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The transcription factor MEF2C is required for craniofacial development

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Summary

MEF2 transcription factors are well-established regulators of muscle development. We have discovered an unanticipated role for MEF2C in the neural crest, where tissue-specific inactivation results in neonatal lethality due to severe craniofacial defects. We show that MEF2C is required for expression of the *Dlx5*, *Dlx6*, and *Hand2* transcription factor genes in the branchial arches, and we identify a novel branchial arch-specific enhancer in the *Dlx5/6* locus, which is activated synergistically by MEF2C and *Dlx5*, demonstrating that these factors interact to induce transcription. *Mef2c* and *Dlx5/6* also interact genetically. Mice heterozygous for either *Dlx5/6* or *mef2c* are normal at birth and survive to weaning. By contrast, heterozygosity for both *mef2c* and *Dlx5/6* results in defective palate development and neonatal lethality. Taken together, the studies presented here define a novel, feed-forward transcriptional circuit between the MADS-box transcription factor MEF2C and the homeodomain transcription factors *Dlx5* and *Dlx6* in craniofacial development.

Introduction

Neural crest (NC) cells originate from the dorsal neural tube at the border between the neural plate and the lateral epidermis (Knecht and Bronner-Fraser, 2002; Trainor, 2005). When induced by contact mediated signals, NC precursor cells undergo an epithelial to mesenchymal transition, migrate throughout the developing embryo, and give rise to various tissues including the dorsal root and sympathetic ganglia, the glial cells of the peripheral and enteric nervous systems, melanocytes, and skeletal elements of the face and head (Knecht and Bronner-Fraser, 2002; Le Douarin et al., 2004; Trainor, 2005). Numerous congenital disorders are due to improper development of the NC and its derivatives, including craniofacial defects, which account for one third of all congenital anomalies (Farlie et al., 2004; Trainor, 2005).

Several transcription factors are known to be required for craniofacial development (Cobourne, 2000; Depew et al., 2005). Among these, the homeodomain transcription factors *Dlx5* and *Dlx6* are expressed in the NC-derived ectomesenchyme of the first and second branchial arches, and inactivation of *Dlx5* and *Dlx6* results in mice that have numerous craniofacial defects (Beverdam et al., 2002; Depew et al., 2002). *Dlx6* is a direct transcriptional activator of the

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Hand2 gene, which encodes a basic helix-loop-helix (bHLH) transcription factor, in the first and second branchial arches via a conserved branchial arch enhancer element (Charite et al., 2001). *Hand2* enhancer null mice exhibit cleft palate and hypoplasia of the mandible, among other craniofacial malformations (Yanagisawa et al., 2003). It is known that expression of both *Dlx6* and *Hand2* in the branchial arch mesenchyme requires endothelin signaling, but other components of this pathway, including the transcription factors required for activation of *Dlx5* and *Dlx6* are unknown (Charite et al., 2001; Clouthier et al., 2000; Ruest et al., 2004; Thomas et al., 1998).

The myocyte enhancer factor 2 (MEF2) family of transcription factors has four members in vertebrates, MEF2A–D (Black and Olson, 1998; McKinsey et al., 2002). MEF2 proteins bind to a consensus DNA-binding element known as a MEF2 site as homo- and heterodimers and interact with other transcription factors to function as both positive and negative regulators of gene expression in part through their association with class II histone deacetylases (HDACs) (McKinsey et al., 2001). *Mef2c* is the first member of the MEF2 family to be expressed during development, and *mef2c* null mice die at E9.5 with severe defects in cardiac and vascular development (Edmondson et al., 1994; Lin et al., 1998; Lin et al., 1997). Although MEF2C is widely appreciated for its role in the development of muscle lineages, its expression adjacent to the neural folds at E8.5 in early NC development has been reported previously (Edmondson et al., 1994), but its function in the NC has not been investigated.

In the present study, we used a conditional gene targeting approach in mice to inactivate *mef2c* in the NC, which results in lethality at birth due to upper airway obstruction. *Mef2c* NC knockout mice exhibit delayed ossification and hypoplasia or loss of the majority of the skeletal elements of the face and skull. We show that the transcription factor genes *Dlx5*, *Dlx6*, and *Hand2* are not expressed in the NC component of the branchial arches in *mef2c* NC conditional knockout embryos, and we identify a MEF2-dependent transcriptional enhancer in the *Dlx5/6* locus that is sufficient to direct expression to the branchial arches *in vivo*, establishing MEF2C as a direct transcriptional regulator of the *Dlx6*-*Hand2* pathway in the NC. Furthermore, the *Dlx5/6* enhancer identified in these studies is activated synergistically by MEF2C and *Dlx5*, indicating that these factors interact to regulate transcription. We also show that *mef2c* and *Dlx5/6* interact genetically. Mice heterozygous for either *Dlx5/6* or *mef2c* are normal, whereas mice heterozygous for both *mef2c* and *Dlx5/6* do not survive and exhibit defective palate development. These studies highlight a previously unappreciated role for MEF2C in craniofacial development and identify a novel feed-forward transcriptional circuit between MEF2C and *Dlx5/6* in craniofacial development.

Results

Mice lacking *mef2c* function in the neural crest die at birth due to upper airway obstruction

To determine the function of MEF2C in the NC, we conditionally inactivated a floxed allele of *mef2c* using *Wnt1-Cre* transgenic mice (Danielian et al., 1998; Vong et al., 2005). Animals lacking *mef2c* in the NC were born at expected Mendelian ratios and were animated and responsive to touch. However, *mef2c* conditional knockout mice quickly became cyanotic, and 100% of these animals died within an hour of birth (Fig. 1A).

The hearts, vasculature, and diaphragms of *mef2c* NC null mice showed no differences in gross morphology, histology, or marker gene expression compared to control littermates (data not shown). However, neonates lacking *mef2c* function in the NC exhibited a posterior cleft of the palate (Fig. 1B), a constricted airway, and defective position of the tongue near the back of the oral cavity compared to control littermates (Fig. 1C, D). These defects occurred with 100% penetrance in knockout animals (n = 63) and in none of the littermate controls. Knockout animals were tracheostomized with a 30G needle to bypass the upper airway, which resulted

in recovery from cyanosis and restoration of viability prior to humane euthanasia (data not shown), demonstrating that mice lacking *mef2c* in the NC die at birth from asphyxiation caused by upper airway obstruction.

***Mef2c* function is required in the neural crest for craniofacial development**

Skulls from *mef2c* NC conditional knockout neonates exhibited several defective or missing craniofacial structures compared to control littermates, including a hypoplastic mandible, zygomatic arch, and temporal bone (Fig. 1, compare panels E, G to F, H). Additionally, the coronoid, condular, and angular processes of the mutant mandibles were severely hypoplastic when compared to wild type littermate controls, and the tympanic ring was absent in mice lacking *mef2c* function in the NC (Fig. 1G, H). At E16.5, wild type skulls displayed normal Meckel's cartilage and extensive ossification in the mandible and maxilla (Fig. 1I). By contrast, *mef2c* NC knockout embryos exhibited hypoplastic Meckel's cartilage and a lack of ossification as well as hypoplasia of other future skeletal elements in the skull (Fig. 1J). The differences in skeletal development could be observed as early as E13.5, a time prior to the onset of ossification, when Meckel's cartilage was already hypoplastic in conditional knockout animals (data not shown). Taken together, these results clearly establish that MEF2C function is required in the NC for proper craniofacial development.

Branchial arch expression of *Dlx5*, *Dlx6*, and *Hand2* requires MEF2C

In order to place MEF2C into a pathway for craniofacial development, we examined the expression of several transcription factor genes required for craniofacial development at E9.5, which is the time when *mef2c* is expressed in the NC component of the branchial arches and developing craniofacial mesenchyme (Fig. 2A, B). Notably, *Dlx5*, *Dlx6*, and *Hand2* expression in the first and second branchial arches was almost completely absent in *mef2c* NC knockout mice compared to control littermates at E9.5 (Fig. 2C–H; also Supplemental Fig. S1, panels A–D). *Prx1* null mice also have a similar craniofacial phenotype to *mef2c* NC conditional mice (Martin et al., 1995). However, no differences were observed in *Prx1* expression in *mef2c* NC knockout and control embryos (Supplemental Fig. S1, panels E, F), indicating that branchial arch development in general was not defective at this stage and that *Prx1* is not a downstream target of MEF2C. No differences were observed in the *Wnt1*-Cre fate map between NC knockout and control embryos in a ROSA26R *lacZ* reporter background (Soriano, 1999), indicating that contribution of NC cells to the branchial arches and viscerocranium was not grossly defective (Fig. 2I, J). Likewise, no obvious changes were observed in proliferation or apoptosis between knockout and control embryos at E9.5 or E10.5 (Supplemental Fig. S1, panels G–J). Taken together, these results suggest that the requirement for *mef2c* is in post-migratory NC and that MEF2C is an upstream regulator of the *Dlx6*-*Hand2* pathway in craniofacial development.

A novel, branchial arch enhancer from the *Dlx5/6* locus is directly activated by MEF2C

Dlx6 is closely linked to *Dlx5* in the mouse genome (Zerucha et al., 2000), and deletion of both genes results in very severe craniofacial defects. Inactivation of *Dlx5/6* in mice results in loss of *Hand2* expression in the branchial arches at E9.5, and *Dlx6* has been shown to be a direct transcriptional regulator of *Hand2* through a conserved enhancer in the *Hand2* locus (Beverdam et al., 2002; Charite et al., 2001; Depew et al., 2002; Ruest et al., 2004). Since *Dlx5*, *Dlx6*, and *Hand2* were down regulated in the absence of *mef2c* function in the NC, we hypothesized that MEF2C might directly regulate these genes. To identify potential transcriptional enhancers from these genes, we analyzed all of the conserved, noncoding sequences in the *Dlx5/6* and *Hand2* loci for MEF2 sites, and we identified a deeply conserved element immediately upstream of the *Dlx6* coding sequence (Fig. 3A) that contained four highly conserved MEF2 sites and multiple potential homeodomain protein (Hox) binding sites

(Fig. 3B). We tested this region of the *Dlx5/6* locus for enhancer activity by cloning it into the *Hsp68-lacZ* reporter plasmid and using this construct to generate transgenic embryos. This novel region of the *Dlx5/6* locus directed expression to the first and second branchial arches at E9.5 (Fig. 3C) in a pattern very similar to the endogenous *Dlx5* and *Dlx6* expression patterns in the arches (Fig. 2C, E).

All four of the candidate MEF2 sites in the *Dlx5/6* locus were bound by MEF2C binding by EMSA, although only the MEF2-3 and MEF2-4 sites exhibited robust binding *in vitro* (data not shown). To determine if the *Dlx6* enhancer was responsive to MEF2 activation via its conserved MEF2 sites, we tested a *Dlx6-TK-lacZ* reporter construct for MEF2C *trans*-activation in 3T3 cells (Fig. 3D). Cotransfection with a MEF2C-VP16 expression plasmid resulted in activation of the *Dlx5/6* reporter more than 10-fold over control cotransfections (Fig. 3D, lane 4) and this activation was dependent on the presence of intact MEF2 sites since mutation of the four sites resulted in a complete loss of activation of the enhancer element by MEF2C (Fig. 3D, lane 6). Taken together, these results demonstrate that the MEF2 sites in this novel *Dlx5/6* branchial arch enhancer are functional and further support the notion that *Dlx5* and *Dlx6* are direct transcriptional targets of MEF2C via this enhancer element.

Cooperative activation of the *Dlx5/6* branchial arch enhancer by MEF2C and Dlx5

The presence of multiple conserved Hox sites, combined with the presence of functional MEF2 sites in the *Dlx5/6* branchial arch enhancer suggested that this enhancer might also be activated by Dlx proteins themselves. We tested this possibility and the possibility that MEF2C might participate in a feed-forward transcriptional circuit with Dlx5/6 by activating their expression and then cooperating with them to amplify *Dlx5/6* expression (Fig. 3E). Indeed, Dlx5 activated expression of the *Dlx5/6* enhancer more than 50-fold (Fig. 3E, lane 5). Furthermore, under conditions in which wild type MEF2C activated the enhancer only 3-fold (Fig. 3E, lane 4), cotransfection of Dlx5 and MEF2C resulted in clear synergy by activating reporter expression more than 400-fold (Fig. 3E, lane 6).

Genetic interaction between *Mef2c* and *Dlx5/6* results in neonatal lethality in compound heterozygous mice

The transcriptional synergy between MEF2C and Dlx5 suggested that these genes might interact. Therefore, we crossed *Dlx5/6*^{+/-} mice with *mef2c*^{+/-} mice to test for genetic interaction. Because *Dlx5* and *Dlx6* are closely linked, the two genes have been deleted together in a single gene targeting event (Depew et al., 2002). Heterozygosity for either *mef2c* or *Dlx5/6* alone did not significantly affect neonatal viability (Fig. 4A). By contrast, 100% of double heterozygous offspring were obviously sick at birth and died on P0 (Fig. 4A), indicating a clear genetic interaction between the *Dlx5/6* and *mef2c* loci. Compared to wild type littermates, *Dlx5/6*^{+/-}; *mef2c*^{+/-} mice exhibited a small, incomplete palate and a displaced tongue (Fig. 4B, C), which is consistent with the palate and displaced tongue phenotype in *mef2c* NC conditional knockout mice (Fig. 1D) and further supports the conclusion that MEF2C functions as a transcriptional activator and partner of Dlx5 and Dlx6 in craniofacial development (Fig. 4D).

Discussion

A MEF2-dependent transcriptional pathway for craniofacial development

We have uncovered a novel role for MEF2C function in the NC as an essential regulator of craniofacial development. The defects in *mef2c* NC knockout mice are similar to those found in other transcription factor knockouts, including *Prx1*, *Dlx5/6*, and *Hand2* knockout animals (Beverdam et al., 2002; Depew et al., 2002; Martin et al., 1995; Yanagisawa et al., 2003). We show that *Dlx5*, *Dlx6*, and *Hand2* expression are dramatically reduced in *mef2c* NC knockout

embryos, and we present evidence that MEF2C is a direct transcriptional regulator of *Dlx5/6* via a novel branchial arch enhancer. Additionally, we show that *mef2c* and *Dlx5/6* genetically interact and that MEF2C functions as a transcriptional partner with Dlx5 in a feed-forward transcriptional circuit by synergistically activating a novel branchial enhancer in the *Dlx5/6* locus (Fig. 4D).

Modular control of branchial arch gene regulation

It has been noted previously that deletion of the *Hand2* branchial arch enhancer does not completely abolish *Hand2* expression in the branchial arches as a small region of distal expression within the arches appears to be governed by a hypothetical, unidentified regulatory element (Charite et al., 2001; Ruest et al., 2004; Yanagisawa et al., 2003). Interestingly, the loss of *Hand2* expression seen in the absence of *mef2c* function in the NC is nearly complete except at the distal end of the first arch, which appears to correspond to the Dlx6-independent *Hand2* expression domain (Fig. 2H; Yanagisawa et al., 2003). Thus, *Hand2* regulation, even within the branchial arches, appears to be controlled by multiple enhancer modules (Charite et al., 2001; Ruest et al., 2004; Yanagisawa et al., 2003). Similarly, a previously identified intergenic *Dlx5/6* enhancer directs expression to the forebrain and to a subset of the endogenous *Dlx5* and *Dlx6* expression patterns in the branchial arches (Ruest et al., 2003; Zerucha et al., 2000). Here, we identify an additional regulatory module immediately upstream of the proposed *Dlx6* transcriptional start site that regulates expression in the branchial arches (Fig. 3). This type of compartmentalized regulation provides the opportunity for independent control of gene expression in discrete lineages or for multiple different upstream pathways to intersect in the same lineage.

MEF2C as a potential downstream effector of endothelin signaling

Dlx6 and *Hand2* expression in branchial arch NC requires endothelin signaling (Charite et al., 2001; Clouthier et al., 2000; Ruest et al., 2004; Thomas et al., 1998). Endothelin signaling regulates a diverse array of normal developmental and physiological processes, including cardiovascular and craniofacial development in the embryo and vascular tone postnatally (Clouthier et al., 1998; Kedzierski and Yanagisawa, 2001; Kurihara et al., 1994). Endothelin signaling is also critical in a number of pathological processes as well, including hypertension, atherosclerosis, and pathological cardiac hypertrophy and heart failure (Kedzierski and Yanagisawa, 2001). Targeted inactivation of several endothelin pathway components, including *Edn1*, *Ednra*, and *Ece1* each result in lethality at birth due to craniofacial defects and mechanical asphyxiation similar to the phenotypes exhibited by *mef2c* NC knockout mice, including loss of *Dlx6* and *Hand2* expression (Charite et al., 2001; Clouthier et al., 1998; Clouthier et al., 2000; Kurihara et al., 1994; Ruest et al., 2004; Thomas et al., 1998; Yanagisawa et al., 1998). Based on these observations, it will be interesting to determine if MEF2C functions as a downstream effector of endothelin signaling during craniofacial development (Fig. 4D).

Implications for MEF2C involvement in congenital craniofacial defects

Craniofacial defects are among the most common serious congenital anomalies in humans, affecting as many as 1 in every 300 births (Stanier and Moore, 2004). However, the affected genes and developmental mechanisms underlying the majority of craniofacial disorders remain unknown (Stanier and Moore, 2004). This is likely due to the fact that many craniofacial anomalies with a genetic component are highly variable in penetrance or severity, several present as part of broader syndromes, and some craniofacial defects are thought to result from combinations of environmental and genetic influences (Farlie et al., 2004; Stanier and Moore, 2004). For example, Pierre Robin sequence (PRS) is characterized by hypoplastic mandible, cleft palate, and obstruction of the upper airway by the tongue (Dinwiddie, 2004; Farlie et al., 2004). The etiology of PRS is thought to be primarily environmental in nature, although there

is evidence for a genetic component to this complex and to broader syndromes that include the PRS phenotypes (Dinwiddie, 2004; Farlie et al., 2004). Mice lacking *mef2c* function in the NC appear to have a similar set of defects in the oral cavity, including a small lower jaw, cleft palate, and a displaced position of the tongue, suggesting the possibility that developmental processes regulated by MEF2C may contribute to this or other similar craniofacial defects in humans.

Experimental Procedures

Cloning and Mutagenesis

The *Dlx6* enhancer fragment described in these studies was amplified from mouse genomic DNA using the primers Dlx6-F 5'-ccaccacacaagcttgctacccccacac-3' and Dlx6R 5'-tgtgtcagaagcaggggcctag-3' and then cloned into plasmids *Hsp68-lacZ* and pTK- β -gal for transgenic and transfection analyses, respectively. Mutagenesis was performed as described previously (Dodou et al., 2003). The sequences of the mutagenic oligonucleotides and other oligonucleotides used for cloning are available on request.

Transgenic Mice

Transgenic mice were generated by oocyte microinjection using standard methods (Hogan et al., 1994) as described previously (Dodou et al., 2003). *Mef2c* mutant mice carrying the *Mef2c*^{fllox} allele and the conventional knockout allele, *mef2c*^{Tm1}, have each been described (Lin et al., 1997; Vong et al., 2005). *Wnt1*-Cre, *Dlx5/6*^{+/-}, and Rosa26R *lacZ* reporter mice have also each been described (Danielian et al., 1998; Depew et al., 2002; Soriano, 1999). Transgenic and knockout alleles were detected by Southern blot. All experiments using animals complied with federal and institutional guidelines and were reviewed and approved by the UCSF Institutional Animal Care and Use Committee.

X-gal staining, *in situ* hybridization, and skeleton preparations

X-gal staining to detect β -galactosidase expression was performed as described previously (Dodou et al., 2003). *In situ* hybridization was performed according to standard methods using digoxigenin-labeled antisense probes as described previously (Rojas et al., 2005). Skeleton and cartilage preparations were performed according to standard procedures (Hogan et al., 1994).

Cell Culture and Transfections

3T3 cells were maintained in DMEM supplemented with 10% FBS. Transfections were performed with FuGene 6 (Roche), according to the manufacturer's recommendations in 35 mm dishes. A total of 1 μ g each of reporter and expression plasmid was used in each transfection so that the quantity of DNA was always held constant at 2 μ g per sample. Cells were cultured for 48 h after transfection, harvested, and assayed using the Luminescent β -gal Detection System (Clontech), as described previously (Dodou et al., 2003). Plasmid pRK5-MEF2C contains the mouse MEF2C cDNA in the pRK-5 mammalian expression vector (BD Pharmingen). Plasmid pRK5-MEF2C-VP16 is the same except that the Herpesvirus VP16 activation domain has been fused in-frame to the C-terminus of the MEF2C coding sequence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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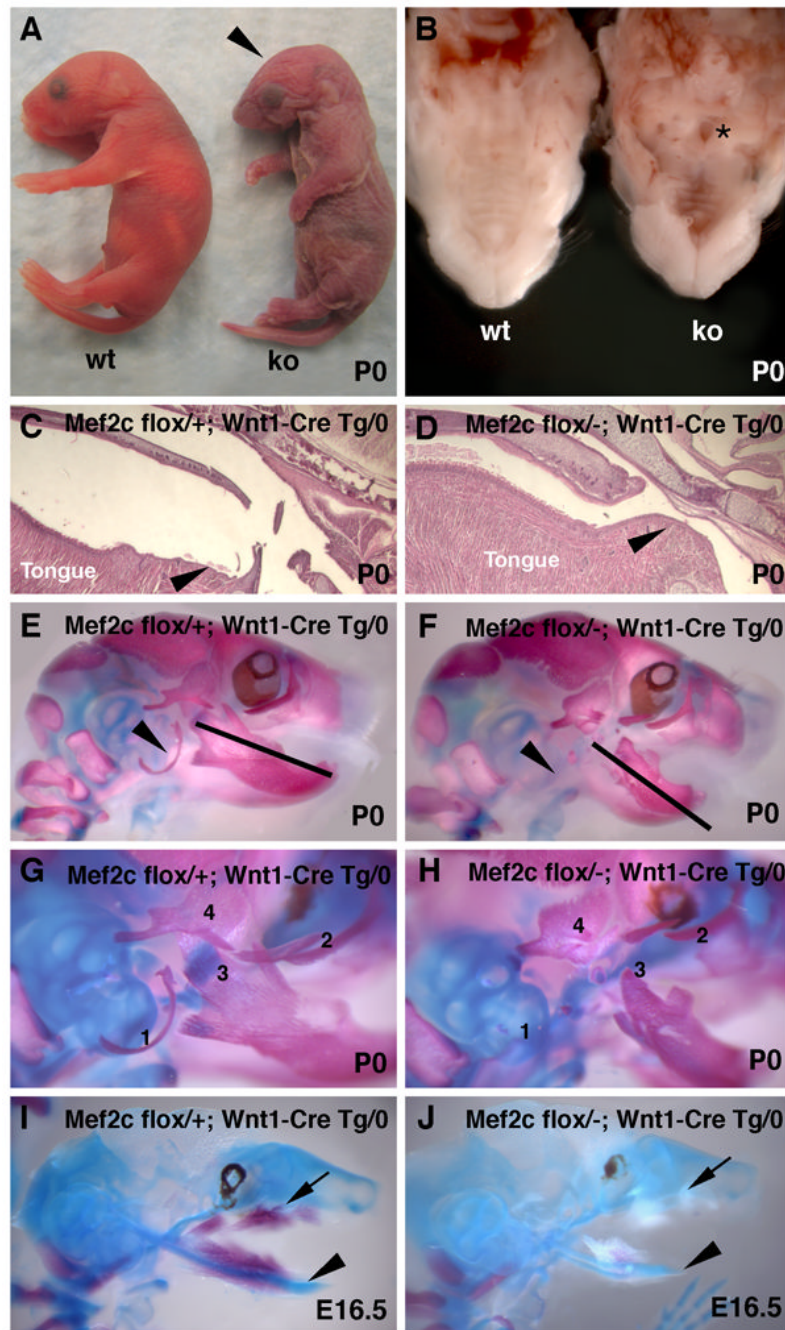


Figure 1.

Mef2c is required for craniofacial development. (A) Mice lacking *mef2c* function in the neural crest have misshapen heads (arrowhead) and die from asphyxiation within an hour of birth. (B) *Mef2c* conditional knockout animals have a cleft of the posterior palate (asterisk). (C, D) Sagittal sections through the heads of *mef2c* neural crest conditional knockout (D) and littermate control (C) neonates show that the upper airway is constricted in the conditional knockout compared to control littermates (arrowheads). (E–J) Skeleton preparations of neonatal and fetal mice stained with alizarin red and alcian blue show that *mef2c* flox^{-/-}; *Wnt1*-Cre Tg/0 skulls (F, H, J) have multiple craniofacial defects compared to littermate controls (E, G, I). Arrowheads mark location of the tympanic ring in a control skull (E) and its

absence in the mutant skull (H). Bars of equal size show that the mutant mandible is markedly shorter than the control (E, F). (G, H) Magnified view of the temporal region of control (G) and mutant (H) skulls showing the tympanic ring (1), zygomatic bone (2), mandibular processes (3), and temporal bone processes (4). Note that each of these structures is hypoplastic or missing in *mef2c* conditional knockout mice. (I, J) Delayed ossification in *mef2c* neural crest knockout mice at E16.5. Note the calcified bone in control (I) but not in mutant (J). In panels I and J, arrowheads mark Meckel's cartilage, and arrows mark the developing maxilla.

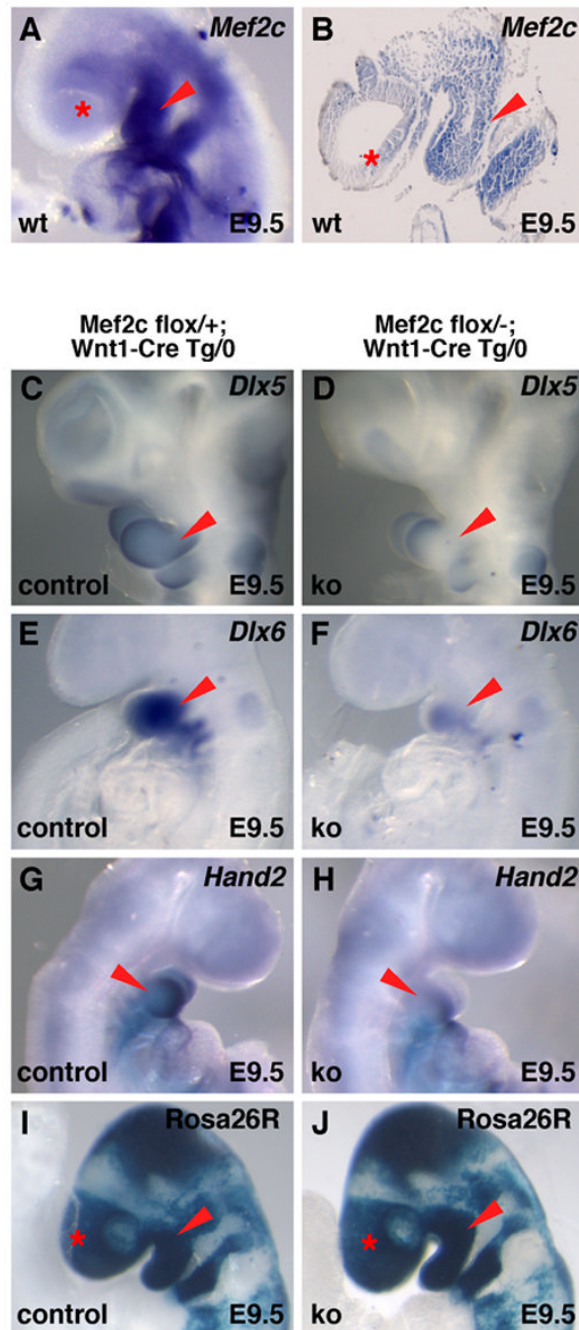
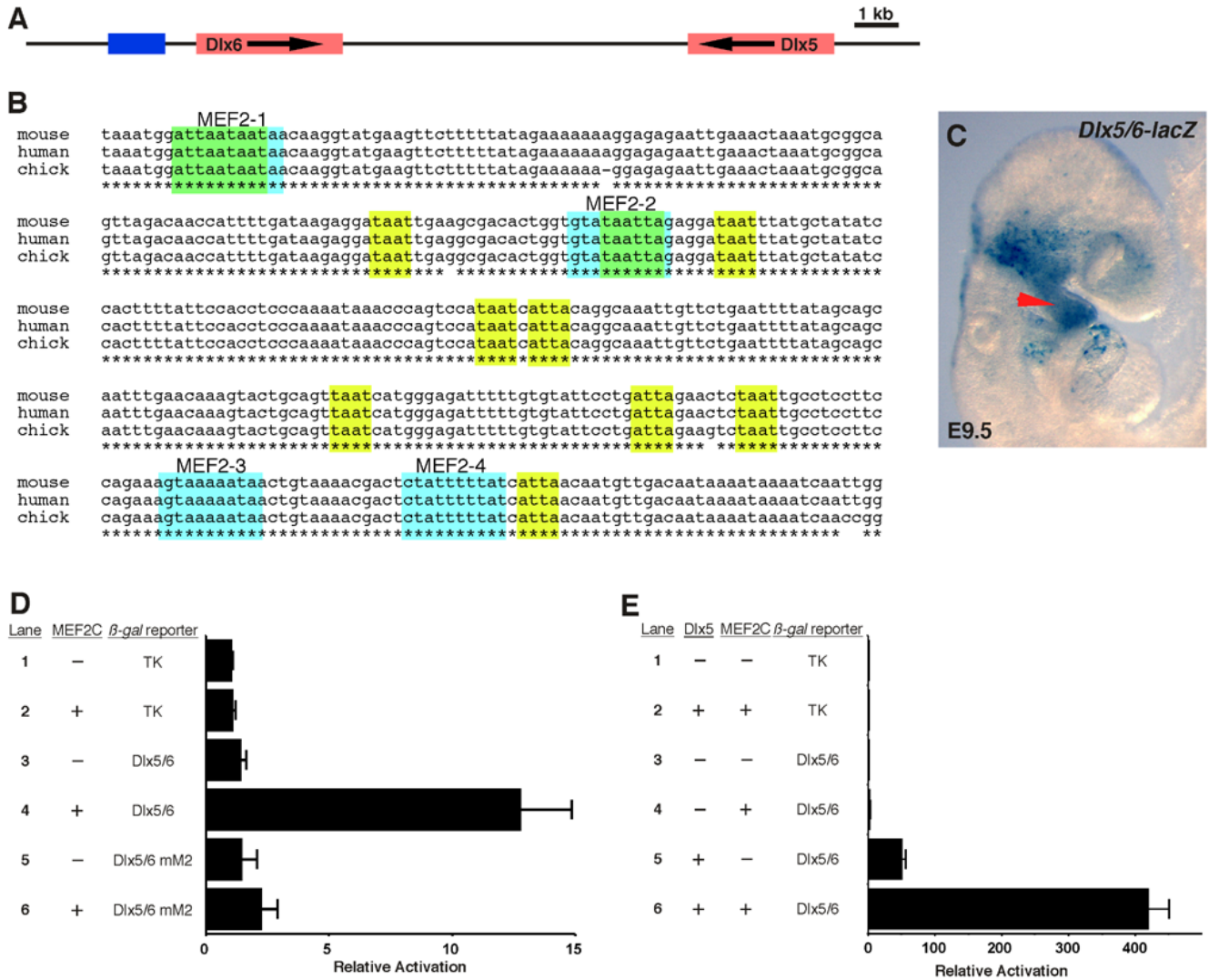


Figure 2.

Dlx5, *Dlx6*, and *Hand2* expression in the branchial arches requires *mef2c*. (A, B) Whole mount (A) and sagittal section (B) *in situ* hybridization showing endogenous *mef2c* expression in wild type embryos at E9.5. Note the robust expression of *mef2c* in the first and second branchial arches. (C–H) *Dlx5*, *Dlx6*, and *Hand2* expression are nearly absent in the branchial arches of *mef2c* neural crest knockout embryos at E9.5. Note that *Dlx6* expression is still faintly detected in the first arch of *mef2c* neural crest knockout embryos (F) and that *Dlx5* and *Hand2* expression appear to be absent except in a small region at the distal tip of the first arch (D, H). (I, J) X-gal staining of E9.5 embryos generated by crossing a ROSA26R *lacZ* reporter allele into the *mef2c* conditional knockout background showed no obvious defects in neural crest contribution

to the branchial arches or craniofacial mesenchyme compared to littermate controls. Red arrowheads mark the first branchial arch in all panels; asterisks mark craniofacial ectomesenchymal expression.

**Figure 3.**

Identification of a MEF2-dependent transcriptional enhancer from the *Dlx5/6* locus. (A) Schematic representation of the *Dlx5/6* locus. Red boxes denote the *Dlx5* and *Dlx6* transcribed regions; arrows show the direction of transcription. The blue box denotes the location of the enhancer identified in this study. (B) ClustalW alignment of the mouse, human, and chicken sequences in the conserved region of the *Dlx6* branchial arch enhancer. Conserved MEF2 consensus elements are highlighted by light blue boxes; conserved Hox/Dlx binding sites are highlighted by yellow-green boxes; asterisks denote conservation across all three species. (C) The *Dlx5/6* enhancer directs *lacZ* expression to the first and second branchial arches in transgenic embryos at E9.5 as shown by whole mount X-gal staining. The arrowhead indicates branchial arch expression of the *Dlx5/6-lacZ* transgene. (D) MEF2C *trans*-activates the *Dlx5/6* branchial arch enhancer. Cotransfection of a MEF2C-VP16 expression plasmid with the *Dlx5/6*-TK-*lacZ* results in 12-fold activation of the reporter (lane 4). This activation is dependent upon the presence of the MEF2 sites since mutation of those elements almost completely abolished MEF2 *trans*-activation (lane 6). No *trans*-activation of the parent reporter by MEF2C-VP16 was observed (lane 2). Data are presented as the mean plus standard error for six independent experiments. (E) MEF2C and Dlx5 synergistically activate the *Dlx5/6*

enhancer. Cotransfection of a MEF2C or Dlx5 expression plasmid results in 3-fold and 50-fold activation of the *Dlx5/6* enhancer, respectively (lanes 4, 5). Cotransfection of both expression plasmids results in strong synergistic activation of over 400-fold (lane 6). No *trans*-activation of the TK parent reporter by MEF2C plus Dlx5 was observed (lane 2). Data are presented as the mean plus standard error for three independent experiments.

A

	<u># born</u>	<u># alive</u>	<u># dead/dying</u>
wild type	19	18	1
<i>Dlx5/6 +/-</i>	13	11	2
<i>Mef2c +/-</i>	18	17	1
<i>Dlx5/6 +/-;Mef2c +/-</i>	9	0	9

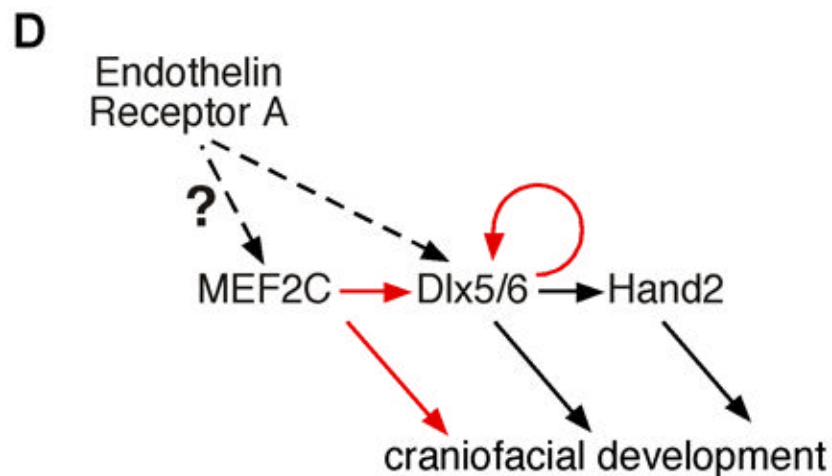
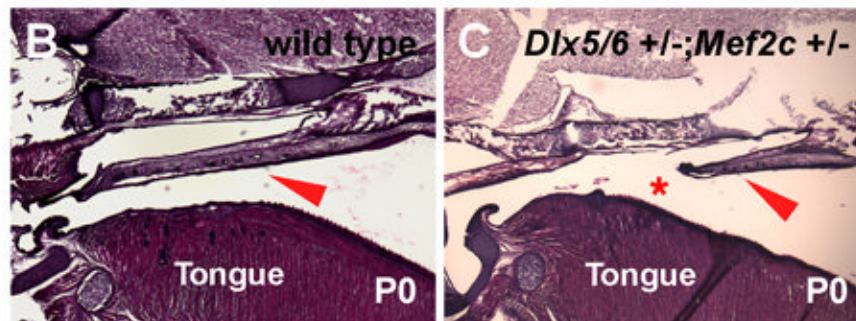


Figure 4.

Mef2c and *Dlx5/6* interact genetically. (A) *Mef2c*^{+/-} mice were crossed to *Dlx5/6*^{+/-} mice and the total number of offspring was scored on P0 (# born). Among the total of each genotype born, animals were scored as either alive and viable (# alive) or as dead or clearly cyanotic and dying (# dead/dying). Nearly all wild type, *Mef2c*^{+/-}, and *Dlx5/6*^{+/-} mice were viable at birth. By contrast, 100% (9/9) *Dlx5/6*^{+/-};*Mef2c*^{+/-} mice died or were clearly cyanotic on P0, indicating a strong genetic interaction. (B, C) Sagittal sections of the heads of wild type (B) or *Dlx5/6*^{+/-};*Mef2c*^{+/-} (C) mice collected on P0. Compared to wild type littermates, *Dlx5/6*^{+/-};*Mef2c*^{+/-} mice exhibit a misshapen (arrowheads) and incomplete (asterisk) palate and an improper position of the tongue at the rear of the oral cavity. (D) A model transcriptional

pathway for craniofacial development in which MEF2C functions downstream of endothelin receptor A signaling to activate Dlx5 and Dlx6, which in turn, activates Hand2 and also reinforces their own expression in a feed-forward fashion. Solid arrows denote direct regulation; dashed arrows denote direct or indirect regulation.