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ARTICLE

Spatio-Temporal Dynamics of Gene Expression of the Edn1-Dlx5/6 Pathway During Development of the Lower Jaw

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Summary: The morphogenesis of the vertebrate skull results from highly dynamic integrated processes involving the exchange of signals between the ectoderm, the endoderm, and cephalic neural crest cells (CNCCs). Before migration CNCCs are not committed to form any specific skull element, molecular signals exchanged in restricted regions of tissue interaction are crucial in providing positional identity to the CNCCs mesenchyme and activate the specific morphogenetic process of different skeletal components of the head. In particular, the endothelin-1 (Edn1)-dependent activation of *Dlx5* and *Dlx6* in CNCCs that colonize the first pharyngeal arch (PA1) is necessary and sufficient to specify maxillo-mandibular identity. Here, to better analyze the spatio-temporal dynamics of this process, we associate quantitative gene expression analysis with detailed examination of skeletal phenotypes resulting from combined allelic reduction of *Edn1*, *Dlx5*, and *Dlx6*. We show that Edn1-dependent and -independent regulatory pathways act at different developmental times in distinct regions of PA1. The Edn1→Dlx5/6→Hand2 pathway is already active at E9.5 during early stages of CNCCs colonization. At later stages (E10.5) the scenario is more complex: we propose a model in which PA1 is subdivided into four adjacent territories in which distinct regulations are taking place. This new developmental model may provide a conceptual framework to interpret the craniofacial malformations present in several mouse mutants and in human first arch syndromes. More in general, our findings emphasize the importance of quantitative gene expression in the fine control of morphogenetic events. genesis 48:362–373, 2010.

Key words: endothelin-1; Dlx; craniofacial development; pharyngeal arches; allelic dosage; cranial neural crest cells; first arch syndromes

INTRODUCTION

Vertebrate jaws are formed through complex morphogenetic processes beginning with the colonization of the first pharyngeal arch (PA1) by *Hox*-negative cephalic neural crest cells (CNCCs) emigrating from the posterior mesencephalic and rhombencephalic neural folds.

Additional Supporting Information may be found in the online version of this article.

Authors' contributions: MV-R carried out mating, pharyngeal arches and skeletal dissections, designed experiments, performed statistical analysis, made figures and prepared the manuscript. SM carried out ISH and quantitative PCR experiments. EH carried out some ISH experiments. OB and SM maintained the animal colony, performed mouse mating and genotyping. GC provided medical expertise and scanners of FAS patients. HK provided Edn1 mutant mice and extensive discussion of the manuscript. GRM carried out ISH, analyzed Real Time PCR data and performed skeletal dissections. GRM and GL designed and coordinated the study, organized the results and prepared the manuscript. All authors read and approved the final manuscript.

Giovanni Levi and Giorgio R. Merlo are co-senior authors.

Abbreviations: CNCCs, cranial neural crest cells; Edn1, endothelin-1; Ednra, endothelin-1 receptor type A; FAS, first arch syndromes; Gsc, goosecoid; PA1, 1st pharyngeal arch; qPCR, quantitative polymerase chain reaction; WT, wild type.

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Whereas CNCCs give rise to most chondrocranial and dermatocranial elements of the jaws (Clouthier *et al*., 1998; Couly *et al*., 2002; Depew and Simpson, 2006; Kontges and Lumsden, 1996; Ruhin *et al*., 2003), they do not possess, before migration, the topographic information needed to carry out the jaw morphogenesis (Couly *et al*., 1993). Surgical removal and grafting of small territories of the foregut endoderm at different developmental stages has shown that this epithelium provides to CNCCs part of the topographic information needed to form jaw structures (Couly *et al*., 1993; Kontges and Lumsden, 1996; Kurihara *et al*., 1994; Le Douarin and Dupin, 2003; Noden and Trainor, 2005; Trainor and Tam, 1995). The molecular nature of the endodermal signals is only partly known, as experimental evidence suggest that FGFs, BMPs, Edn1, and Shh are surely involved (Benouaiche *et al*., 2008; Ozeki *et al*., 2004; Vieux-Rochas *et al*., 2007).

In this study, we have analyzed mice with combined and/or partial loss of *Edn1* and *Dlx5;Dlx6* alleles. The Edn1→Dlx5/6→Hand2 signaling is a relevant model to study the spatio-temporal dynamics of gene expression in the PA1 and the consequences for CNCCs specification. Indeed *Edn1* is expressed in the endoderm and in the mesodermal core of the mandibular prominence of PA1, whereas *Ednra* (*Edn1 receptor-type A*) is broadly expressed by the CNCC-derived PA1 ectomesenchyme and *Dlx5;Dlx6* are only expressed in the mesenchyme of the mandibular prominence (Abe *et al*., 2007; Clouthier *et al*., 1998, 2000; Ozeki *et al*., 2004; Ruest *et al*., 2004, 2005). Loss of Edn1→Ednra signaling results in the down regulation of the two members of the *distalless* homeobox gene family *Dlx5* and *Dlx6* (Merlo *et al*., 2002a; Panganiban and Rubenstein, 2002), and in a homeotic-like transformation of lower into upper jaw structures, similar to that observed upon double inactivation of *Dlx5* and *Dlx6* (Beverdam *et al*., 2002; Depew *et al*., 2002; Fukuhara *et al*., 2004; Ruest *et al*., 2004). The constitutive activation of the Edn1→Ednra signaling in the entire PA1 induces a partial transformation of the upper jaw suggesting that PA1 CNCCs are competent to respond to Edn1 signaling.

Within the PA1 of E10.5 mouse embryos *Dlx* genes are expressed in nested proximo/distal domains: *Dlx1* and *Dlx2* in the proximal and distal maxillary and mandibular prominences, *Dlx5* and *Dlx6* in the entire mandibular prominence, while *Dlx3* only in a medio/distal territory of the mandibular prominence (Depew *et al*., 2002; Merlo *et al*., 2000). The most informative data on the role of *Dlx* genes in PA1 patterning come from the analysis of mice carrying single or multiple inactivating mutations for *Dlx1, Dlx2*, *Dlx5*, and *Dlx6*. In *Dlx5;6* double mutant mice, lower jaw cartilages and bones are transformed and acquire the shape typical of upper jaw elements. Furthermore, in *Dlx5;6* double null mice, *vibrissae* and palatine rugae are symmetrically present in the upper and lower jaw, suggesting that an homeotic transformation has taken place (Beverdam *et al*., 2002; Depew *et al*., 2002). In Dlx1;2 double null mice the proximal maxillary region develops abnormal skeletal elements reminiscent of the reptilian upper jaw (Depew *et al*., 2005; Qiu *et al*., 1997). These observations have led to the proposition that the combinatorial expression of Dlx genes by PA1 CNCCs determine their relative position and their capacity to give rise to different skeletal elements (Depew and Simpson, 2006; Depew *et al*., 2005; Merlo *et al*., 2000).

Several genes have been shown to act downstream of *Dlx5* and *Dlx6*, including *Gsc*, *Pitx1*, *Wnt5a*, *Dlx3*, *Meis2*, and the bHLH transcription factor *Hand2* (Beverdam *et al*., 2002; Depew *et al*., 1999, 2002; Merlo *et al*., 2000, 2002a). A further set of candidate targets of Dlx5/6 have been recently identified (Jeong *et al*., 2008). Several of the proposed targets might be directly regulated by Dlx5/6 (e.g., *Gbx2, Hand2*) as their promoters harbor Dlx-binding regulatory elements (Charite *et al*., 2001; Jeong *et al*., 2008).

Integrating quantitative gene expression data with observed phenotypes we propose that Edn1 signaling occurs in two phases: (1) early in development, Edn1 activates the *Dlx5/6→Hand2* pathway in postmigratory CNCCs. (2) Late in development, distinct regulations can be recognized in distinct regions of the mandibular prominence: in a more proximal region *Dlx5/6* are activated independently from Edn1 and their expression is not associated with *Gsc*. More distally *Dlx5/6* expression depends on Edn1 signaling and results in the activation of downstream genes including *Gsc* and *Pitx1. Hand2* is expressed only in the medio/distal region of the mandibular prominence and its expression depends upon at least three different, regionally restricted, regulations. We conclude that the organization of latero/proximal PA1 structures depends on the quantitative, gene-dosage dependent, regulation of the Edn1→Dlx5/6→(Gsc, Pitx1, etc…) pathway, while medio/distal lower jaw morphology depends on *Hand2* expression. Our findings may also provide the developmental framework in which to elucidate and functionally characterize the molecular lesions, yet to be identified, causing or associated with those human first arch syndromes (FAS) affecting the proximal arch.

RESULTS

Edn1 Allelic Dosage and Dynamics of *Dlx* and *Hand2* Expression in PA1

To better define the role of Edn1/Ednra signaling in the control of mandibular morphogenesis, we examined the effects of allelic reduction of *Edn1* on the expression of key regulators of PA1 patterning. First, we measured by RT-qPCR the abundance of *Dlx2, 3, 5, 6*, and *Hand2* transcripts in the dissected mandibular prominence (the ventral segment of the PA1) of WT and $Edn1^{+/-}$ E9 embryos. At this stage of development CNCCs are still migrating, but most of them have already colonized the mandibular region (Couly *et al*., 2002; Couly *et al*., 1993; Le Douarin *et al*., 2004). In $Edn1^{+/-}$ mandibular

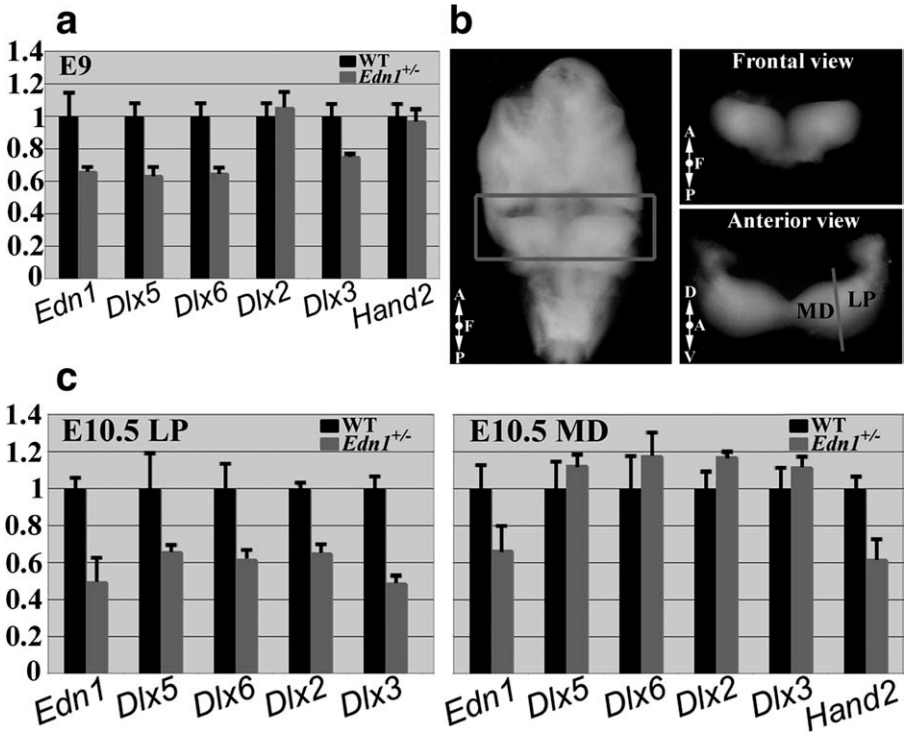

FIG. 1. Effects of allelic reduction of *Edn1* on *Dlx* and *Hand2* gene expression levels in PA1 at E9 and E10.5. (**a**) RT-qPCR measurement of mRNA abundance of *Edn1, Dlx5, Dlx6, Dlx2, Dlx3* and *Hand2* in mandibular prominences from WT (black bars) or $Edn1^{+/-}$ (red bars) at E9. (**b**) Dissection procedure used to separate the LP from the MD part of the mandibular prominence of PA1 at E10.5. The whole mandibular process was first isolated from the embryo and the latero-proximal and medio-distal portions were then separated with a single sharp cut (red line). (**c**) Quantification of the mRNA abundance of *Edn1, Dlx5, Dlx6, Dlx2, Dlx3*, and *Hand2* in LP (left) and MD (right) dissected mandibular prominences from WT (black bars) or $Edn1^{+/-}$ (red bars) at E10.5. WT is set = 1. Axis orientation: A, anterior; D, dorsal; F, frontal; P, posterior; V, ventral. LP, latero-proximal; MD, medio-distal. Black bars represent standard deviation between two independent samples.

prominences, *Edn1* expression was reduced by 38% compared to WT, while *Dlx5, Dlx6*, and *Dlx3* levels were reduced respectively of 35, 36, and 24%. *Dlx2* and *Hand2* were virtually unchanged (Fig. 1a).

Then, we carried out a similar analysis on dissected mandibular prominences obtained from E10.5 WT and $Edn1^{+/-}$ embryos. In this case we further subdivided the mandibular prominence into a latero/proximal (LP) and a medio/distal (MD) segment (as shown in Fig. 1b). In the LP segment, *Dlx2, Dlx3, Dlx5*, and *Dlx6* levels were reduced by 35, 50, 35, and 39%, respectively; *Hand2* expression was very low and was therefore not considered. In the MD segment the levels of expression of *Dlx2*, *Dlx3*, *Dlx5*, and *Dlx6* were not detectably different, while *Hand2* transcripts were reduced by 40% (Fig. 2b). In the LP and MD segments of $Edn1^{+/-}$ mandibular prominences, *Edn1* transcripts were reduced, respectively by 60 and 40% (Fig. 1c).

Thus, loss of one *Edn1* allele reduces the expression levels of *Dlx* genes in E9 mandibular prominences while at E10.5 *Dlx* expression is only reduced in the LP part of the mandibular prominence but not in the MD. However, in the MD portion of the E10.5 mandibular prominence, *Hand2* expression is detectably reduced, suggesting that Edn1 can regulate *Hand2* expression independently from *Dlx* genes.

Expression of *Dlx* Target Genes in the Mandibular Prominence of *Dlx5;Dlx6* Mutant Embryos

In different regions of the mandibular prominence of PA1 Edn1 and Dlx5/6 signaling could act independently. This led us to analyze the quantitative effects of *Dlx5/6* allelic reduction. We first examined how the loss of *Dlx5*;*Dlx6* alleles affected their own level of mRNA expression. In the mandibular prominence of $Dlx5^{+/-}$;$Dlx6^{+/-}$ embryos *Dlx5* and *Dlx6* mRNAs were reduced, respectively, by 40 and 45%, while in that of $Dlx5^{-/-}$;$Dlx6^{-/-}$ embryos *Dlx5* and *Dlx6* mRNAs were nearly undetectable (Fig. 2a). To further confirm this finding, we performed in situ hybridization. In $Dlx5^{+/-}$;$Dlx6^{+/-}$ embryos we observed a reduced *Dlx5* and *Dlx6* signal in the first and second PA, and in the otic vesicle (Fig. 2b). These results confirm that each allele contributes to the pool of transcripts and that mRNA abundance directly reflects allele dosage.

It has been shown that in the mandibular prominence of $Dlx5^{-/-}$;$Dlx6^{-/-}$ embryos, the expression of many target genes is either up- or down-regulated (Beverdam *et al*., 2002; Depew *et al*., 2002; Jeong *et al*., 2008); in particular it appears that Dlx6 directly activates the transcription of *Hand2* by binding at its promoter (Charite *et al*., 2001). We determined the expression level of putative *Dlx5;Dlx6* target genes on whole PA1s and on dissected LP and MD segments from embryos with different *Dlx5;Dlx6* allelic

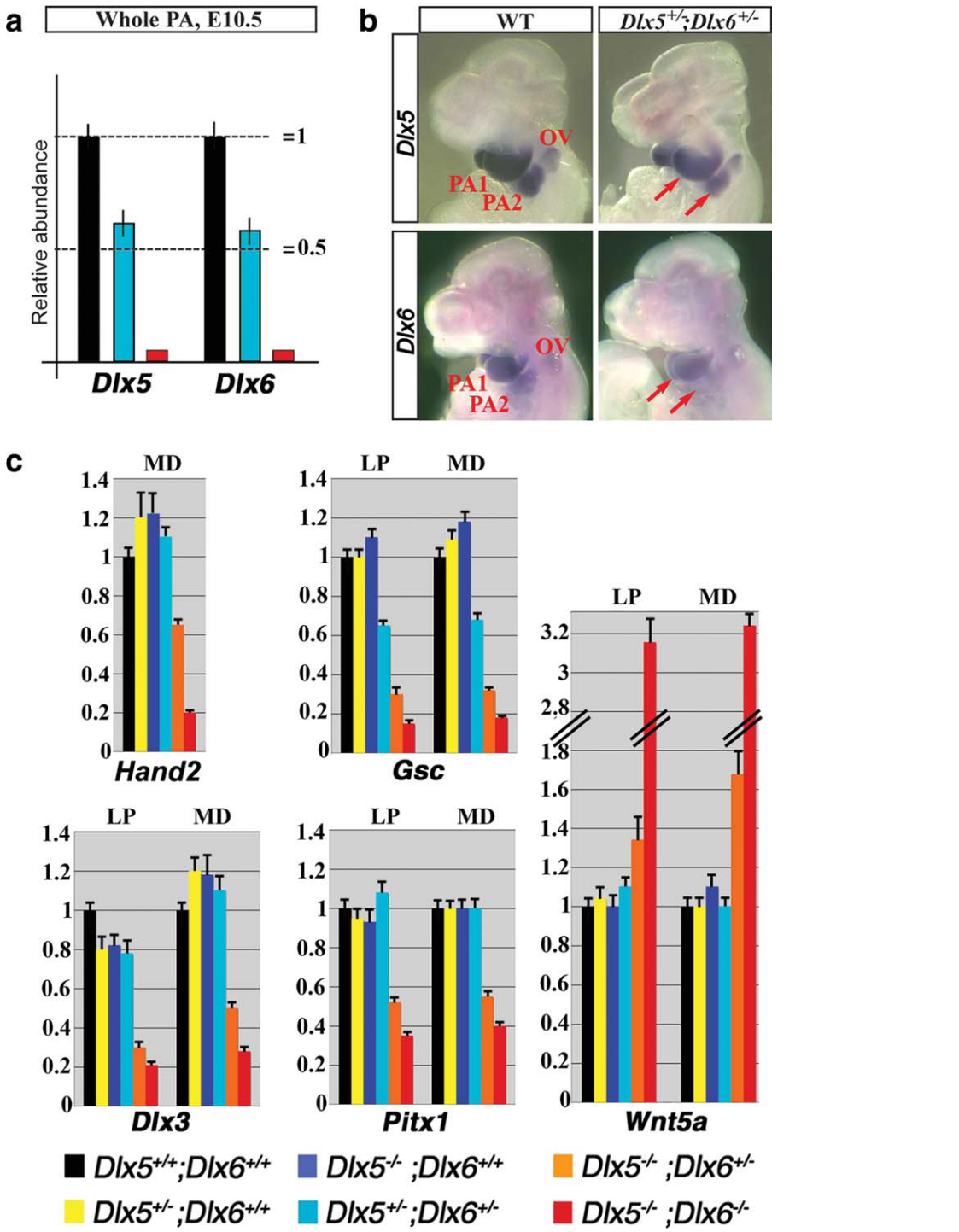

FIG. 2. Effect of allelic reduction of *Dlx5* and *Dlx6* on gene expression levels of target genes in PA1 at E10.5. (**a**) RT-qPCR measurement of *Dlx5* and *Dlx6* transcripts abundance in PA1 of E10.5 WT (black bars), $Dlx5^{+/-};Dlx6^{+/-}$ (blue bars) or $Dlx5^{-/-};Dlx6^{-/-}$ (red bars) embryos. The WT is set = 1, standard deviation is reported. (**b**) In situ hybridization with *Dlx5* (top) and *Dlx6* (bottom) probes on E10.5 WT (left) and $Dlx5^{+/-}$;Dlx6$^{+/-}$ (right) embryos, showing reduction in mRNA levels in the PAs and otic vesicle of heterozygous embryos. (**c**) PA1s were dissected from E10.5 embryos with progressive loss of *Dlx5* and *Dlx6* alleles and further divided into LP and MD regions (see Fig. 1), and stored individually. Samples of similar genotype were pooled. The levels of expression of the *Dlx* targets *Hand2, Pitx1, Dlx3, Gsc*, and *Wnt5a* were measured by RT-qPCR. The results are color-coded by the number of absent *Dlx* alleles. WT is set = 1.

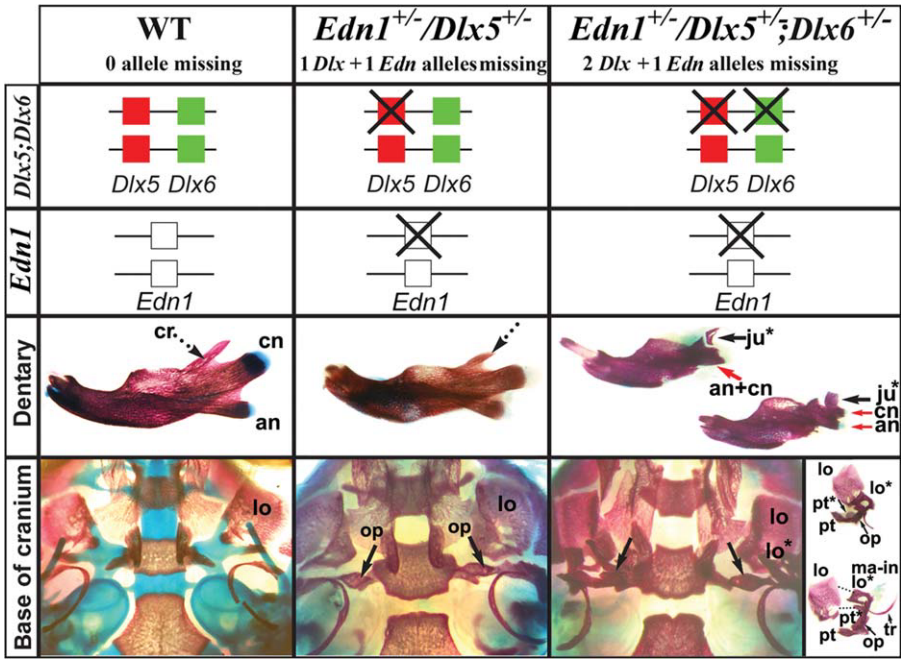

FIG. 3. Allelic reduction of *Dlx5*;*Dlx6* and *Edn1* results in specific proximal defects. WT, $Edn1^{+/-}/Dlx5^{+/-}$ and $Edn1^{+/-}/Dlx5^{+/-};Dlx6^{+/-}$ newborn mice. Loss of one *Dlx* and one *Edn1* allele results in reduction of the coronoid process of the dentary (dashed arrows) and in the appearance of an extra bone between the pterygoid bone and the middle ear (*os paradoxicum*; green arrows). In $Edn1^{+/-}/Dlx5^{+/-};Dlx6^{+/-}$ mice (right), we observe fusion of the condylar and angular processes (red arrows) of the dentary bone; appearance of duplicated jugal bones in the proximal mandibular arch (black arrows) and the appearance of the *os paradoxicum* (green arrows). Note also the appearance of duplicated structures (asterisks) in the lamina obturans/pterygoid region of the base of the cranium, (further dissociated and shown on the right). *Abbreviations*: an, angular process; cn, condylar process; cr, coronoid process; ds, dentary-squamosal articulation; ju, jugal bone; LO, Lamina Obturans; op, *os paradoxicum*; pt, pterygoid; tb; tympanic bone; tr, tympanic ring; zy, zygomatic arch.

dosage (Fig. 2c, Supporting Information Fig. 1). Both up- (*Wnt5a, Meis2*) and down-regulated (*Hand2*, *Pitx1*, *Dlx3*, and *Gsc*) transcripts were examined.

Similar regulations were observed in the LP and MD subregions, with the exception of *Hand2* whose expression in LP was very low and could not be analyzed by RT-qPCR. Allelic reduction of only one or two *Dlx5/6* alleles did not have detectable effects with the exception of *Gsc*, which was reduced of 35% in both LP and MD regions. Inactivation of three out of four *Dlx5/6* alleles ($Dlx5^{-/-};Dlx6^{+/-}$) resulted in more pronounced regulations: *Hand2* (−35%), *Pitx1* (−45%), *Dlx3* (−50%), *Gsc* (−65%), and *Wnt5a* (+170%). In $Dlx5^{-/-};Dlx6^{-/-}$ embryos: *Hand2* was reduced of 80%, *Pitx1* of 60%, *Dlx3* of 75% *Gsc* of 85% while *Wnt5a* was increased three folds (Fig. 2c). *Meis2* expression was slightly increased (+30%) in the PA1 of $Dlx5^{-/-};Dlx6^{-/-}$ embryos (Supporting Information Fig. 1a) but did not change in all the other genotypes.

The progressive reduction in mRNA abundance of *Hand2* and *Dlx3* in the mandibular prominence of embryos with three or four *Dlx5/6* alleles missing was verified by in situ hybridization. While in $Dlx5^{-/-};Dlx6^{-/-}$ embryos *Hand2* and *Dlx3* expression was below detection, in $Dlx5^{-/-};Dlx6^{+/-}$ embryos we observed a reduced hybridization signal compared to WT embryos (Supporting Information Fig. 1b). These findings suggest that a threshold level of *Dlx5* and *Dlx6* mRNA is necessary to activate target gene transcription.

Craniofacial Phenotypes of Mice With Combined Loss of *Edn1* and *Dlx5;Dlx6* Alleles

Dlx and *Hand2* genes play important roles in the control of craniofacial morphogenesis. As the loss of one *Edn1* allele reduces the expression levels of *Dlx* and *Hand2* genes, we analyzed the skulls of $Edn1^{+/-}$ newborn mice, but no obvious malformation could be detected (not shown); this finding is not surprising as $Dlx2^{+/-}$, $Dlx5^{+/-}$, $Dlx5^{+/-}$; $Dlx6^{+/-}$ and $Hand2^{+/-}$ mice also show only minor craniofacial defects (Acampora *et al*., 1999; Beverdam *et al*., 2002; Depew *et al*., 1999; Robledo *et al*., 2002; Yanagisawa *et al*., 2003). As the loss of one *Edn1* allele could further reduce the level of *Dlx5/6* and/or *Hand2* expression, we examined the craniofacial skeleton of combined *Edn1/Dlx* mutant mice. We therefore crossbred $Edn1^{+/-}$ mice with either $Dlx5^{+/-}$ or $Dlx5^{+/-};Dlx6^{+/-}$ mice.

When both one *Edn1* and one *Dlx5* allele were lost, we observed a slightly shorter coronoid process of the dentary and the appearance of an *os paradoxicum* at the base of the cranium, highly reminiscent of that observed in $Dlx5^{+/-};Dlx6^{+/-}$ or in $Dlx5^{-/-}$ or $Dlx6^{-/-}$ mutants; in each of these allelic configurations two *Dlx*5/6 alleles are missing (Fig. 3, Supporting Informa-

tion Fig. 4 and Table 1; Jeong *et al*., 2008). No other obvious defect was observed.

In *Edn1*$^{+/-}$/*Dlx5*$^{+/-}$;*Dlx6*$^{+/-}$ mice we observed a more severe phenotype. The distance between the condylar and angular processes of the dentary was reduced and often these two processes fused into a single large structure, similar to the zygomatic process of the maxilla. The coronoid process was missing and an additional skeletal element was often observed between the abnormal condylar process (lower jaw) and the jugal bone (upper jaw). This new structure could be interpreted as a duplicated jugal bone. At the base of the cranium, the pterygoid and the *ala temporalis* were duplicated and fused with the *os paradoxicum* and positioned ventrally to overlap with the normal structure (see Fig. 3). Collectively, these phenotypes closely resemble those observed in *Dlx5*$^{-/-}$;*Dlx6*$^{+/-}$ animals (three *Dlx* alleles missing; Supporting Information Fig. 2; Beverdam *et al*., 2002; Depew *et al*., 2005). Indeed *Dlx5*$^{-/-}$;*Dlx6*$^{+/-}$ mice also display reduced distance or fusion of the condylar and angular processes of the dentary and the lateral extension of the fused processes giving rise to a structure similar to the zygomatic process of the maxilla. In these mice an extra element is also present, which can be interpreted as a duplicated jugal bone and duplication of the pterygoid-ala temporalis-lamina obturans on the mandibular side. Thus, the anomalies seen in *Dlx5*$^{-/-}$;*Dlx6*$^{+/-}$, and in *Edn1*$^{+/-}$/*Dlx5*$^{+/-}$;*Dlx6*$^{+/-}$ newborns affect derivatives of the proximal region of the mandibular segment, while derivatives of the distal region such as the body of the dentary show no major defects. In most embryos these defects were asymmetric, namely the left side of the mandible was more severely affected than the right one (data not shown). In summary: (1) the gradual changes observed in the levels of expression of Dlx5/6 targets correlate well with the progressive onset of specific skeletal anomalies affecting the proximal lower jaw and 2) the reduction of *Edn1* level of transcription, in combination with the loss of one or two *Dlx5;Dlx6* alleles, has phenotypic consequences similar to the loss of one additional *Dlx* allele (Figs. 2 and 3, and Supporting Information Fig. 3).

Remarkably, the defects caused by allelic reduction of *Edn1* and *Dlx5;Dlx6* resemble those present in patients affected by first arch syndromes (FAS) in which only proximal derivatives of PA1 are affected and which often show the presence of ectopic bones positionally homologous to a duplicated jugal (see Discussion).

Effect of *Ednra* and *Dlx5/6* Inactivation on Downstream Targets Expression Pattern

In the mandibular prominence of normal E10.5 embryos, *Dlx5* and *Dlx6* are expressed in a large and coherent territory extending distally from a proximal limit corresponding to the maxillo/mandibular boundary. The distal-most region of PA1, including the medial fusion, does not, however, express *Dlx5* and *Dlx6* (Fig. 4a,c,e,g). Inactivation of *Ednra* completely prevents the expression of *Dlx5* and *Dlx6* in the E9.5 PA1 (Ozeki *et al*., 2004); in these same mutants at E10.5, however, *Dlx5* and *Dlx6* are expressed in an Edn1-independent territory in the proximal part of PA1 (Fig. 4b,d,f,h black arrows; Ozeki *et al*., 2004). In normal E10.5 embryos, *Gsc* expression is limited to a distal region of PA1 overlapping in part with the Edn1-dependent territory of *Dlx* activation. *Gsc* expression is abolished in both *Ednra* and *Dlx5/6* mutant mice (Fig. 4i–n). Careful analysis of our embryos revealed an additional territory of *Gsc* expression in the proximal endoderm of PA1 (red arrows, Fig. 5i–k,n), this small territory of expression is independent from both Ednra and Dlx5/6.

DISCUSSION

An emerging theme in developmental biology is the importance of quantitative functions shared by related and coexpressed genes. Examples of these are the signaling functions of FGFs expressed in the apical ectodermal ridge (Mariani *et al*., 2008), the gene-dosage dependent functions of *Msx1* and *Msx2* for osteogenic differentiation of CNCCs (Han *et al*., 2007), and the progressive limb phenotypes associated with the combined loss of posterior *HoxD* alleles and with a gradual increase of expression of the HoxD target Epha3 (Cobb and Duboule, 2005). Our study offers a new example in this direction. One implication of this is that the function of individual genes is best examined upon partial and cumulative gene losses, and within the context of expression of related genes. Indeed, the examination of developmental phenotypes in mice homozygous for recessive mutant mice, although widely used, has serious limitations. One of these is the inability to recognize late-occurring regulations (or phenotypes), in case an early event severely affects morphogenesis, patterning or embryonic viability. Second, we cannot appreciate the phenotypic consequences of reduced gene expression; third we may fail to recognize the dynamics of cell–cell signaling and interactions, as these often require a nearly normal context. Such is the case of Edn1→Dlx signaling at the basis of the homeotic lower jaw transformation, to investigate which many studies have been carried out based on either loss-of-function (Acampora *et al*., 1999; Beverdam *et al*., 2002; Clouthier *et al*., 1998; Depew *et al*., 1999, 2002; Kurihara *et al*., 1994; Ozeki *et al*., 2004; Sato *et al*., 2008a; Thomas *et al*., 1998; Yanagisawa *et al*., 2003) or gain-of-function mutants (Sato *et al*., 2008b). Here we provide quantitative data on the effects of allelic reduction of *Edn1* and *Dlx5;Dlx6* at different developmental stages. Our findings are complementary to those recently reported by Ruest and Clouthier (2009) using CNCC-specific gene deletion and receptor antagonism, and corroborate and extend their major conclusions. We show that, during PA1 development, different Edn1-dependent regulatory pathways act at diverse developmental times in distinct regions of the mandibular prominence. We also show that upon partial

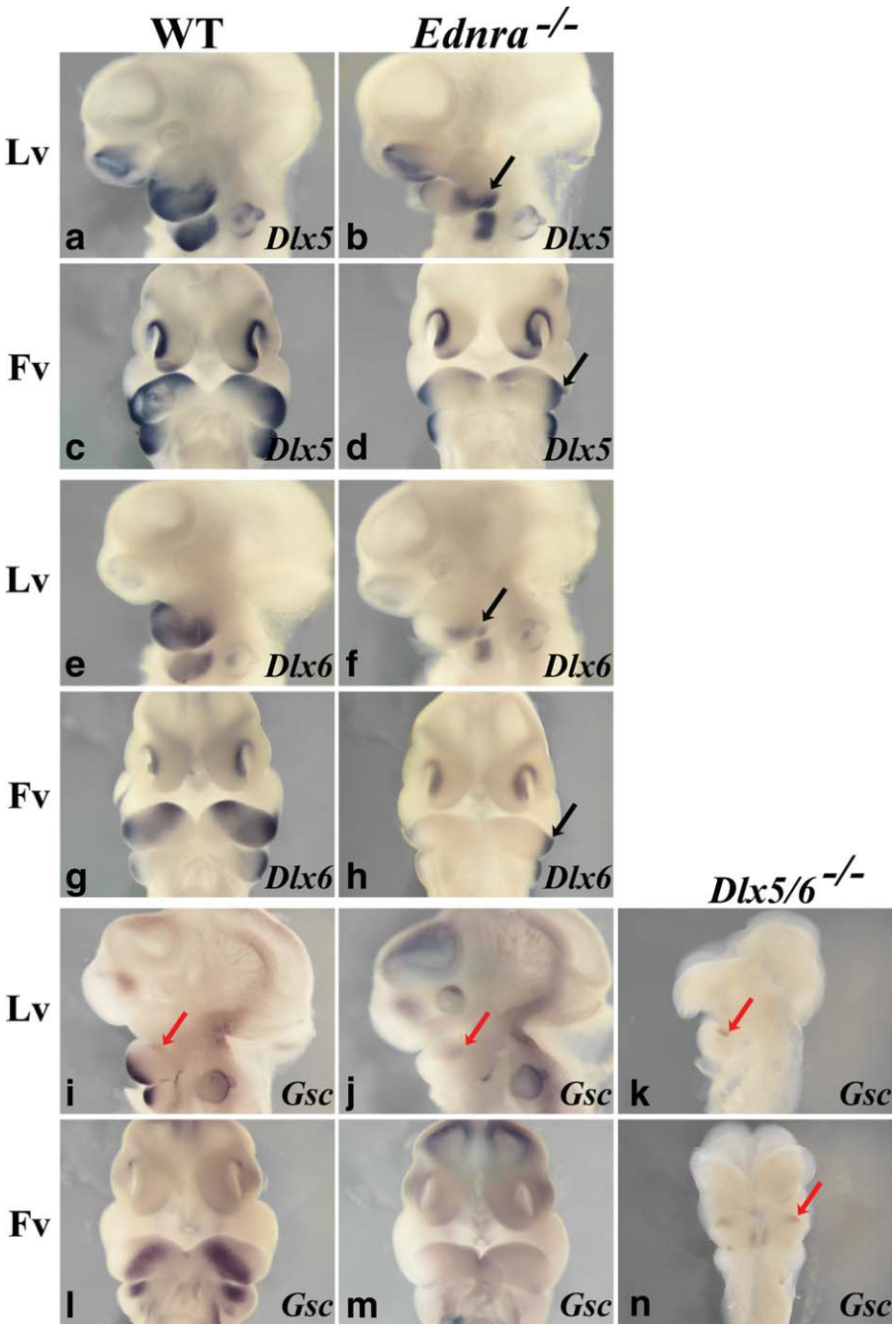

FIG. 4. *Dlx5*, *Dlx6* and *Gsc* expression in *Ednra* and *Dlx5/6* mutant mice. Whole-mount in situ hybridization on wild-type (**a**,**c**,**e**,**g**,**i**,**l**), *Ednra*$^{-/-}$ (**b**,**d**,**f**,**h**,**j**,**m**) or *Dlx5/6*$^{-/-}$ (**k**,**n**) E10.5 embryos using *Dlx5* (a–d), *Dlx6* (e–h) and *Gsc* (i–n) probes. *Dlx5* and *Dlx6* are expressed in the mandibular part of the PA1 and in the PA2 of wild-type embryos (a,b,e,f). In Ednra homozygous mutants, distal expression of *Dlx5* and *Dlx6* is lost in PA, but is still maintained in the proximal part of PA1 (black arrow) and PA2 (c,d,g,h). In normal embryos, *Gsc* is expressed in a latero-distal region within PA1 and PA2 and in a small endodermal territory located at the mandibulo-maxillary junction (red arrow) (i,l). In *Ednra* and *Dlx5/6* mutant embryos, *Gsc* expression is lost in the distal PA1 and PA2 whereas is still maintained in the endoderm at the mandibulo-maxillary junction (j–n). Fv, Frontal view; Lv, Lateral view.

allele loss, the proximal territory of mandibular prominence is the region mainly affected.

At early stages of CNCCs colonization, Edn1 signaling activates *Dlx5/6* expression in CNCCs; accordingly *Dlx5/6* mRNAs are reduced at E9 in the mandibular prominence of *Edn1* heterozygotes (see Fig. 1). If early Edn1 signaling is abrogated (i.e., in *Edn1* or *Ednra*-null mice), *Dlx5/6* fail to be activated in the entire PA1 at least up to E9.5 (Abe *et al*., 2007; Ozeki *et al*., 2004; see Fig. 5). This implies that signals that pattern *Dlx* expres-

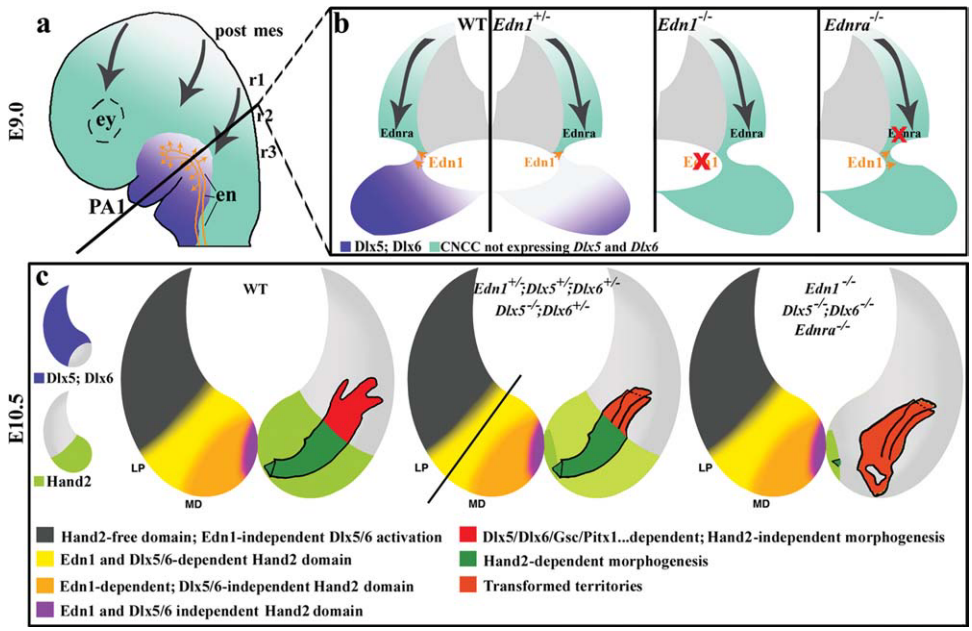

FIG. 5. Summary diagram of Edn1-dependent regulations occurring during PA1 development and hypothetical model for the origin of first arch syndromes. (**a**) Schematic view illustrating CNCCs migration on a lateral view of an E9 mouse embryo. In orange is indicated the endoderm from which *Edn1* signaling originates, in purple the postmigratory CNCCs expressing *Dlx5/6*, in light blue the territory of migration of CNCCs (arrows). (**b**) Drawings represent transverse sections through the embryo in (A), the same color code is used. Sections corresponding to WT, *Edn1*$^{+/-}$, *Edn1*$^{-/-}$ and *Ednra*$^{-/-}$ embryos are shown. Note the reduced level of *Dlx5/6* in *Edn1*$^{+/-}$ embryos and the absence of early *Dlx5/6* activation when Edn1 signaling is disrupted. (**c**) Summary scheme representing different modes and territories of gene activation in E10.5 mandibular prominence. Small inserts on the left represent the territories of expression of *Dlx5/6* (upper, purple) and *Hand2* (lower, light green) respectively in WT embryos. The diagram on the left represents the frontal view of the mandibular part of PA1 of a WT E10.5 embryo. The central diagram refers to three *Dlx/Edn1* alleles missing, either *Dlx5*$^{-/-}$;*Dlx6*$^{+/-}$ or *Edn1*$^{+/-}$/*Dlx5*$^{+/-}$;*Dlx6*$^{+/-}$. Finally the right diagram refers to homozygous mutants for either *Edn1*, *Ednra* or *Dlx5/6*. The color code used in the left side of each drawing indicates the four different regulations observed in PA1 at this stage and are detailed in the caption of this panel. The large grey area corresponds to the Hand2-independent part of PA1, while in all colored, distal regions Hand2 regulation is important for correct morphogenesis. The right part of the diagram depicts the levels of *Hand2* expression encountered in the different mutants as well as the morphogenetic defects occurring in the proximal and distal part of the dentary. The subdivisions of different regions of the mandibular prominence are not divided by actual borders, but represent partially overlapping expression/regulation territories. Color-coded region have faded borders to indicate this. *Abbreviations*: en, endoderm; ey, eye; LP, latero proximal; MD, medio-distal; post mes, posterior mesenchephalon; r1, rhombomere 1; r2, rhombomere 2; r3, rhombomere 3.

sion, such as Edn1 or FGF8 likely act on CNCCs prior to E10.5; for example *Dlx5* expression in response to Edn1 initiates around E8.5-E9 in CNCCs migrating to the distal PA1 (Vieux-Rochas *et al*., 2007).

At later stages (E10.5) the situation is more complex. Combining our data with results reported in the literature, we propose a model in which the E10.5 mandibular arch is subdivided into four adjacent territories, in which distinct timing and patterns of gene expression are linked to the onset of specific phenotypes (Fig. 5c): (1) in the distal-most region (purple in Fig. 5c), *Hand2* expression is independent from both Edn1 and Dlx5/6. Indeed, *Hand2* expression is retained in a small distal territory in *Edn1*-null, *Ednra*-null and *Dlx5;6*-null animals (Beverdam *et al*., 2002; Clouthier *et al*., 2000; Fukuhara *et al*., 2004; Ozeki *et al*., 2004; Ruest *et al*., 2004), possibly associated with the specific fate of this region to undergo midline fusion (Barbosa *et al*., 2007). (2) in the MD region of PA1, *Hand2* expression can be activated by Edn1 independently of Dlx5/6 as seen from the fact that *Edn1* allelic reduction affects *Hand2*, but not *Dlx5/6* expression (see Fig. 1). This Dlx-independent *Hand2* regulation could well take place in the distal Dlx5/6-free region of the E10.5 PA1 (orange in Fig. 5c). (3) In the medial region of PA1 at E10.5 (yellow in Fig. 5c), *Hand2* is activated through the established Edn1→Dlx6 pathway most probably involving the reported Dlx6-dependent *Hand2* enhancer (Charite *et al*., 2001). Notably, inactivation of this enhancer results in defects in the medio-distal part of PA1 as suggested by our model (Yanagisawa *et al*., 2003) and by timed inhibition of Edn1 signaling using Ednra antagonists (Ruest and Clouthier, 2009). (4) Finally, in the proximal part of the E10.5 PA1 (grey in Fig. 5c), *Hand2* is never expressed. Within subterritories of this same region *Dlx5* and *Dlx6* can be activated even in the absence of an Edn1 inducing signal. These different sub-territories could confer a regional selectivity, in turn required for the correct unfolding of the lower jaw morphogenetic program.

Allelic reduction of *Edn1* results in lower *Dlx5/Dlx6* (and *Dlx2* and *Dlx3*) expression in the proximal, but not

in the medio-distal part of the mandibular prominence at E10.5. Therefore, the expression of *Dlx* genes in the distal PA1 (at early stages) is independent from Edn1. A possible interpretation of these findings is that an initial Edn1 signal is necessary to activate *Dlx5/6* expression in incoming CNCCs and that, at later stages, the distal expression of these genes is maintained independently of Edn1 (Fig. 1c). In support of this, Dlx5/6 expression in the proximal PA1 is reactivated at E10.5 even in the absence of Edn1 (Fig. 5a–h; Ozeki *et al*., 2004), indicating the existence of an Edn1-independent mechanism of *Dlx5/6* activation or maintenance in the LP region. The reduced expression of *Dlx2* and *Dlx3* in the presence of only one *Edn1* allele may indicate the possibility of a global Edn1→Dlx control, or of Dlx5;6 regulating the expression of *Dlx2* and *Dlx3*. This hypothesis, however, would need to be specifically tested.

Allelic reduction of *Edn1* affects *Hand2* expression in the MD territory suggesting an Edn1-dependent, Dlx-independent regulation of *Hand2* which might take place in the Dlx-free region of the distal PA1. Hand2 is expressed in the distal mandibular prominence and its inactivation causes loss of distal skeletal elements of the lower jaw (Yanagisawa *et al*., 2003). Analysis of the regulatory regions of *Hand2* has revealed the presence of an *Edn1*-responsive enhancer whose activation depends upon binding of Dlx6, although other, yet unspecified, Edn1-dependent proteins could bind to this enhancer (Charite *et al*., 2001). On the basis of these results, it has been proposed that Hand2 is the final effector of the Edn1→Dlx5/6 regulatory cascade and its level of expression could determine the shape of the distal lower jaw (Sato *et al*., 2008b; Yanagisawa *et al*., 2003). However, targeted inactivation of the Edn1/Dlx6-dependent enhancer does not completely abrogate *Hand2* expression in the distal part of PA1 suggesting that other, not yet identified, regulatory elements might activate *Hand2* expression in PA1 (Yanagisawa *et al*., 2003). As *Hand2* is expressed only in the medio-distal portion of PA1 while *Edn1*, *Ednra* and *Dlx* genes are expressed both in proximal and distal parts of PA1 an active suppression mechanism for *Hand2* expression might be acting in the proximal territory.

Considering *Hand2* expression and regulation, and the loss of the distal lower jaw in Hand2 null mice (Thomas *et al*., 1998; Yanagisawa *et al*., 2003), we conclude that the mandibular arch is subdivided into two Hand2-independent and dependent parts corresponding to the proximal and distal part of the dentary, respectively. This notion is supported by the fact that, forced expression of *Hand2* in the whole PA1, including the maxillary arch, induces only transformation of maxillary derivatives into distal mandibular structures (Sato *et al*., 2008b).

The phenotypes of mice carrying combined *Dlx* gene mutations, and the nested expression of *Dlx* genes within the PAs at E10.5 have led to the proposal that *Dlx* genes might establish maxillo-mandibular identity by providing a *Hox*-like proximo/distal and upper/lower combinatorial code (Depew *et al*., 2002, 2005). A more sophisticated model, known as the "hinge-caps" organization of the PA1, has been proposed (Depew and Compagnucci, 2008). Both of these models, however, do not take in account the dynamics of gene expression and cell migration during PA1 development. In our view, the nested *Dlx* gene expression pattern is likely to be the consequence of patterning events occurring at much earlier stages, as by E10.5 most CNCCs have already migrated to their final position, have initiated expression of PA-specific genes and are fate-committed (Couly *et al*., 1998; Le Douarin *et al*., 2004; Le Douarin and Dupin, 2003).

CNCCs of the proximal mandibular prominence appear more sensitive to variations in the genetic environment, than are distal ones: inactivation or allelic reductions of *Edn1, Ednra, Dlx5* (Acampora *et al*., 1999; Depew *et al*., 1999), *Dlx6* (Jeong *et al*., 2008), *Gsc* (Yamada *et al*., 1995), *Pitx1* (Bobola *et al*., 2003; Lanctot *et al*., 1999), *Gbx2* (Byrd and Meyers, 2005) all lead to proximal defect of the dentary or of the middle and external ear whereas derivatives of the distal part of the first arch are not affected. Interestingly, *Dlx5;Dlx6* are expressed at higher level distally (Figs. 2 and 4) and even allelic reduction of *Edn1* results in maintaining their distal expression levels. These findings suggest the existence of a threshold level of expression of *Dlx* for the activation of targets genes.

Human first arch syndromes (FAS) include a wide spectrum of congenital anomalies characterized by defects of CNCC derivatives, and in most cases proximal and not distal jaw structures are affected (Gorlin, 2001). The abnormal traits are associated with different conditions including for example oculo-auriculo-vertebral spectrum (OAVS, OMIM 164210), hemifacial microsomia, mandibulofacial dysostosis, Goldenhar or Franceschetti syndromes. The consequence is a lateral deviation of the mandible accompanied by an anomaly of the dentary occlusion and hearing deficiency. The phenotypes of FAS are strongly suggestive of a defect of CNCCs, and interestingly, targeted inactivation of genes involved in patterning CNCCs often results in proximal defects of the dentary and/or of the middle and external ear (for a recent review see: Gitton *et al*., 2010). Based on morphological similarities with mouse mutant models, the involvement of Edn1 and putative targets in FAS has been suggested (Kelberman *et al*., 2001; Masotti *et al*., 2008; Singer *et al*., 1994), but not experimentally proven. Our observation on partial allele losses of the Edn1-Dlx pathway might help explain why human FAS affect proximal, rather than distal, derivatives of PA1.

A final general conclusion of our study is that early morphogenetic signals seem to define "large" territories of the craniofacial anlage while subsequent regulations coordinate much more spatio-temporally defined and diversified structures, to specify more "local" shapes of individual elements of the jaw. Distinct time-specific levels of regulation might help to explain the apparent contradiction between data suggesting that CNCCs specification

requires external signals (Benouaiche *et al*., 2008; Couly *et al*., 2002; Le Douarin *et al*., 2004; Le Douarin and Dupin, 2003; Vieux-Rochas *et al*., 2007) and data suggesting that CNCCs are instead endowed with cell-autonomous information to generate craniofacial structures (Schneider and Helms, 2003). As expected, early signals (Edn1, FGF8, others) appear more conserved in different animal classes, while subsequent complex regulations might considerably vary from genome to genome and could contribute to jaw diversification in vertebrates.

METHODS

Mouse Mutants

Animal procedures were approved by National and Institutional ethical committees. Mouse strains were maintained on B6/D2 F1 hybrid genetic background. *Edn1* mutant mice were genotyped as indicated (Kurihara *et al*., 1994). Mice with targeted disruption of *Dlx5* or *Dlx5;Dlx6* were genotyped as previously reported (Acampora *et al*., 1999; Beverdam *et al*., 2002; Merlo *et al*., 2002b). The genotypes of embryos obtained from mixed *Dlx* heterozygous parents were determined using the *Dlx5-lacZ* or the *Dlx5;Dlx6*-mutant allele-specific forward primers L-proF and G-proF, respectively, and the *lacZ* reverse primer, with the following sequence:

- *L-proF (Dlx5* allele) 5′CGCAGTAGAAGAACAGC CAC
- *G-proF (Dlx5;Dlx6*-mutant allele) 5′GAGCTATGAC AGGAGTGTTTG
- *KO6 RT-R2 (lacZ* reverse) 5′GGCGATTAAGTTGG GTAACG

$Edn1^{+/-}$ animals were crossbred with $Dlx5^{+/-}$ and $Dlx5^{+/-}$;$Dlx6^{+/-}$ to generate double and triple heterozygotes, and from these $Edn1^{+/-}$;$Dlx5^{-/-}$ and $Edn1^{+/-}$; $Dlx5^{-/-}$;$Dlx6^{+/-}$ animals were obtained.

Skeletal Preparations and In Situ Hybridization

Skeletal staining of E14.5 embryos and newborn animals (Alcian Blue for E14.5 embryos, Alizarin Red/Alcian Blue for newborns) was carried out as previously described (Vieux-Rochas *et al*., 2007). A minimum of 4, with a maximum of 10, embryos/newborns per genotype were analyzed for skeletal phenotypes, per each genotype.

In situ hybridization was done with DIG-labeled RNA probes corresponding to the antisense sequence of murine *Dlx3*, *Dlx5*, *Dlx6, Gsc* and *Hand2* (all previously reported: (Charite *et al*., 2001; Perera *et al*., 2004; Radoja *et al*., 2007), using the procedure described by Wilkinson and Nieto (1993). For each probe, at least three normal and three mutant specimens were examined. For semi-quantitative comparisons, all the procedures were carried out in the same vials on littermate embryos; the time of chromogenic reaction was reduced to avoid signal saturation.

Tissue Collection, RNA Extraction, and RT-qPCR

E9 or E10.5 embryos were genotyped by PCR on DNA extracted from extra-embryonic tissues. The PA1s were dissected under stereomicroscope using fine scissors, further separated into a proximal and a distal part (see Fig. 5c). The anatomic hallmark was the bulge formed at the PA1 end. Sections were carried out vertically in a rostro-caudal way. Tissues were collected in RNA later (Ambion), pooled according to the genotype, transferred in Tripure Reagent (Roche) and processed for RNA extraction as indicated by the manufacturer. A minimum of three PA1s per genotype were pooled in one sample, two biological replicates were prepared. Each sample was analyzed in duplicates (technical replicates). RNA quality, primer efficiency and correct product size were verified by RT-PCR and agarose gel electrophoresis. qPCR was performed with LightCycler (Roche) using FastStart DNA MasterPLUS SYBR-Green I (Roche). Five microliter of cDNA were used in each reaction, standard curve were done using WT cDNA with four calibration points: TQ; 1:3; 1:9; 1:27. Specificity and absence of primer dimers was controlled by denaturation curves. *GAPDH* mRNA was used for normalization. Results of mutant tissues are expressed as fold-change relative to the corresponding WT. For each target, the mRNA abundance was calculated relative to GADPH, using the LightCycler Software 3.5.3, based on the general formula Δ (Δ CT). Because of the limited sample size (two replicates) and the two steps of normalization, the Student *t*-test could to determine statistical significance could not be done.

- GAPDH Sens 5′TGTCAGCAATGCATCCTGCA
- GAPDH Antisens 5′TGTATGCAGGGATGATGTTC
- Hand2 Sens 5′CCAGCTACATCGCCTACGTC
- Hand2 Antisens 5′TTGCTGCTCACTGTGCTTTT
- Wnt5a Sens 5′AGGAGTTCGTGGACGCTAGA
- Wnt5a Antisens 5′ACTTCTCCTTGAGGGCATCG
- Bmp7 Sens 5′GCGATTTGACAACGAGACCT
- Bmp7 Antisens 5′AGGGTCTCCACAGAGAGCTG
- Dlx3 Sens 5′CGTTTCCAGAAAGCCCAGTA
- Dlx3 Antisens 5′CGTGGAATGGGAAGATGTGT
- Dlx5 Sens 5′CTGGCCGCTTTACAGAGAAG
- Dlx5 Antisens 5′CTGGTGACTGTGGCGAGTTA
- Dlx6-5F Sens 5′CTCAATACCTGGCCCTTCC
- Dlx6-5R Antisens 5′AGAGCGCTTATTCTGAAACCAT
- Meis2 Sens 5′ATCTCAAGGCAAGGGGAAGT
- Meis2 Antisens 5′GAGTAGGGTGTGGGGTCATC
- Pitx1 Sens 5′ATCGTCCGACGCTGATCT
- Pitx1 Antisens 5′CTTAGCTGGGTCCTCTGCAC
- Gsc Sens 5′ACCGATGAGCAGCTCGAA
- Gsc Antisens 5′GCGGTTCTTAAACCAGACCTC
- Edn1 Sens 5′TCCTTGATGGACAAGGAGTGT
- Edn1 Antisens 5′TCGTACCGTATGGACTGGG

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