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Deletion of the endothelin-A receptor gene within the developing mandible

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Abstract

Signaling from the endothelin-A (Ednra) receptor is responsible for initiating multiple signaling pathways within neural crest cells (NCCs). Loss of this initiation is presumably the basis for the craniofacial defects observed in *Ednra*$^{-/-}$ embryos. However, it is not known whether continued Ednra signaling in NCC derivatives is required for subsequent development of the lower jaw. To address this question, mice containing *lox*P recombination sequences flanking a portion of the *Ednra* gene were bred with transgenic mice that express *Cre* recombinase under control of a *Dlx5/6* enhancer element. We find that while *Ednra* gene inactivation within the mandibular arch of these Ednra conditional knockout embryos is detectable by embryonic day (E) 10.5, mandibular arch-specific gene expression is normal, as is overall mandible development. These results suggest that while Ednra receptor signaling is crucial for early NCC patterning, subsequent Ednra signaling is not essential for mandible bone development.

Keywords

Craniofacial; Neural crest; *lox*P; Cre recombinase; Knockout mice; Transgenic mice

Introduction

Lower jaw formation is arguably one of the more amazing morphological achievements of embryogenesis. Much of the facial skeleton is formed by cephalic neural crest cells (NCCs),

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which migrate to the pharyngeal arches from the midbrain/hindbrain region, subsequently giving rise to bone and cartilage (Noden 1983; Lumsden et al. 1991; Serbedzija et al. 1992). NCCs appear to be patterned once in the arches by environmental signals from the surrounding tissues (Le Douarin et al. 1993; Couly et al. 2002; Trainor et al. 2002; Schneider and Helms 2003). However, the ability of NCCs and their derivatives to respond to these signals appears to decrease with time. In E9.0 explanted mandibles, Fgf8-soaked beads can induce widespread *Lhx7* expression, though by E11.0, *Lhx7* expression is independent of Fgf8 (Tucker et al. 1999). These findings illustrate that NCC patterning signals may only be necessary for the initiation of developmental pathways within NCCs but not during subsequent mesenchymal differentiation.

Endothelin-1 (Edn1), expressed by the surrounding ectoderm, core paraxial mesoderm and pharyngeal pouch endoderm, is also crucial for NCC patterning, acting through the endothelin-A receptor (Ednra) located on NCCs (for review, see Clouthier and Schilling 2004). Targeted inactivation of either *Edn1* or *Ednra* results in severe craniofacial and cardiovascular defects that are attributable to aberrant NCC development (Kurihara et al. 1994; Clouthier et al. 1998; Yanagisawa et al. 1998). These include an apparent homeotic transformation of lower jaw structures to more maxillary-like derivatives (Ozeki et al. 2004; Ruest et al. 2004). While these findings indicate a role for Ednra signaling in initiating early crest cell patterning, it is not clear if continued Ednra signaling is required to maintain patterning mechanisms and whether Ednra signaling is required for later differentiation of the crest derived mesenchyme. To address these questions, we specifically inactivated the *Ednra* gene in a subset of cells within the mandibular arch using Cre/*lox*P technology.

Materials and methods

Animals

Ednra$^{flox/flox}$ (Kedzierski et al. 2003) and *Dlx5/6-Cre* (Ruest et al. 2003) mice were generated as previously described. *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* embryos were generated by crossing *Ednra*$^{flox/+}$; *Dlx5/6-Cre* female mice with *Ednra*$^{flox/flox}$ male mice. We also generated conditional knockout mice carrying one conventional *Ednra* mutant allele (*Ednra*$^{+/-}$; Clouthier et al. 1998) and one conditional allele (*Ednra*$^{flox/-}$).*Ednra*$^{flox/-}$ female mice were bred with *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* male mice to generate *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos. Genotyping was performed by PCR using genomic DNA prepared from tail biopsies or amniotic sacs. *Dlx5/6-Cre* mouse genotyping was performed as previously described (Ruest et al. 2003). The genotyping of the *Ednra* conditional allele was performed with the following primers: 5′-ACACAACCATGGTGTCGA-3′ and 5′-CGGTTCTTATCCATCTCATC-3′. These primers flank the 5′ *lox*P site located between the fifth and sixth exons of the conditionally targeted *Ednra* gene (Kedzierski et al. 2003), thus producing ~420 bp and ~380 bp bands in *Ednra*$^{flox/+}$ animals, a single ~420 bp band in *Ednra*$^{flox/flox}$ animals and a single ~380 bp band in *Ednra*$^{+/+}$mice. Reaction products were visualized on a 1.5% agarose gel. *Ednra* mutant genotyping was performed as previously described (Clouthier et al. 1998).

To determine whether the conditional *Ednra* gene had undergone recombination, genomic DNA was extracted from the mandibular pharyngeal arch of E9.5 and E10.5 embryos. To obtain bone DNA, the mandible bone was dissected from 3-month-old *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* females and cleaned of all muscle and tendon tissue. Mandibles were ground in liquid nitrogen and incubated for 48 h in 1 ml of 0.5 M EDTA (pH 8.0)/2% sarkosyl with agitation at room temperature. After addition of TRIS (pH 8.0) and NaCl to 20 mM and 400 mM final concentration, respectively, samples were incubated with proteinase K (10 mg/ml) at 55°C overnight. Following phenol/chloroform extraction, samples were dialyzed in Spectra Float A Lyzer tubes (3500 MWcut-off) for 20 h in TE buffer. After addition of sodium acetate and isopropyl alcohol, DNA was precipitated and washed with 75% ethanol. DNAwas used in a

recombinant PCR reaction with the following primers: 5′-ACACAACCATGTTGTCGAGGTCGA-3′ and 5′-GAGAACCTACAACTGGGGACACAAACAC-3′. Recombination of the conditional *Ednra* allele gives rise to a 1.2 kb band.

Skeleton staining and histology

Skeleton staining was performed as previously described (McLeod 1980). Skeletons were preserved in glycerol or in 25% glycerol/75% ethanol. Skeletons were photographed with an Olympus DP11 digital camera mounted on an Olympus SZX12 stereomicroscope. For histological analysis, E18.5 embryos were fixed in 10% neutral buffered formalin (Sigma), dehydrated in graded ethanols and then embedded in paraffin. Eight-μm sagittal sections were then collected onto Plus-coated slides. Every other section was then counterstained with hematoxylin and eosin (H&E), dehydrated in graded ethanols and then coverslipped using DPX mounting medium (BDH). Sections were examined and photographed on an E600 Nikon microscope fitted with a Spot-RT digital camera. To examine the extent of calcification in the mandible of embryos, contiguous slides to those used for H&E analysis were deparaffinized and rehydrated and then subjected to Van Kossa's method. Briefly, sections were incubated in 5% silver nitrate for 1 h at room temperature under a 60-W bulb. Slides were then rinsed 3 times in distilled water and incubated in 5% sodium thiosulfate. Sections were rinsed in water and then counterstained with nuclear fast red before dehydrating and mounting as described above. We analyzed more than 20 *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* embryos and ten *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos.

In situ hybridization

Whole-mount in situ hybridization analysis was performed as previously described (Clouthier et al. 2000). Embryos were hybridized with digoxigenin (DIG)-labeled cRNA riboprobes against *dHAND* (Srivastava et al. 1997) and *Dlx5* (Depew et al. 1999). Riboprobe labeling was performed using the DIG labeling kit (Roche). Stained embryos were photographed in whole-mount as described above.

Results

Generation of Ednra$^{flox/flox}$; Dlx5/6-Cre conditional knockout mice

Mice carrying a conditionally targeted *Ednra* allele (referred to as *ET*$_A^{flox/flox}$) have been described previously (Kedzierski et al. 2003). Briefly, these mice carry two *lox*P sites that flank the last three exons of the *Ednra* gene (hence the term "flox"). We have also previously described the generation and characterization of *Dlx5/6-Cre* transgenic mice (Ruest et al. 2003). Cre expression in these mice is restricted to the mandibular first arch, with expression first detected at embryonic day (E) 9.5 before being down regulated by E10.5. *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* embryos, generated by crossing *Ednra*$^{flox/+}$; *Dlx5/6-Cre* with *Ednra*$^{flox/flox}$ mice, were collected at both E9.5 and E10.5 and mandibular arch DNA isolated to verify recombination of the conditional *Ednra* allele. At E9.5, recombination of the conditional allele was not detected by PCR in either *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* (Fig. 1, lane 4) or *ETA*$^{flox/+}$; *Dlx5/6-Cre* embryos (data not shown). However, by E10.5, recombination of the conditional *Ednra* allele was observed in embryos carrying both an *Edmr*^{aflox} allele and the *Dlx5/6-Cre* transgene (lanes 5 and 6). Recombination was not observed in *Ednra*^{flox} embryos (lanes 2 and 3) or in amniotic sac DNA from *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* (neither *Dlx5* nor *Dlx6* is expressed in the amniotic sac; Beverdam et al. 2002; Robledo et al. 2002; Ruest et al. 2003). To quantify the extent of recombination, we counted the number of labeled cells in multiple sections through the mandibular arch of E10.5 *R26R*; *Dlx5/6-Cre* embryos, finding that 3.2% (±0.5) of cells were labeled (data not shown). While this indicates a low level of recombination, we have shown that Dlx5/6 daughter cells are restricted to the mandibular bone of E16.5 *R26R*; *Dlx5/6-*

Cre embryos. To determine if this restriction was detectable by recombination PCR, we examined the extent of *Ednra* gene recombination in the adult mandible bone, comparing it to surrounding tissue. We found that recombination was only present in bone DNA but not in skin or muscle DNA (Fig. 1d).

Lower jaw development in Ednra$^{flox/flox}$; Dlx5/6-Cre embryos

The mandible of *Ednra*$^{-/-}$embryos has significant defects in NCC-derived structures of the first mandibular arch (Clouthier et al. 1998; Ruest et al. 2004). To examine whether similar defects were present in *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* embryos, we examined the skulls of E18.5 embryos. We focused our attention on the mandible bone, since β-galactosidase-labeled cells in *Dlx5/6-Cre*; *R26R* embryos, representing *Dlx5/6* daughter cells, are found almost exclusively in the mandible bone of E16.5 embryos (Ruest et al. 2003). In *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* embryos (Fig. 2c,d), no obvious differences were apparent in the mandible bone when compared with either *Ednra*$^{flox/+}$; *Dlx5/6-Cre* (Fig. 2a,b) or *Ednra*$^{flox/flox}$ (data not shown) control littermates. The absence of defects in *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* embryos could reflect insufficient recombination of the *Ednra* conditional allele, resulting in genetic mosaicism (Nagy 2000; Kwan 2002). To address this issue, we also examined lower jaw structures in *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos. Since one *Ednra* allele of *Ednra*$^{flox/-}$ animals already contains a traditional mutation, recombination would only have to occur once to result in an *Ednra* mutant genotype. However, defects were also not present in *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos (Fig. 2e, f).

Histological analysis of E18.5 embryos also did not reveal any structural differences in the mandible bone between *Ednra*$^{flox/flox}$ (control; Fig. 3a), *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* (data not shown) and *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos (Fig. 3b). The size of the dental papilla and the extent of early dentin matrix formation around the teeth also appeared normal and suggested that odontogenesis was unaffected in *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos.We also examined calcification of the mandibular bone at E18.5 using Van Kossa's method, which results in a black deposit of reduced silver in the presence of calcium. Calcified matrices along the mandible bone appeared similar between *Ednra*$^{flox/flox}$ (control; Fig. 3c) and *Ednra*$^{flox/-}$; *Dlx5/6-Cre* (Fig. 3d) embryos. The absence of defects was reflected in adult *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* mice, which are viable and fertile at least to 15 months, indicating normal dentition, growth, musculature, innervation and tendon connections.

Normal gene expression within the mandibular arch of Ednra$^{flox/flox}$; Dlx5/6-Cre embryos

Ednra signaling is required for normal signaling of at least eight transcription factors involved in neural crest cell development, including the bHLH transcription factor dHAND/HAND2 and the *Distal-less* homeobox family member Dlx5 (Clouthier et al. 1998,Clouthier et al. 2000; Thomas et al. 1998; Ivey et al. 2003;Park et al. 2004; Ruest et al. 2004). Since defects were not observed in either *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* or *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos, we examined whether this reflected normal gene expression within the mandibular arch. In both *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* and *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos, expression of both *Dlx5* (Fig. 4a–c) and *dHAND* (Fig. 4d–f) was normal in the pharyngeal arch mesenchyme. This suggests that inactivation of the conditional *Ednra* gene is occurring after activation of the signaling cascade(s) involving both Dlx5 and dHAND.

Discussion

Our previous analysis of *Dlx5/6-Cre*; *R26R* embryos demonstrated that *Cre* expression in *Dlx5/6-Cre* embryos occurs between E9.5 and E10.5, with Dlx5/6 daughter cells restricted to the mandible bone of E18.5 embryos (Ruest et al. 2003). However, when crossed into the conditional *Ednra*$^{flox/flox}$ background, neither *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* nor *Ednra*$^{flox/-}$;

Dlx5/6-Cre embryos develop defects in lower jaw structures. The *Ednra*^{flox} line has been used previously to inactivate *Ednra* expression specifically in myocardial cells of the adult heart, with Edn1 binding assays illustrating loss of Ednra receptor function (Kedzierski et al. 2003). While the size of E9.5 and E10.5 pharyngeal arches make binding assays unfeasible, our PCR analysis shows that the conditional *Ednra* allele is recombined by E10.5 in *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* embryos. While we do not observe recombination at E9.5, the time period that *Cre* transgene expression first appears, this absence may simply reflect a very limited recombination of the conditional *Ednra* allele at that time. However, even at E10.5, recombination is only observed in 3.2% of the arch mesenchyme cells, which could suggest that inefficient recombination leads to an absence of mandibular defects. Yet, our results with *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos argue against inefficient recombination of the conditional *Ednra* allele. Rather, we believe that we are targeting a small group of cells that will later participate in mandible bone formation. A lack of phenotype is probably due to the timing of recombination rather than the extent of recombination, as we have shown using chimera analysis that the *Ednra* mutation acts in a cell autonomous manner (Clouthier et al. 2003). This argues against rescue of mutant cells by neighboring cells within the arches. This is also supported by a lack of change in gene expression patterns. Taken together, these aspects indicate, though do not prove, that an absence of phenotype is more probably due to the timing of gene recombination rather than an absence of recombination.

Ednra signaling is crucial for NCC development in mouse (Clouthier et al. 1998), rat (Spence et al. 1999), zebrafish (Miller et al. 2000) and chick (Kempf et al. 1998). We have clarified the function of Ednra receptor signaling in this study, illustrating that it does not appear to be required for mandible bone development after NCC patterning. However, it is still possible that Ednra receptor function is required within the adult mandibular bone. Ednra receptors are located on osteoblasts (Stern et al. 1995; Suzuki et al. 1997), including those in craniofacial bone (Kitano et al. 1998). Further, recent evidence suggests that Ednra signaling may play a role in the bone formation observed during osteoblastic bone metastases (Yin et al. 2003). These findings may indicate that Ednra receptor signaling is required for adult bone remodeling. It is plausible that such a mechanism could be mediated by Dlx5, as *Dlx5* expression is observed in broken bones of adult mice (Miyama et al. 1999). It will be interesting to examine whether the absence of the *Ednra* gene in *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* or *Ednra*$^{flox/-}$; *Dlx5/6-Cre* mice affects mandible fracture repair.

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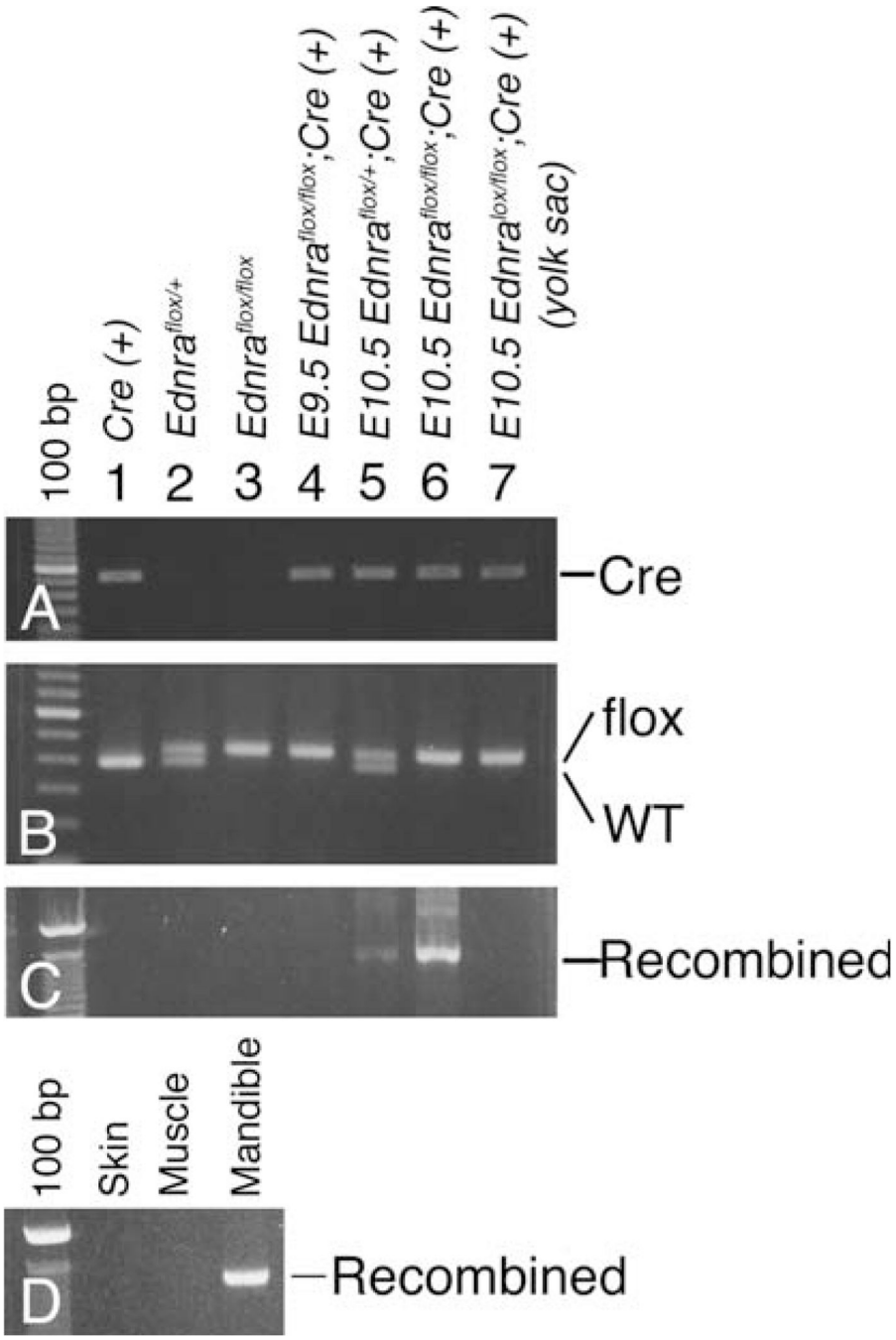

Fig. 1.

Analysis of the *Ednra* gene recombination in *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* embryos. Genomic PCR analysis of DNA extracted from adult mouse tails (*lanes 1–3*), embryonic mandibular pharyngeal arches (*lanes 4–6*) or embryonic yolk sac (*lane 7*). The genotype of each animal is listed above the lane number. **a** *Cre*PCR amplification generates a band ~500 bp. **b** *lox*P PCR reveals a 375 bp band, representing the wild type (*WT*) allele, a 425 bp band, representing the mutant allele (flox), or both. **c** Recombinant PCR detects the recombined *Ednra*^{flox} allele and generates a 1200 bp band. **d** Recombinant PCR of DNA extracted from the skin, muscle and bone of the lower jaw of adult *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* mice

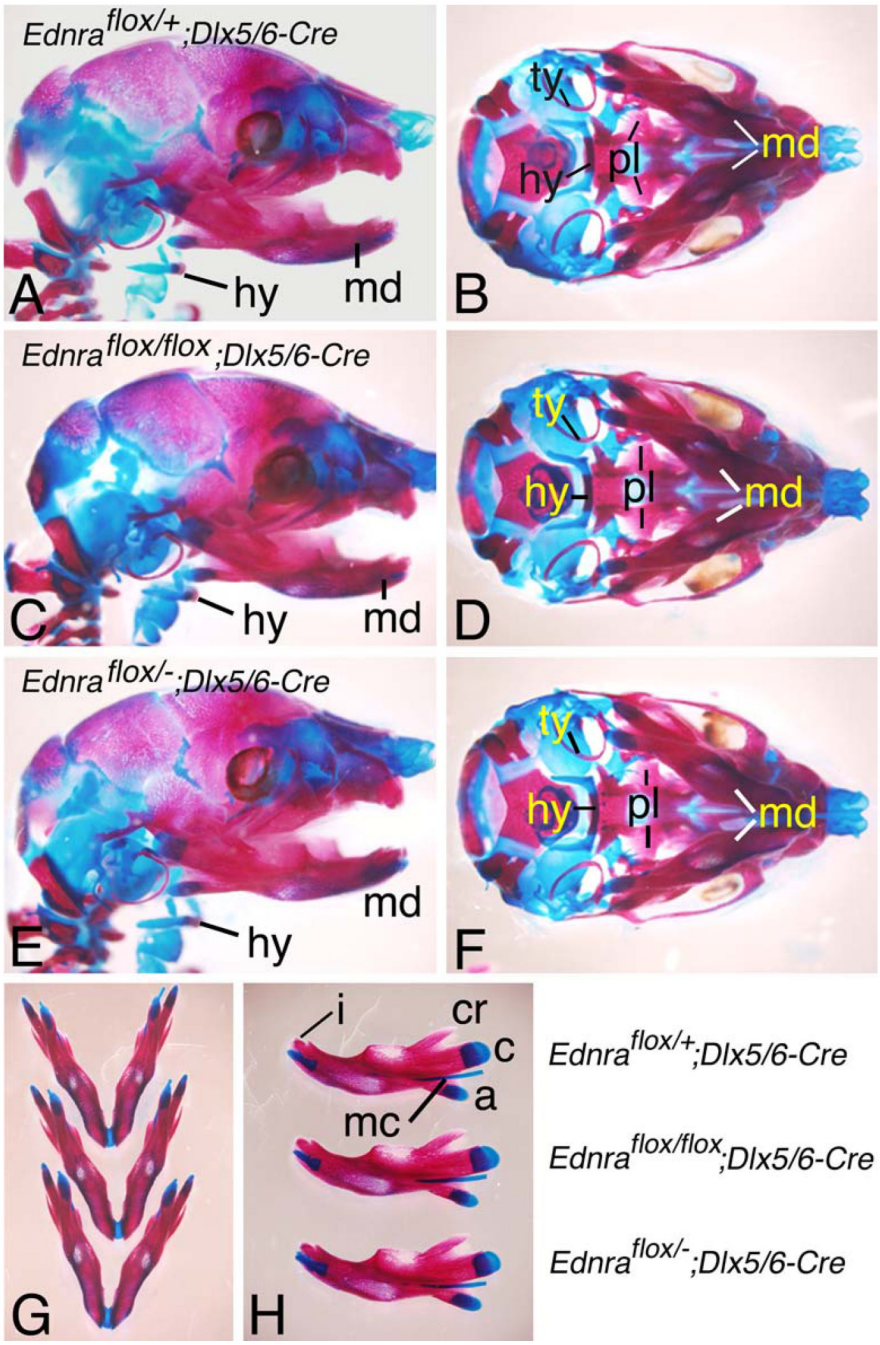

Fig. 2.

Analysis of jaw development in E18.5 *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* and *Ednra*$^{flox/-}$; *Dlx5/6-Cre* conditional knockout embryos. Lateral (**a,c,e**), ventral (**b,d,f,g**) and intralateral (**h**) views of *Ednra*$^{flox/+}$; *Dlx5/6-Cre* (control; **a,b**), *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* (**c,d**) and *Ednra*$^{flox/-}$; *Dlx5/6-Cre* (**e,f**) conditional knockout embryos stained with alizarin red and alcian blue. **a,f** Regardless of the genotype, defects are not observed in any skeletal structures, including the mandible, Meckel's cartilage, malleus and incisors. **g,h** In comparison to a control mandible, *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* and *Ednra*$^{flox/-}$; *Dlx5/6-Cre* conditional knockout mandibles do not display any morphological differences in their shape, length or processes. *a* Articular process;

c condylar process; *cr* coronoid process; *i* incisor; *md* mandible; *mc* Meckel's cartilage; *hy* hyoid; *ty* tympanic ring

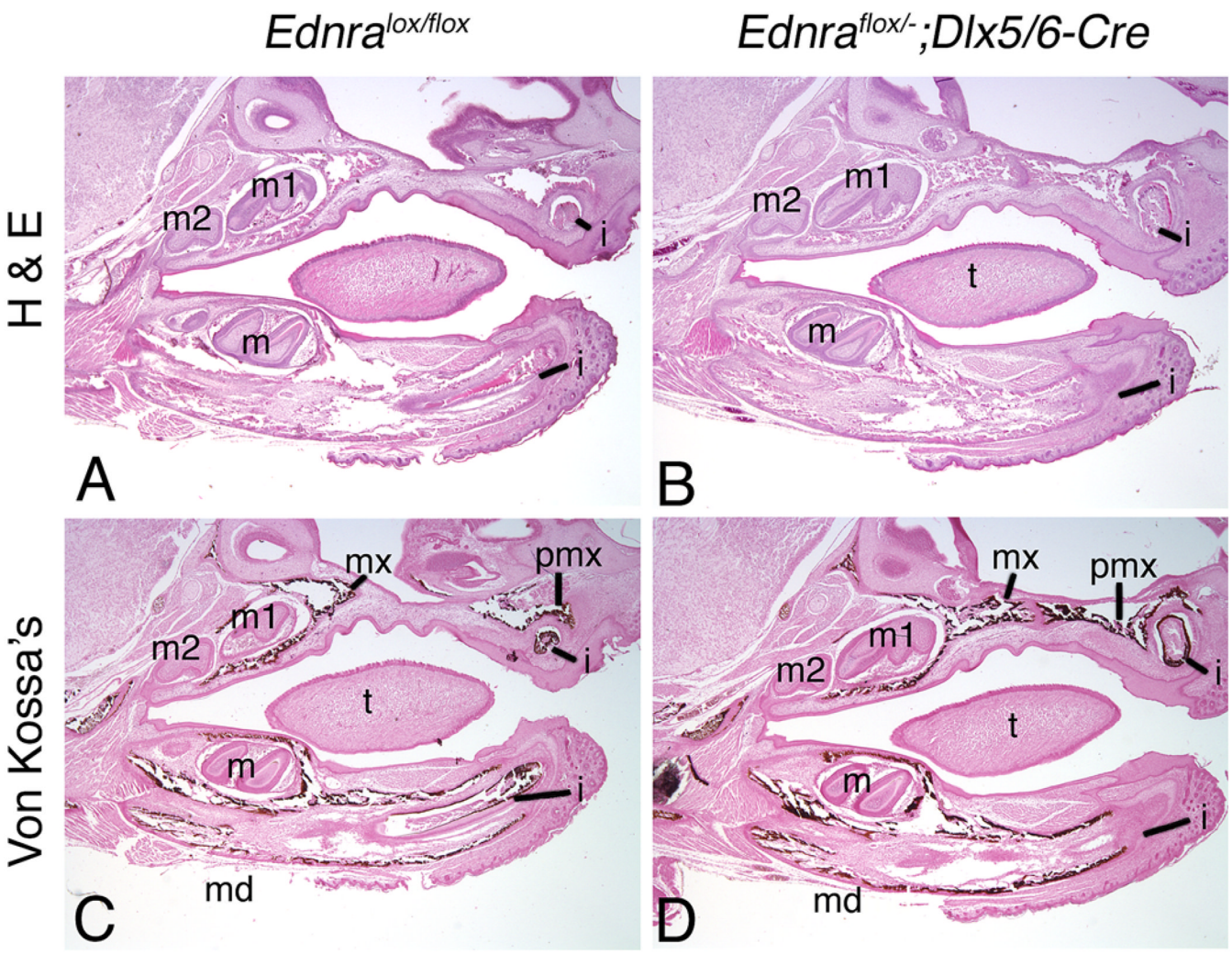

Fig. 3.

Analysis of general histology and calcification in E18.5 *Ednra*$^{flox/-}$;*Dlx5/6-Cre* conditional knockout mandibles. Sagittal paraffin sections through the head of E18.5 *Ednra*$^{flox/flox}$ (used as control; **a,c**) and *Ednra*$^{flox/-}$;*Dlx5/6-Cre* (**b,d**) embryos stained with either hematoxylin and eosin (H&E; **a,b**) or Van Kossa's method counterstained with nuclear fast red (*c,d*). **a,b** Histological analysis of H&E-stained sections illustrate that differences are not observed in either the mandibular/alveolar bone structure or incisors of control (**a**) and *Ednra*$^{flox/-}$;*Dlx5/6-Cre* (**b**) embryos. **c,d** Analysis of calcification by Van Kossa's method, which produces a precipitate of reduced silver metal in the presence of calcium, reveals no differences between control (**c**) and *Ednra*$^{flox/-}$;*Dlx5/6-Cre* (**d**) embryos. Silver metal deposits were also observed along the incisors of *Ednra*$^{flox/-}$; *Dlx5/6-Cre* embryos, though this is not apparent in the section shown in **d**. *i* Incisor; *m*; molar; *m1* first molar; *m2* second molar; *md* mandible; *mx* maxilla; *pmx* pre-maxilla; *t* tongue

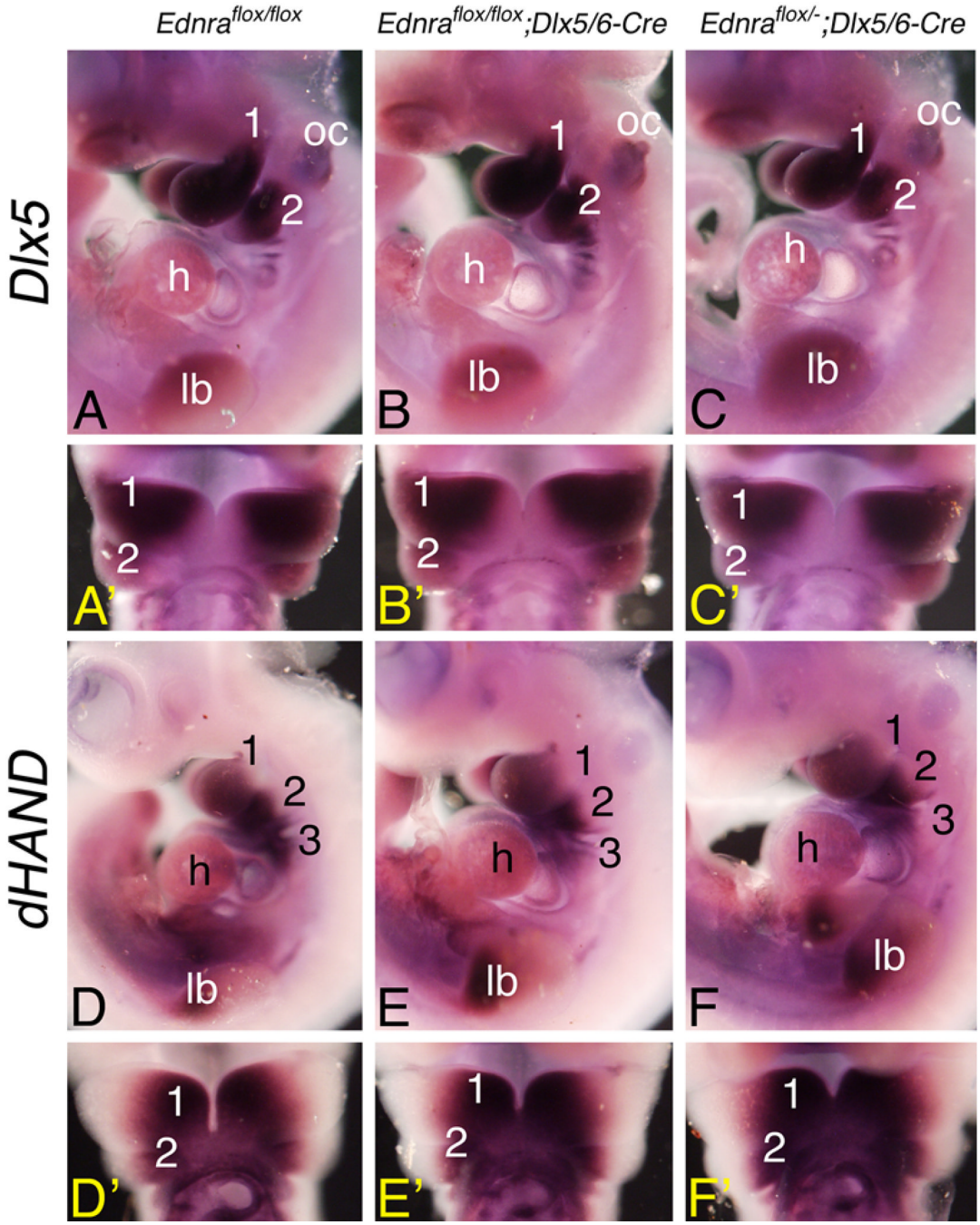

Fig. 4.

Analysis of gene expression in E10.5 conditional knockout embryos. Whole-mount in situ hybridization analysis of *Dlx5* (**a–c**) and *dHAND* (**d–f**) expression in E10.5 *Ednra*$^{flox/flox}$ (control; **a,d**), *Ednra*$^{flox/flox}$; *Dlx5/6-Cre* (**b,e**) and *Ednra*$^{flox/-}$; *Dlx5/6-Cre* (**c,f**) embryos, presented in lateral (**a–f**) and ventral (**a′–f′**) views. (**a–f**) No differences in *Dlx5* and *dHAND* expression are observed in the mandibular (*1*) and second (*2*) pharyngeal arches between the different genotypes. *oc* Otic capsule; *h* heart; *lb* limb bud; *3* third pharyngeal arch