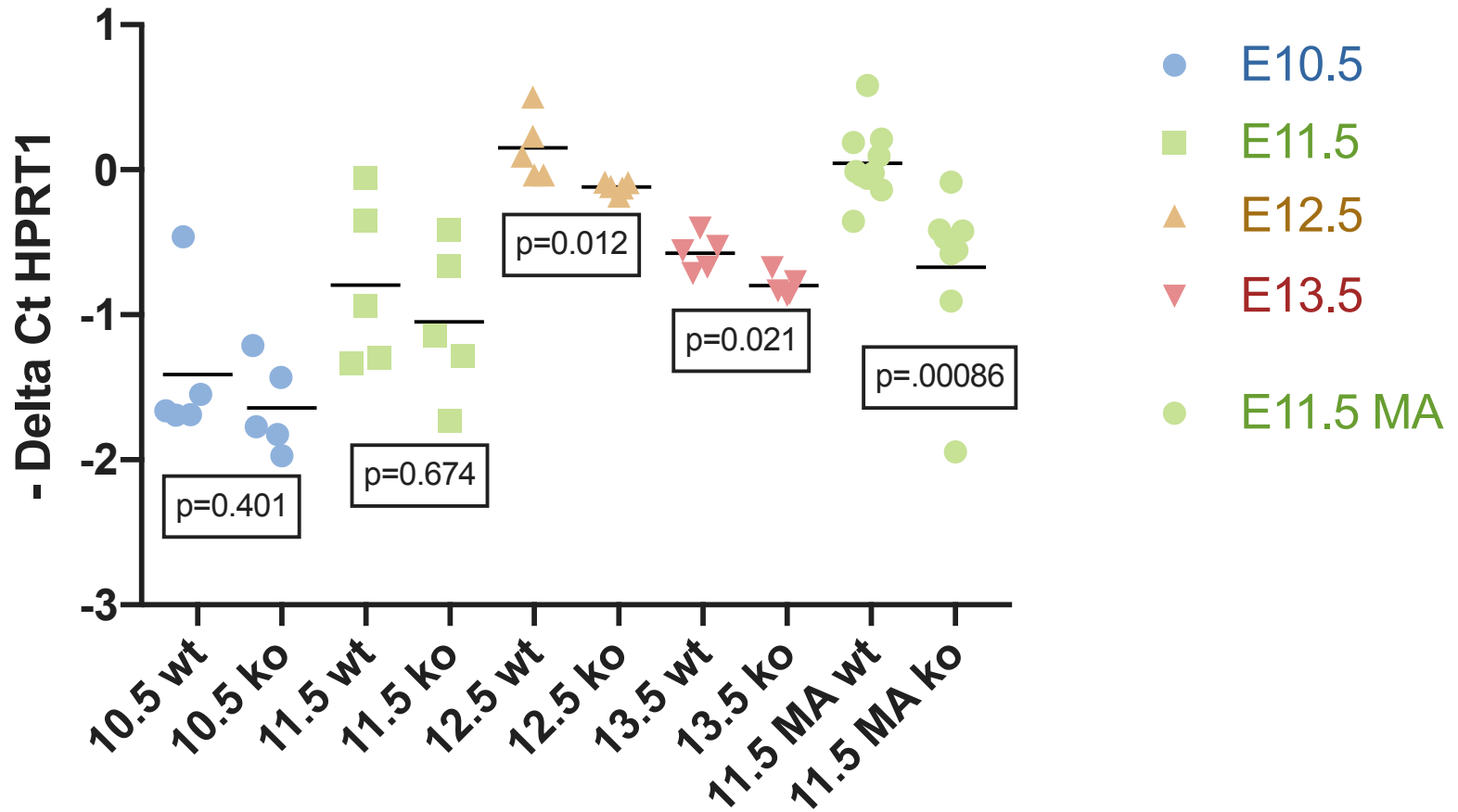


Figure S4. Comparing *Pitx1* expression in hindlimb and mandibular arch in litter-matched wild type and *PDE*^{-/-} mice.

Pitx1 expression was compared across samples using *Hprt1* expression as an internal reference. The vertical axis shows $-(Ct)$ values, calculated as $-(Ct_{Pitx1} - Ct_{HPRT1})$. Ct values were converted to $-(Ct)$ values for purposes of illustration, but were not used for statistical testing. Each data point at each developmental stage represents a litter-matched, one-to-one measure of *Pitx1* expression in wild type ('wt') or *PDE*^{-/-} ('ko') mouse. These measurements were used to generate the summary data shown in Figure 2. Differential expression P values were calculated for each tissue and timepoint using a Mann-Whitney U test.

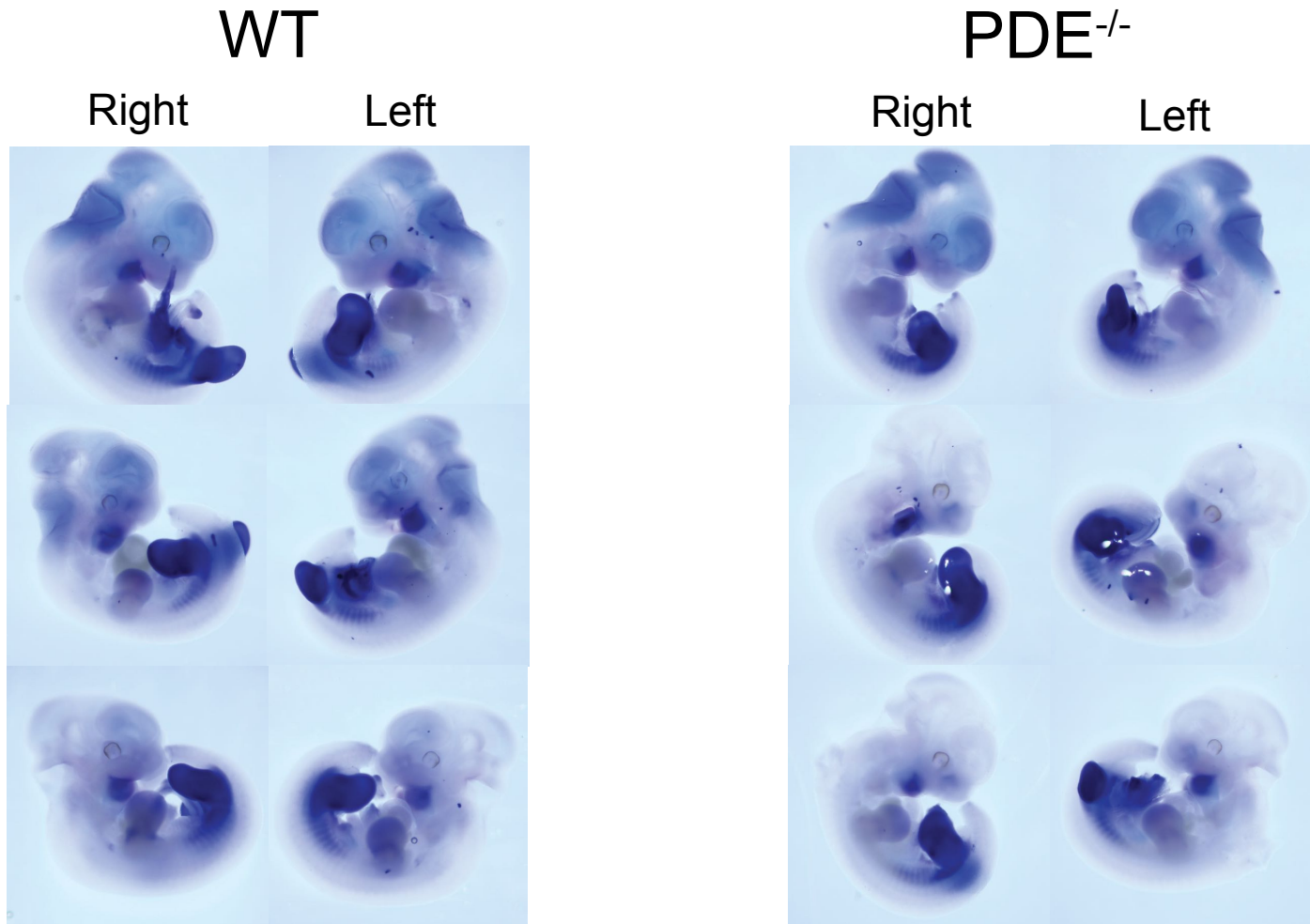


Figure S5 Comparison of *Pitx1* expression in wild type and PDE^{-/-} E11.5 embryos using whole mount *in situ* hybridization. Additional examples presented in support of results shown in Figure 3.

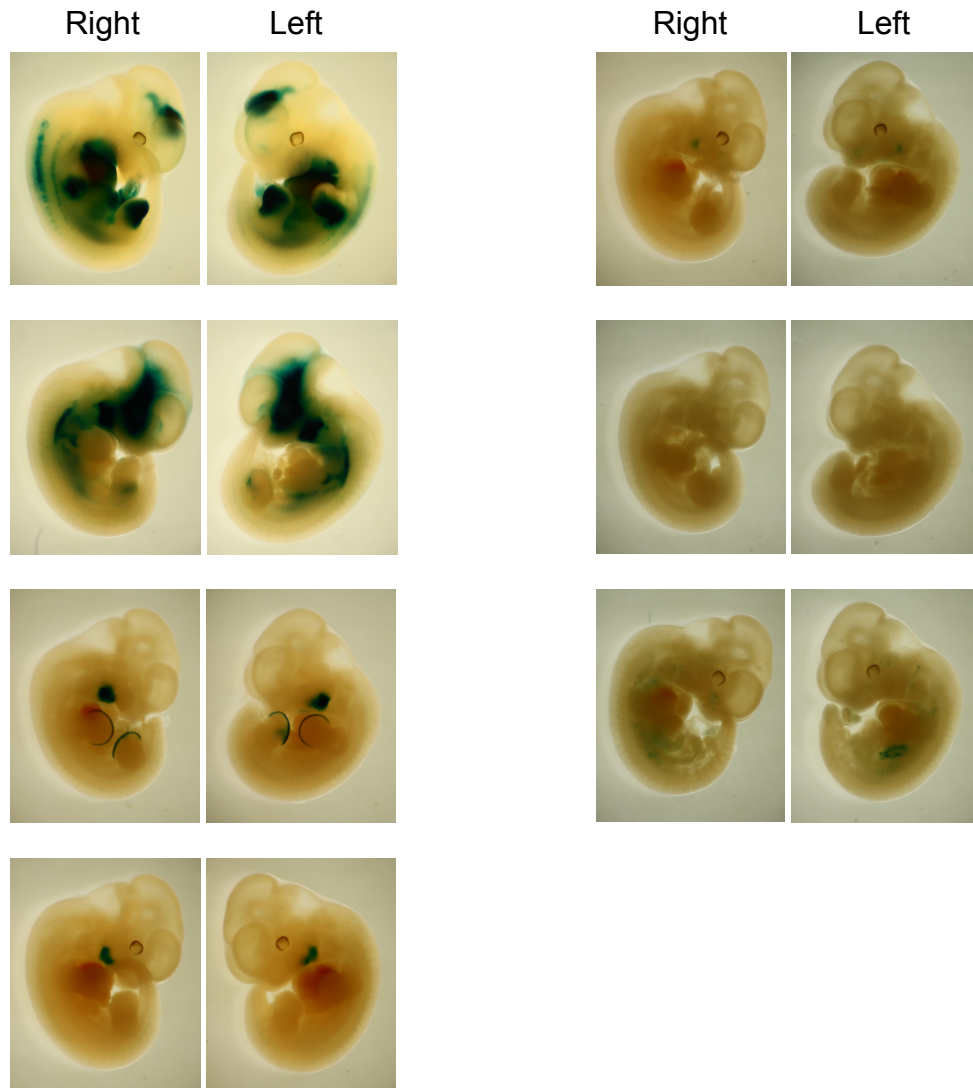


Figure S6. Enhancer activity of the PDE in a transgenic mouse LacZ reporter assay. The assay was performed in E11.5 embryos. Sagittal views of each LacZ positive embryo are shown.

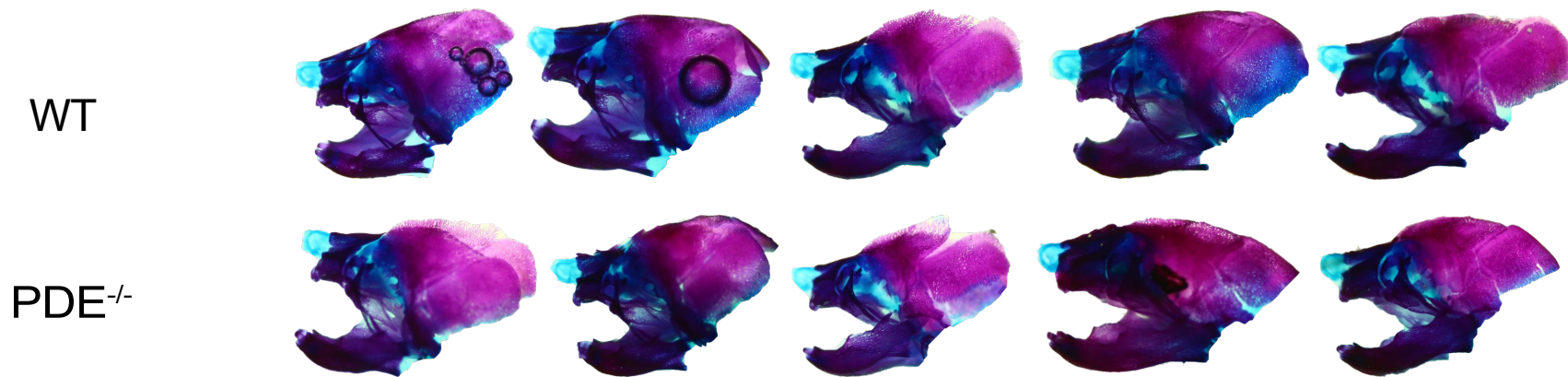


Figure S7. Comparison of E18.5 skull morphology in wild type and PDE^{-/-} mice using alcian blue (cartilage) and alizarin red (bone) staining. Additional examples presented in support of results shown in Figure 5.

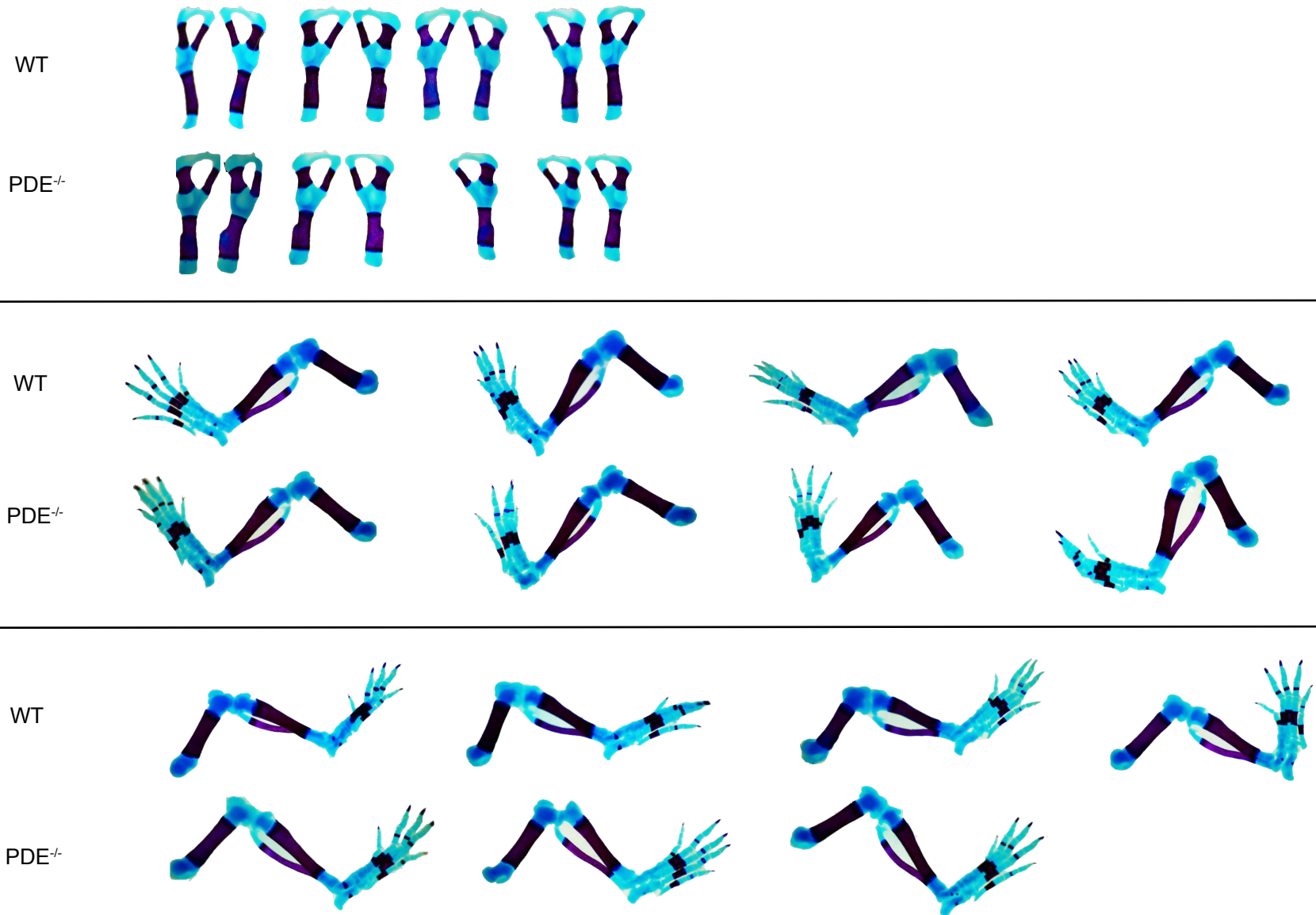


Figure S8. Comparison of E18.5 hindlimb and pelvic bone morphology in wild type and PDE^{-/-} mice. Additional examples presented in support of results shown in Figure 5.

Supplemental methods: 4C Protocol

Richard Sarro

Protocol adapted from Naumova et al. 2012, van de Werken et al. 2012, and Demare et al. 2013.

Day 1: Harvest of embryonic tissues and crosslinking. (Collect ~50-100ug of chromatin)

Dissect pregnant mother and place embryos in Petri dish with PBS on ice.

Remove the tissues of interest from embryo and transfer to fresh tube and keep on ice in PBS.

Disrupt tissue with blue plastic pestle.

Add ice cold PBS to final volume of 1mL.

Add 37% formaldehyde to final of 1% (27 uL in this case) and invert quickly several times.

Place on rotator and incubate at room temp for 15 minutes.

Add 2.5 M glycine to 150 mM final (63 uL in this case) and invert quickly several times.

Place on rotator and incubate at room temp for 10 minutes.

Harvest cells by centrifugation (2000g, 5 min, 4C).

Remove supernatant and resuspend pellet in 1mL of fresh, cold PBS by flicking the tube.

Harvest cells by centrifugation as above.

Wash 2 more times with PBS and harvest by centrifugation.

Remove all buffer from pellet.

Flash freeze pellet in liquid nitrogen and store at -80C until further use.

** for cultured cells collect 10^7 cells and follow and the above crosslinking protocol

Day 2: Digest chromatin

Resuspend pellet in 6 volumes of pelleted cells (vpc) of Lysis buffer I (+protease inhibitor) by pipetting up and down (~500uL).

Transfer cells to dounce and homogenize (if necessary, i.e. very clumpy) with pestle A (5-20 strokes).

Incubate on ice for 15 to 20 minutes.

Homogenize 20 to 30 strokes with pestle B.

Transfer solution to fresh tube. Rinse dounce with fresh swelling buffer and add to tube.

Harvest nuclei by centrifugation (2500g, 5min, 4C).

Make up 5mL of 1X NEB restriction buffer.

Wash in 500 ul of 1X restriction nuclease buffer and harvest by centrifugation.

Resuspend in 500 ul 1X NEB restriction nuclease buffer and add 15uL of 10%SDS.

Shake for 10 min at 65C at 800 rpm.

Add 150uL of 10% TritonX-100

Mix for 10 mins at 37C on nutator.

Add 16.5uL 10X NEB buffer and split in to two tubes.

Save 5uL in 40uL water for undigested control

Add 200U 4-cutter restriction enzyme to each tube and place on nutator at 37 O/N.

*****Make sure this enzyme can digest intact chromatin as can be deactivated by heat*****

Day 3: Ligate and reverse crosslinking

Next morning add 150U of enzyme and place on 37C nutator for 2 hours.

Heat inactivate enzyme at 65C stationary for 30' (SDS can also be used

Save 5ul in 40uL water for digestion control

Set up ligation reactions on ice (3 tubes per sample):

745 ul 10X T4 ligase buffer (NEB)

745 ul 10% TritonX-100

8 ul 100mg/ml BSA (or 80 uL BSA 10mg/ml)

5.5 ml H₂O

Add 200ul heat inactivated digestion reaction to each ligation mix.

Add 1ul of Thermo T4 DNA ligase (30 Weiss U/ul) and mix gently.

Incubate at 16C for 2 hr. (O/N ok too)

Add 50ul 25mg/ml Proteinase K and incubate O/N at 65C.

For undigested and digested controls:

Add 1 ul 10mg/ml RNase A and incubate for 1 hr at 37C.

Add 10 ul 25mg/ml Proteinase K and incubate O/N at 65C.

Day 4: Purify DNA

Add additional 25ul 25 mg/ml proteinase K and incubate 2 hr at 65C

Prepare 6 MaxTract tubes by centrifuging 1-2 min at 1500g.

Add ligation reactions to MaxTract tubes and add equal volume of phenol:chloro:isoamyl alcohol, mix by inverting several times

Spin 5' at 1500g. Decant aqueous phase into 30 ml glass centrifuge tubes

Add 1/10 vol 3M sodium acetate pH 5.2 and mix by inverting (cover with parafilm)

Add 0.7 vol isopropanol, cover tubes with aluminum foil, and place in -80C > 1 hr.

Let tubes thaw ~30 at RT, then spin 14000rpm at 4C for 45'

Resuspend pellets and combine into 1ml TE total

Add 10ul 10mg/ml RNAase A and incubate at 37C for 1 hour.

Prep 2 x 2ml phase lock tubes by spinning 30 seconds at 16000g

Split sample into 2 x 500 ul and add to phase lock tube and add equal volume of phenol:chloro:isoamyl alcohol, mix by inverting several times and spin 5' at 16000g

Add equal volume of chloroform, mix by inverting, spin 5' at 16000g

Pipet off aqueous phase into fresh tube, add 1/10 vol sodium acetate and 2.5 vol cold ethanol

Mix and place at -80C for > 1 hr.

Spin 45' max speed at 4C.

Wash 5 x 1ml 70% EtOH

Resuspend pellet in TE (100ul -1 ml depending on expected yield)

Save 5uL in 40uL water for ligation control

Day 5: Secondary RE digestion

Set-up digestion reaction:

150ul 3C library

50uL 10X RE Buffer

50 Units of secondary 4-cutter restriction enzyme
295uL water

Incubate o/n at 37C

Day 6: Secondary Ligation

Heat inactivate enzyme at 65C for 25 min

Take 5ul in 40uL water to serve as secondary digestion control

Transfer sample to a 50mL falcon tube on ice and add:

1.4mL 10X T4 ligase buffer (NEB)

12.6mL Cold Water

Add 3.3ul of Thermo T4 DNA ligase (30 Weiss U/ul) and mix gently.

Incubate for >2hrs (O/N ok) at 16C

Day 7: Purify DNA

Split sample in to 2 glass tubes and add to each tube:

0.7 mL of 3M NaAc pH 5.2

7 uL glycogen

17.5 mL of EtOH

Store at -80C until completely frozen.

Let thaw at RT and spin for 45 min at 9000 rpm (8300g) at 4C.

Remove supernatant and wash with 15 mL of cold 70% EtOH.

Spin for 15 min at 3650 rpm (3300g) at 4C.

Remove supernatant and allow pellet to dry.

Dissolve pellet in 150uL of TE at 37C.

Purify with 3 qiaquick pcr purification columns and re-suspend each column in 50uL TE, pool, and quantify on nanodrop.

Day 8: PCR and DNA Size Selection

Divide 1ug of purified 4C template over 10 (100ng/rxn) concurrent individual 50ul PCR reactions:

Use previously designed 4C primers and a long-template polymerase.

Run the reaction for 28 cycles.

Pool the 10 reactions and PCR purify on a qiagen column.

Combine 75uL of purified PCR product DNA and combine with .45x ampure bead solution (33.75ul).

Vortex and allow to sit for ~5 mins.

Place tube on magnet for 2 mins.

Collect 100ul of supernatant and mix with an additional 38ul of ampure bead solution in a new tube.

Follow ampure bead protocol as normally directed:

Vortex solution and allow to sit for ~5 mins.

Place tube on magnet for 2 mins.

Remove supernatant and wash beads twice (while still on magnet) with 200uL 80% EtOH.

Air dry beads until majority of EtOH is gone, but pellet not entirely dry, ~15 mins.

Remove from magnet and elute DNA by vortexing in 50uL TE.

Place back on magnet for 2 mins and remove supernatant.

Quantify DNA concentration with nanodrop and check for size selection on an agarose gel.

This size selected product can be submitted for library prep and sequencing.

*During sequencing, phiX DNA doping may be required if all submitted samples utilize the same primer set.

Table S1. FourCseq analysis of 4C results

[Click here to Download Table S1](#)

Table S2. Count of wild-type, PDE^{+/-} and PDE^{-/-} offspring by litter

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Table S3. Differential gene expression analysis in wild-type and PDE^{-/-} E11.5 hindlimb

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Table S4. Differential gene expression analysis in wild-type and PDE^{-/-} E11.5 mandibular arch

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Table S5. GO enrichment analyses of all genes exhibiting expression changes between wild-type and PDE^{-/-} E11.5 limb or mandibular arch at P<0.05 prior to multiple testing correction

[Click here to Download Table S5](#)

Table S6. Comparison of H3K27ac levels in wild-type and PDE^{-/-} E11.5 hindlimb

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Table S7. Comparison of H3K27me3 levels in wild-type and PDE^{-/-} E11.5 hindlimb

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Table S8. Oligonucleotides and primers used for genome editing and PCR

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