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Supplemental Information

Gene Regulatory and Expression Differences between Mouse and Pig Limb Buds Provide Insights into the Evolutionary Emergence of Artiodactyl Traits

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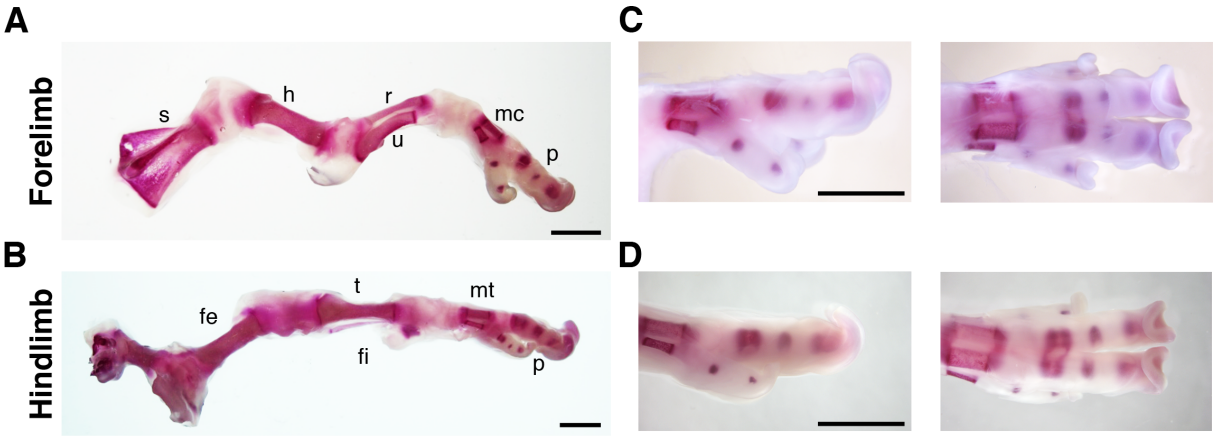

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Mouse Stage	Pig Stage	Morphological criteria used to compare mouse and pig limb development
E9.5 (25-27 somites)	D18	Prominent forelimb buds. First evidence of hindlimb bud formation.
E10.5 (34-36 somites)	D21 (D23HL)	Prominent forelimb bud outgrowth along the PD axis. No evidence of the AP expansion of the distal mesenchyme giving rise to the handplate. Prominent hindlimb buds.
E11.25 (43-44 somites)	D23	Handplate present. Footplate not yet formed.
E11.5-E11.75 (45-49 somites)	D24	Handplate fully expanded along the AP axis. Onset of footplate formation.
E13.25	D30	All digital rays present as cartilaginous condensations. Interdigit indentations starting to form.
E13.75	D33	Cartilaginous templates of metacarpals and proximal phalanges clearly distinguished. Mineralization starting in the humerus.
E17.5	D55	Advanced fetal stages (prenatal in mice; midgestation in pig). Used to illustrate limb skeletal pattern

Figure S1. Skeletal analysis of pig fore- and hindlimbs. Related to Figure 1. (A, B) Fore- and hindlimbs of a pig foetus at gestational day 55. Mineralized bone is stained with Alizarin red. s: scapula; h: humerus; r: radius; u: ulna; mc: metacarpals; p: phalanges; pg: pelvic girdle; fe: femur; fi: fibula; mt: metatarsals. Note that the carpal and tarsal bones of the wrist and ankle are not yet ossified. (C, D) Lateral (C) and dorsal (D) views of pig fore- and hindlimb autopods at D55. Anterior is to the top. Scale bars: 5mm; n=2 (E) Equivalent stages of mouse and pig limb bud development. Note that due to the temporal delay in fore- and hindlimb development, D23 pig hindlimb (HL) buds are comparable to D21 forelimb buds (see STAR Methods).

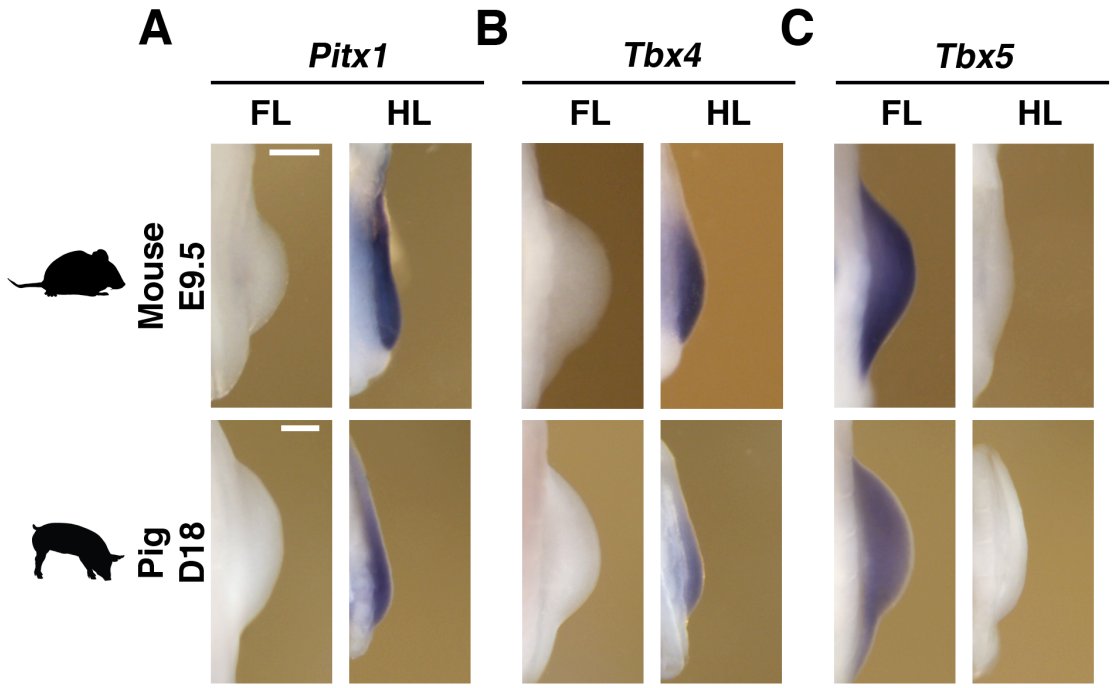

Figure S2. Spatial distribution of *Pitx1, Tbx4* and *Tbx5* transcripts in early mouse and pig limb buds. Related to Figure 1. (A-C) Spatial distribution of *Pitx1* (A), *Tbx4* (B) and *Tbx5* (C) transcripts in equivalent stages of mouse (E9.5) and pig (D18) fore- and hindlimb buds. Scale bars: 0.25mm. n=2 per stage for all pig ISH probes; n=3 per stage for all mouse ISH probes.

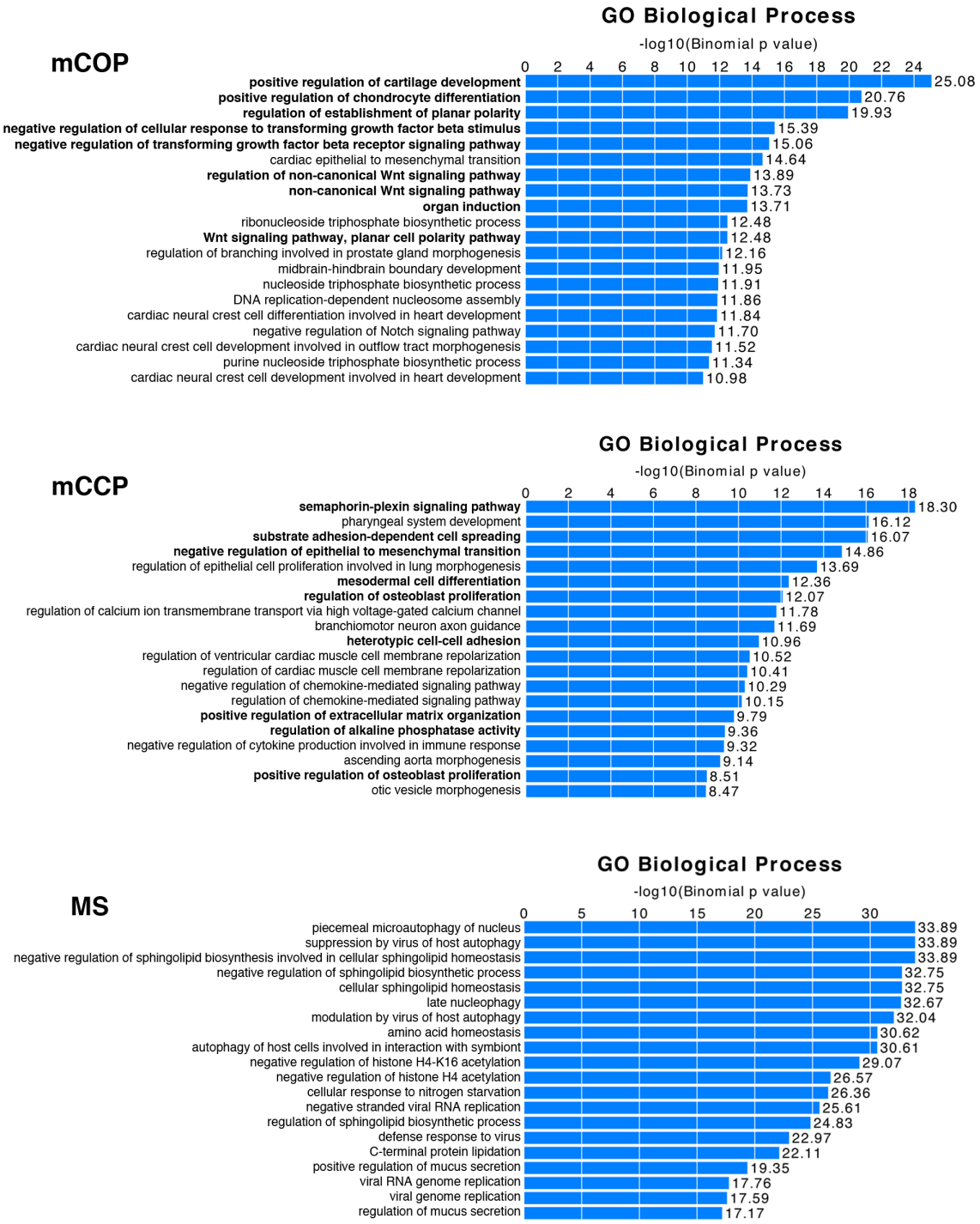

Figure S3. Biological processes associated with the mCOP, mCCP and MS categories of regulatory regions. Related to Figure 4. Gene ontology (GO) GREAT analysis reveals that regulatory regions belonging to the mCOP and mCCP categories associate with genes that function in cartilage and skeletal development (GO terms indicated in bold). Top 20 biological processes are shown.

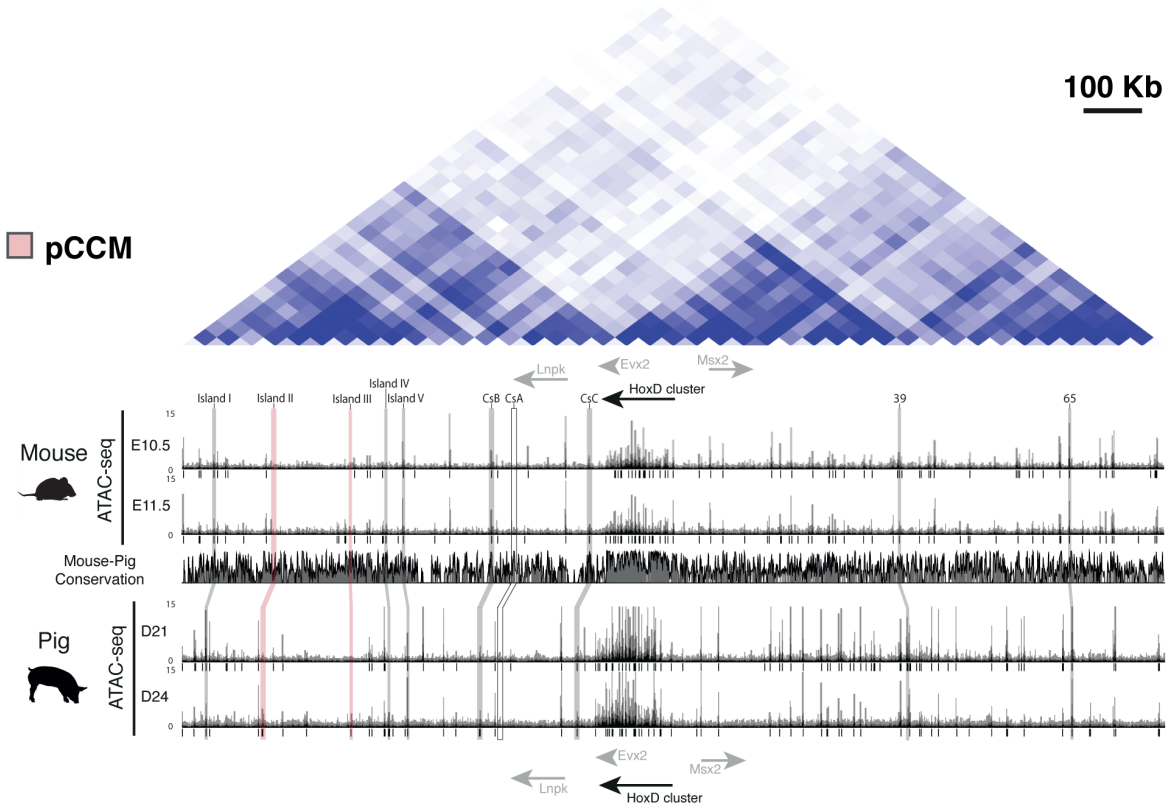

Figure S4. Mouse and pig *cis*-regulatory landscapes encoding the *HOXD* gene clusters. Related to Figure 4. HiC contact profile (Dixon et al., 2012) of the two TADs flanking the mouse *Hoxd* cluster (top panel). Non-*Hoxd* genes located in the two TADs are indicated in grey. Shown below are the ATAC-seq profiles for mouse (mm10; chr2:73,921,944-75,601,943) and pig (susScr11; chr15:81,111,890-82,911,114) limb buds at E10.5/D21 and E11.5/D24. Called peaks are indicated by black bars. The previously identified mouse *Hoxd* limb regulatory regions CNS 39, CNS65 (Andrey et al., 2013), CsB, CsC (Gonzalez et al., 2007) and islands I, IV and V (Montavon et al., 2011) are evolutionary conserved and accessible (mCOP and pCOM; in grey) in limb buds of both species. Islands II and III belong to the pCCM category (indicated in pink) and function as enhancers in mouse limb buds at E12.5 (Montavon et al., 2011). In mouse limb buds at E11.5, these chromatin regions are still closed, while they are accessible in pig limb buds at D24, which points to possible heterochrony. CsA, which functions as a neural tube enhancer (Gonzalez et al., 2007) is neither accessible in mouse nor pig limb buds.

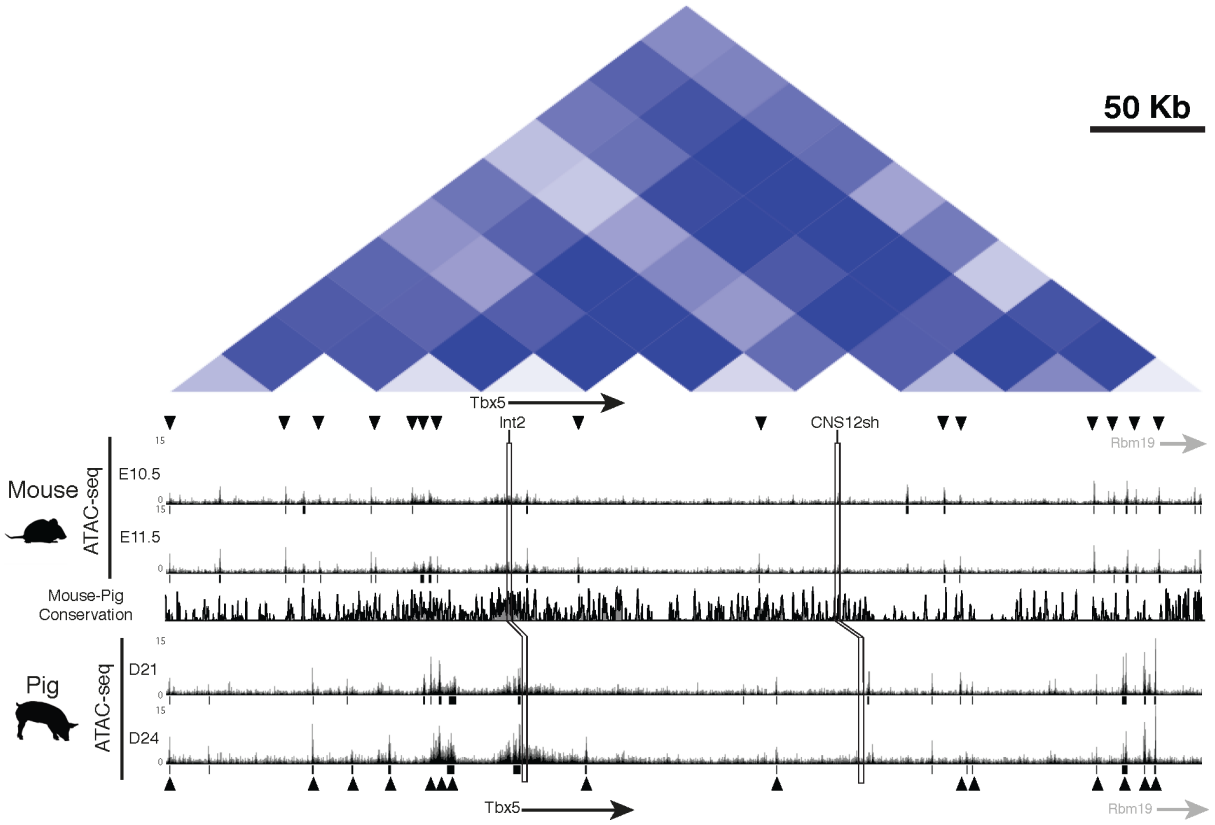

Figure S5. Mouse and pig *cis*-regulatory landscapes encoding the *Tbx5* locus. Related to Figure 4. HiC contact profile (Dixon et al., 2012) of the mouse *Tbx5* sub-TAD (top panel), defined on the basis of SMC1A ChIA-PET data (Dowen et al., 2014). Additional genes located in the same landscape are indicated in grey. Shown below are the ATAC-seq profiles for both stages in mouse (mm10; chr5:119,690,014-120,134,958) and pig (susScr11; chr14:37,602,722-38,104,628) limb buds. Called regions are indicated as black bars. The position of the putative *Tbx5* forelimb enhancers Int2 (Minguillon et al., 2012) and CNS12sh (Adachi et al., 2016; Cunningham et al., 2018) is indicated by open rectangles. None of these two conserved regulatory elements overlap regions of accessible chromatin in mouse or pig limb buds. Int2 has enhancer activity in the mouse forelimb field and early E9.5 forelimb buds (E8.5-E9.5; Minguillon et al., 2012). Our analysis indicates that Int2 might be no longer active during progression of mouse and pig limb bud outgrowth. CNS12sh was identified in fish (Adachi et al., 2016), and deletion of these two putative *Tbx5* forelimb enhancers in mouse embryos causes neither limb bud patterning nor skeletal defects (Cunningham et al., 2018). Conserved regions of open chromatin in both mouse and pig limb buds (mCOP/pCOM) are indicated with black arrowheads. Some of these may encode additional *cis*-regulatory regions active both in mouse and pig limb buds at the stages analysed.

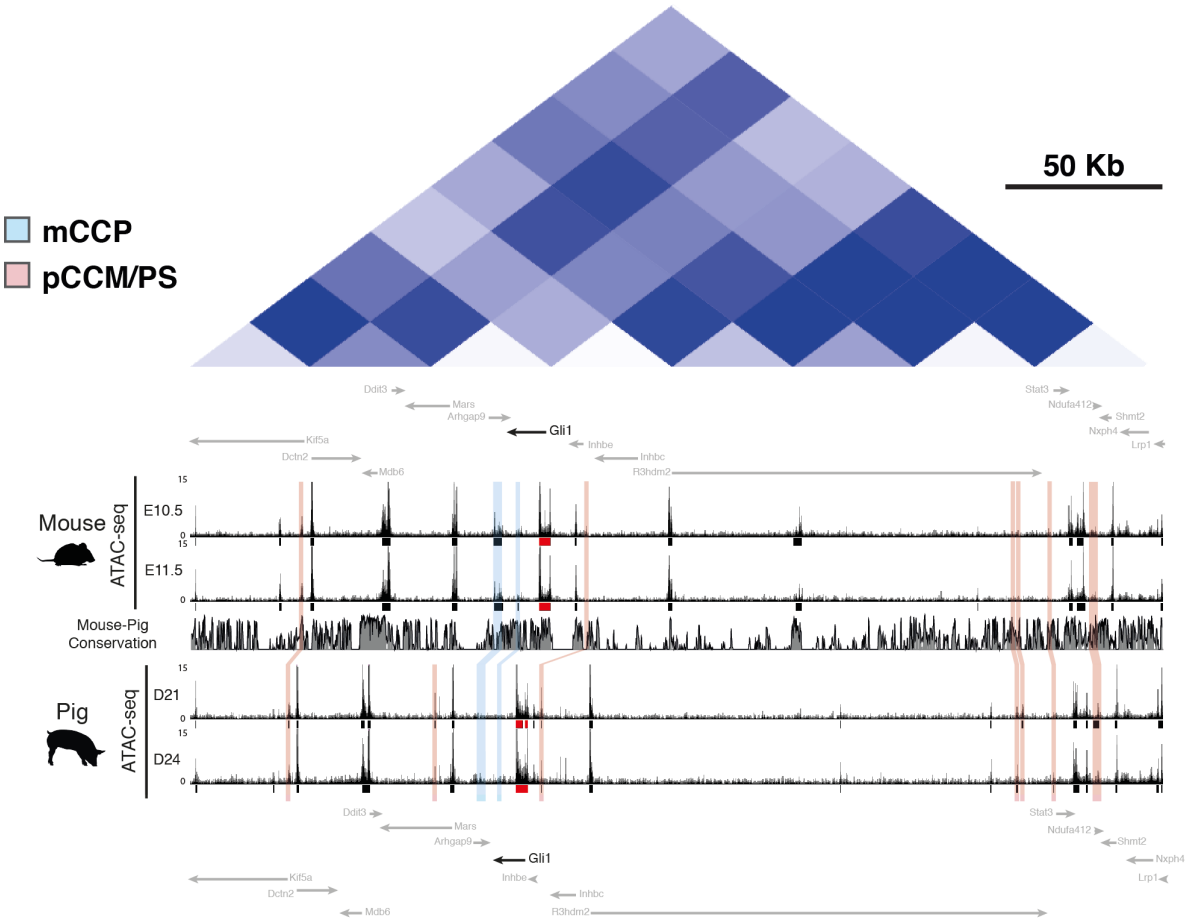

Figure S6. Mouse and pig *cis*-regulatory landscapes encoding the *Gli1* locus. Related to Figure 4. HiC contact profile (Dixon et al., 2012) of the mouse *Gli1* sub-TAD (top panel) defined on the basis of SMC1A ChIA-PET data (Dowen et al., 2014). Additional genes located in the same TAD are indicated in grey. Shown below are the ATAC-seq profiles for both stages in mouse (mm10; chr10:127,227,311-127,538,540) and pig (susScr11; chr5:22,518,876-22,850,852) limb buds. All called regions are indicated as black bars. mCCP and pCCM/PS regions are labelled in blue and pink, respectively. No MS regions are present. The only region enriched in GLI chromatin complexes in mouse limb buds corresponds to the *Gli1* promoter and the first exon/intron (Vokes et al., 2008). Interestingly, this element (labelled in red and conserved and open in both species) is not sufficient to drive transgenic reporter expression in a domain matching the endogenous *Gli1* expression domain in mouse limb buds (Vokes et al., 2008).