Crucial and Overlapping Roles of *Six1* and *Six2* in Craniofacial Development

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Abstract

SIX1 and *SIX2* encode closely related transcription factors of which disruptions have been associated with distinct craniofacial syndromes, with mutations in *SIX1* associated with branchiootic syndrome 3 (BOS3) and heterozygous deletions of *SIX2* associated with frontonasal dysplasia defects. Whereas mice deficient in *Six1* recapitulated most of the developmental defects associated with BOS3, mice lacking *Six2* function had no obvious frontonasal defects. We show that *Six1* and *Six2* exhibit partly overlapping patterns of expression in the developing mouse embryonic frontonasal, maxillary, and mandibular processes. We found that *Six1*^{−/−}*Six2*^{−/−} double-mutant mice were born with severe craniofacial deformity not seen in the *Six1*^{−/−} or *Six2*^{−/−} single mutants, including skull bone agenesis, midline facial cleft, and syngnathia. Moreover, whereas *Six1*^{−/−} mice exhibited partial transformation of maxillary zygomatic bone into a mandibular condyle-like structure, *Six1*^{−/−}*Six2*^{+/−} mice exhibit significantly increased penetrance of the maxillary malformation. In addition to ectopic *Dlx5* expression at the maxillary-mandibular junction as recently reported in E10.5 *Six1*^{−/−} embryos, the E10.5 *Six1*^{−/−}*Six2*^{+/−} embryos showed ectopic expression of *Bmp4*, *Msx1*, and *Msx2* messenger RNAs in the maxillary-mandibular junction. Genetically inactivating 1 allele of either *Ednra* or *Bmp4* significantly reduced the penetrance of maxillary malformation in both *Six1*^{−/−} and *Six1*^{−/−}*Six2*^{+/−} embryos, indicating that *Six1* and *Six2* regulate both endothelin and bone morphogenetic protein-4 signaling pathways to pattern the facial structures. Furthermore, we show that neural crest-specific inactivation of *Six1* in *Six2*^{−/−} embryos resulted in midline facial cleft and frontal bone agenesis. We show that *Six1*^{−/−}*Six2*^{−/−} embryos exhibit significantly reduced expression of key frontonasal development genes *Alx1* and *Alx3* as well as increased apoptosis in the developing frontonasal mesenchyme. Together, these results indicate that *Six1* and *Six2* function partly redundantly to control multiple craniofacial developmental processes and play a crucial neural crest cell-autonomous role in frontonasal morphogenesis.

Keywords: BMP4, frontonasal dysplasia, median facial cleft, neural crest, transcription factor, *Alx1*

Introduction

Frontonasal dysplasia (FND) consists of a group of disorders characterized by ocular hypertelorism, midline facial cleft affecting the nose and/or upper lip and palate, notching or clefting of the alae nasi, and it is sometimes associated with anterior cranium bifidum and other malformations (Wu et al. 2007; Kayserili et al. 2009; Twigg et al. 2009). Although it has long been recognized that FND results from abnormal development of the embryonic frontonasal prominence, which forms from cranial neural crest cells populating in between the forebrain and surface ectoderm and ultimately gives rise to the forehead, nose, philtrum, and premaxillary component of the upper jaw, the causes and molecular mechanisms of FND pathogenesis are not well understood (Farlie et al. 2016). Mutations in 5 genes, including *ALX1*, *ALX3*, *ALX4*, *EFNB1*, and *ZSWIM6*, have been identified in a small number of patients with FND disorders (Twigg et al. 2004; Wieland et al. 2004; Kayserili et al. 2009; Twigg et al. 2009; Uz et al. 2010; Smith et al. 2014; Farlie et al. 2016). Remarkably, loss of function of each of the *ALX* family genes, which encode homeodomain-containing transcription factors, has been associated with distinct autosomal recessive FND syndromes (Kayserili et al. 2009; Twigg et al. 2009; Uz et al. 2010; Farlie et al. 2016). Gene knockout studies in mice showed that *Alx1* function is

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required for survival of embryonic forebrain mesenchyme cells, whereas *Alx3* and *Alx4* play redundant roles in survival of embryonic nasal mesenchyme cells (Zhao et al. 1996; Beverdam et al. 2001). Loss of function of the X-linked *EFNB1* gene caused severe hypertelorism with a grooved nasal tip and craniosynostosis in heterozygous females, whereas hemizygous males showed mildly affected hypertelorism (Twigg et al. 2004; Twigg et al. 2013), due to mosaicism in *EFNB1* expression caused by random X-inactivation in heterozygous females (Davy et al. 2006; Twigg et al. 2013). A single recurrent missense mutation in *ZSWIM6* has been associated with severe frontonasal and limb malformations (Smith et al. 2014), but the mechanism involving *ZSWIM6* in frontonasal and limb development is unknown.

Recently, heterozygous microdeletions of the *SIX2* gene and flanking noncoding sequences have been associated with an FND syndrome in patients of 2 unrelated families, characterized by frontal bossing, a large anterior fontanelle, hypertelorism, a broad nasal bridge, and conductive hearing loss (Hufnagel et al. 2016; Henn et al. 2018). Although *Six2* messenger RNAs (mRNAs) are highly expressed in frontonasal mesenchyme in mouse embryos, *Six2*-null mice exhibit kidney dysgenesis and premature fusion of the bones in the cranial base but no obvious defects in frontonasal development (Oliver et al. 1995; Self et al. 2006; He et al. 2010). However, mice homozygous for an X-irradiation-induced mutation, *Brachyrrhine* (*Br*), which was mapped to the *Six2* locus and caused significantly reduced *Six2* mRNA expression in developing kidney and craniofacial tissues, exhibited a large median facial cleft (Fogelgren et al. 2008). *Six2* belongs to the *SIX* gene family that consists of 6 members (*Six1*–*Six6*), each coding for a DNA-binding protein containing a homeodomain and an adjacent SIX domain (Kumar 2009). In both mouse and human genomes, the *Six2* and *Six3* genes are physically linked immediate neighbors (Fogelgren et al. 2008; Hufnagel et al. 2016; O'Brien et al. 2018). Recent genome sequencing revealed that the *Br* mutation caused inversion of the *Six2/Six3* locus, resulting in aberrant *Six3* gene expression in developing craniofacial and kidney tissues in addition to loss of *Six2* expression. Thus, whether and how *Six2* regulates frontonasal development require further investigation.

In this study, we investigated whether *Six2* function in mouse craniofacial development is compensated by *Six1*, which shares the highest sequence similarity with *Six2* and is also highly expressed in developing frontonasal and other craniofacial tissues (Oliver et al. 1995; Laclef et al. 2003). Mutations in *SIX1* have been associated with autosomal dominant branchiootoc syndrome 3 (BOS3), characterized by preauricular pits, hearing loss, branchial cleft fistula or cyst, and renal dysplasia (Ruf et al. 2004; Sanggaard et al. 2007). Mice lacking *Six1* function display malformation of several craniofacial structures, including the nasal cavity, ear, and maxillary and mandibular skeletons, in addition to kidney agenesis (Laclef et al. 2003; Li et al. 2003; Xu et al. 2003; Ozaki et al. 2004; Tavares et al. 2017). We report here that mice lacking both *Six1* and *Six2* function exhibit more severe defects in multiple craniofacial tissues than in the single mutant mice and

that *Six1* and *Six2* have a crucial but overlapping neural crest cell-autonomous function in frontonasal development.

Materials and Methods

Mouse Strains and Mouse Breeding

This study used mice carrying previously reported *Bmp4*^{null} (Liu et al. 2004), *Six1*^{Cre/hpAP} (Guo et al. 2011), *Six1*^{lox} (Le Grand et al. 2012), and *Six2*^{GCE} (Kobayashi et al. 2008) alleles as well as the *Ell1a-Cre* and *Wnt1Cre* transgenic mouse lines (Lakso et al. 1996; Danielian et al. 1998). The *Ednra*^{+/-} mice, heterozygous for the *Ednra*^{tm1b(EUCOMM)Hmgu} allele, were obtained from The Jackson Laboratory (JAX Stock 027942). *Six1*^{Cre/hpAP} is functionally a *Six1* null allele, whereas *Six2*^{GCE} is functionally a *Six2* null allele (Kobayashi et al. 2008; Guo et al. 2011). *Six1*^{Cre/hpAP} heterozygous mice were crossed with *Six2*^{GCE} heterozygous mice to generate *Six1*^{+/-}*Six2*^{+/-} mice, which were subsequently crossed to the ICR strain to establish the *Six1*^{+/-}*Six2*^{+/-} colony. The *Six1*^{lox} allele (Le Grand et al. 2012) is referred to as *Six1*^c in this report. *Six1*^{c/c} and *Six1*^{c/+}*Wnt1Cre* mice were separately crossed with *Six2*^{GCE/+} mice to generate *Six1*^{c/+}*Six2*^{+/-} and *Six1*^{c/+}*Six2*^{+/-}*Wnt1Cre* mice, which were subsequently used to generate *Six1*^{c/c}*Six2*^{+/-}*Wnt1Cre* compound mutant pups.

All animal work procedures were performed following recommendations in the Guide for Care and Use of Laboratory Animals by the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) at Cincinnati Children's Hospital Medical Center. This study conformed with ARRIVE guidelines for preclinical animal studies.

Histology, In Situ Hybridization, and Skeletal Preparations

Embryos were collected and processed for histology or in situ hybridization as described previously (Li et al. 2017). Skeletal preparations of newborn and late-term fetuses were processed and stained with alizarin red and Alcian blue as described previously (Ovchinnikov 2009).

Immunofluorescence Detection

Immunofluorescent staining was performed using paraffin sections following standard protocols (Xu et al. 2014). The following primary antibodies were used: rabbit antiactive capase 3 (cat. 559565, 1:300; BD Biosciences), rabbit anti-SIX1 (D4A8K, cat. 12891S, 1:200; Cell Signaling Technology), rabbit anti-SIX2 (cat. 11562-1-AP, 1:200; Proteintech), and guinea pig anti-SIX3 (cat. 200-201-A26S, 1:100; Rockland).

Quantitative Real-Time Polymerase Chain Reaction Analysis

Quantitative real-time polymerase chain reaction (RT-qPCR) was performed as previously described (Li et al. 2017). The

Figure 1. Expression of *Six1* and *Six2* in the developing frontonasal, maxillary, and mandibular processes. (A) Whole-mount view of the pattern of *Six1* messenger RNA (mRNA) expression in E10.5 wild-type (WT) embryo. (B–E) Representative sagittal (B, C) and frontal (D, E) sections of E10.5 WT embryos showing immunofluorescent staining (green) using the anti-SIX1 antibody. DAPI counterstaining is shown in blue. (F) Representative frontal section of E10.5 *Six2*^{−/−} embryo showing immunofluorescent staining (green) using the anti-SIX1 antibody. (G) Whole-mount view of the pattern of *Six2* mRNA expression in E10.5 WT embryo. (H–K) Representative sagittal (H, I) and frontal (J, K) sections of E10.5 WT embryos showing immunofluorescent staining (green) using the anti-SIX2 antibody. (L) Immunofluorescent staining of frontal sections of E10.5 *Six2*^{−/−} embryos using the same anti-SIX2 antibody. Multiple serial sections from 3 wild-type and 3 *Six2*^{−/−} embryos were used for immunofluorescent staining using each antibody. hm, head mesenchyme; mdp, mandibular process; mnp, medial nasal process; mxp, maxillary process; np, nasal pit; Rp, Rathke's pouch. Scale bar in panel B, 100 μ m.

relative levels of mRNAs in each sample were normalized to that of *Hprt* mRNAs. Student's *t* test was used to analyze differential expression data. The gene-specific PCR primers are *Alx1F* (ACGATACGGCCAAATACAGC) and *Alx1R* (TAAGGTGTCATGCAGGAGGA), *Alx3F* (AGCGTTATGGGAA GATGCAG) and *Alx3R* (ATGCCCTCTGGAGACATGAG), and *Alx4F* (CTGCGCATCTCTACTTGCAG) and *Alx4R* (CACCCAGGTTGCTCTCTTTG).

Results

Patterns of Expression of *Six1* and *Six2* Exhibit Extensive Overlap in Developing Frontonasal, Maxillary, and Mandibular Processes

To investigate whether *Six1* might complement *Six2* function in frontonasal development, we compared expression patterns of *Six1* and *Six2* mRNAs and proteins in developing mouse embryos. Whole-mount in situ hybridization analysis showed that both *Six1* and *Six2* mRNAs were highly expressed in the nasal and maxillary processes at E10.5 (Fig. 1A, G). Immunofluorescent analyses showed that *Six1* protein was strongly expressed in medial nasal, maxillary, and mandibular mesenchyme, as well as in the nasal pit epithelium and Rathke's pouch (Fig. 1B–E). Strong *Six2* protein expression was detected in the medial nasal and maxillary mesenchyme, whereas lower levels of *Six2* protein were detected in mandibular mesenchyme and nasal epithelium (Fig. 1H–K). We validated specificity of the antibodies by directly comparing immunofluorescent staining of wild-type and *Six2*^{−/−} embryo samples and found that the anti-SIX2 antibody did not detect any specific signal in *Six2*^{−/−} embryos, whereas the patterns of

anti-SIX1 immunostaining of the developing facial tissues were unaltered (Fig. 1F, L; compare with Fig. 1E, K, respectively). These data indicate that *Six1* and *Six2* expression exhibits extensive overlap in the developing facial tissues, particularly in medial nasal and maxillary processes. Since *Br/Br* mutant mouse embryos showed altered pattern of *Six3* expression in addition to disruption of *Six2* expression (O'Brien et al. 2018), we also examined *Six3* expression in *Six2*^{−/−} mutant embryos and control littermates. Our results show that *Six3* expression in the developing facial tissues was unaltered in *Six2*^{−/−} mutant embryos (Appendix Fig. 1), indicating that the alteration in *Six3* expression in *Br/Br* mutant embryos was not due to loss of *Six2* function.

Mice Lacking Both *Six1* and *Six2* Exhibit Severe Craniofacial Malformations Not Seen in *Six1*^{−/−} or *Six2*^{−/−} Mutants

No overt craniofacial defects were detected in *Six1*^{+/−}*Six2*^{+/−} double heterozygous mice. We intercrossed *Six1*^{+/−}*Six2*^{+/−} mice and analyzed craniofacial phenotypes of compound mutant pups. At E18.5, *Six1*^{−/−} pups (*n* = 28) exhibited a domed head, smaller mandible, and a characteristic curvature between the forehead and snout (Fig. 2B, B'). *Six2*^{−/−} pups (*n* = 9) showed shortened snout (Fig. 2C, C'). In contrast, *Six1*^{−/−}*Six2*^{−/−} pups showed severe craniofacial defects, including midline facial cleft, widely open eyes lacking upper eyelid, absence of external ear, and severely shortened mandible (Fig. 2D, D') (*n* = 8). Skeletal preparations showed that, whereas *Six1*^{−/−} pups had defects in nasal and mandibular structures and *Six2*^{−/−} pups showed premature fusion of the presphenoid and basisphenoid bones, the *Six1*^{−/−}*Six2*^{−/−} mutants exhibited

agenesis of frontal and parietal bones of the skull, significantly reduced nasal bones, bony fusion of the maxilla and mandible (syngnathia), midline cleft of the nasal capsule, and absence of the anterior cranial base, with the basioccipital bone aberrantly fused anteriorly with the merged pre/basisphenoid and laterally with the exoccipital bones (Fig. 2E–H'). These results indicate that *Six1* and *Six2* play crucial and partly redundant roles in the development of multiple craniofacial structures.

Six2 Acts in Concert with *Six1* to Pattern the Maxillary/Mandibular Junction

Tavares et al. (2017) recently reported that mice homozygous for one of the *Six1* knockout alleles, *Six1^{tm1kvw}* (Ozaki et al. 2004), exhibited a maxillary defect in which the zygomatic bone was transformed to a rod-like structure resembling the mandibular condyle. However, craniofacial skeletal preparations reported for mice homozygous for other *Six1* knockout alleles did not show this maxillary defect (Laclef et al. 2003; Guo et al. 2011). Since we detected strong expression of both *Six1* and *Six2* mRNAs and proteins in the developing maxillary mesenchyme (Fig. 1), we carefully examined maxillary phenotypes in *Six1^{-/-}* and *Six1^{-/-}Six2^{-/-}* mutant pups. We found that 11 of 28 (39.3%) *Six1^{-/-}* pups showed partial mandibular transformation of the zygomatic bone, with 6 of them showing the defect unilaterally (Fig. 3B; Table). Notably, 16 of 18 (88.9%) *Six1^{-/-}Six2^{+/-}* pups showed the zygomatic bone defect, with 11 being bilateral (Fig. 3C; Table). These data, together with the syngnathia phenotype in *Six1^{-/-}Six2^{-/-}* mutants, indicate that *Six1* and *Six2* regulate maxillo-mandibular patterning in a dose-dependent manner.

Tavares et al. (2017) showed that *Six1^{-/-}* mutant embryos had ectopic expression of *Edn1* in the mandibular arch epithelium and ectopic expression of the EDNRA signaling target gene *Dlx5* in the maxillomandibular junction at E10.5. We also detected ectopic *Dlx5* mRNA expression in the maxillomandibular junction in E10.5 *Six1^{-/-}* embryos and found that *Six1^{-/-}Six2^{+/-}* embryos exhibited stronger ectopic *Dlx5* expression in the maxillary prominences (Fig. 3D–F). Furthermore, inactivating 1 *Ednra* allele significantly reduced the percentage of *Six1^{-/-}* and *Six1^{-/-}Six2^{+/-}* mutant pups with the maxillary bone defect: only 1 of 8 *Six1^{-/-}Ednra^{+/-}* and 5 of 9 *Six1^{-/-}Six2^{+/-}Ednra^{+/-}* mutant pups showed a unilateral transformation of the zygomatic bone, and none of the pups of these genotypes showed bilateral maxillary defects (Table).

The incomplete rescue of the maxillary defect in *Six1^{-/-}Six2^{+/-}* pups by *Ednra* heterozygosity, together with the finding that *Six1^{-/-}Six2^{-/-}* mutants exhibited much more severe craniofacial defects than that previously reported of mice with ectopic activation of *Edn1*-EDNRA signaling throughout the cranial neural crest lineage (Tavares et al. 2017), prompted us

Figure 2. Craniofacial skeletal defects in the *Six1* and *Six2* compound mutant mice. (A–D') Lateral view (A–D) and frontal view (A'–D') of E18.5 heads of the *Six1^{+/+}Six2^{+/+}* (A, A'), *Six1^{-/-}Six2^{+/+}* (B, B'), *Six1^{+/+}Six2^{-/-}* (C, C'), and *Six1^{-/-}Six2^{-/-}* (D, D') embryos. The yellow arrowhead in B points to the abnormal shape of the forehead. The black arrowhead in D and the white arrowheads in D' point to widely open eyes. Asterisk in D' marks midline facial cleft. Scale bar in A', 1 mm. (E–H') Lateral view (E–H) and ventral view (E'–H') of alizarin red- and Alcian blue-stained E18.5 head skeletons of the *Six1^{+/+}Six2^{+/+}* (E, E'), *Six1^{-/-}Six2^{+/+}* (F, F'), *Six1^{+/+}Six2^{-/-}* (G, G'), and *Six1^{-/-}Six2^{-/-}* (H, H') embryos. The thick arrow in F points to the abnormal shape of the forehead. The thin arrow in H points to lack of frontal bones, and the arrowhead points to the severely reduced parietal bones. The arrowhead in H' points to the midline facial cleft, and the arrow points to abnormal zygomatic bone. Scale bar in E, 1 mm. bo, basioccipital; bs, basisphenoid; ea, ear; eo, exoccipital; fb, frontal bone; md, mandible; mx, maxilla; pb, parietal bone; ps, presphenoid; syn, syngnathia; zmx, zygomatic process of maxilla. The asterisks in the ps*/bs* and zmx* labels in Panel H point to severe malformation of those structures in the double homozygous mutants.

to investigate whether *Six1* and *Six2* regulate additional signaling pathways important in maxillomandibular patterning. At E10.5, *Bmp4* mRNAs were expressed in the distal maxillary and mandibular epithelium, whereas no morphogenetic protein (BMP) target genes *Msx1* and *Msx2* were expressed in distal regions of the maxillary and mandibular mesenchyme in control embryos (Fig. 3G, J, M). Whereas *Six1^{-/-}* embryos showed similar patterns of expression of *Bmp4* and *Msx2*, respectively, in the maxillary and mandibular prominences as in control embryos (Fig. 3H, N), 1 of 4 E10.5 *Six1^{-/-}* embryos showed ectopic expression of *Msx1* mRNAs at the maxillomandibular junction (Fig. 3K). Furthermore, expression of *Bmp4* was increased in the maxillary region, and expression of both *Msx1* and *Msx2* mRNAs was expanded to the maxillomandibular junction in all E10.5 *Six1^{-/-}Six2^{+/-}* embryos examined ($n = 4$ for each of the *Bmp4* and *Msx1* probes and $n = 3$ for *Msx2* probe) (Fig. 3I, L, O). Moreover, inactivation of 1 *Bmp4* allele dramatically reduced the percentage of *Six1^{-/-}* and *Six1^{-/-}Six2^{+/-}* mutant pups with the maxillary defect: only 1 of 9 (11.1%) *Six1^{-/-}Bmp4^{+/-}* and 4 of 11 (36.4%) *Six1^{-/-}Six2^{+/-}Bmp4^{+/-}* mutant pups showed the zygomatic bone defect (Table). These data indicate that *Six1* and *Six2* control maxillomandibular patterning through regulating both *Edn1* and *Bmp4* signaling pathways.

Table. Comparison of the Maxillary Defect in E18.5 *Six1*^{-/-}, *Six1*^{-/-}*Six2*^{+/-}, *Six1*^{-/-}*Ednra*^{+/-}, *Six1*^{-/-}*Six2*^{+/-}*Ednra*^{+/-}, *Six1*^{-/-}*Bmp4*^{+/-}, and *Six1*^{-/-}*Six2*^{+/-}*Bmp4*^{+/-} Mutant Mice.

Genotype	Total Number	Unilateral Maxillary Defect	Bilateral Maxillary Defect	Percentage with Maxillary Defect ^a
<i>Six1</i> ^{-/-}	28	6	5	39.3
<i>Six1</i> ^{-/-} <i>Six2</i> ^{+/-}	18	5	11	88.9
<i>Six1</i> ^{-/-} <i>Ednra</i> ^{+/-}	8	1	0	12.5
<i>Six1</i> ^{-/-} <i>Six2</i> ^{+/-} <i>Ednra</i> ^{+/-}	9	5	0	55.6
<i>Six1</i> ^{-/-} <i>Bmp4</i> ^{+/-}	9	1	0	11.1
<i>Six1</i> ^{-/-} <i>Six2</i> ^{+/-} <i>Bmp4</i> ^{+/-}	11	3	1	36.4

^aStudent's *t* test analyses showed significant increase in the frequency of the maxillary defect from *Six1*^{-/-} to *Six1*^{-/-}*Six2*^{+/-} mice ($P < 0.05$) and significant reduction in frequency of the phenotype from *Six1*^{-/-} to *Six1*^{-/-}*Ednra*^{+/-} and *Six1*^{-/-}*Bmp4*^{+/-} mice, respectively ($P < 0.05$). The frequency of the maxillary defect also significantly decreased from *Six1*^{-/-}*Six2*^{+/-} to *Six1*^{-/-}*Six2*^{+/-}*Ednra*^{+/-} and *Six1*^{-/-}*Six2*^{+/-}*Bmp4*^{+/-} mice, respectively ($P < 0.05$).

Figure 3. Maxillary defects in the *Six1* and *Six2* compound mutant mice. (A–C) Ventral view of alizarin red- and Alcian blue-stained E18.5 head skeletons of the *Six1*^{+/-}*Six2*^{+/-} (A), *Six1*^{-/-}*Six2*^{+/-} (B), and *Six1*^{-/-}*Six2*^{-/-} (C) embryos. Arrow in C points to the ectopic cartilage at the proximal end of the zygomatic bone. Scale bar in A, 1 mm. The zmx* label in Panels B and C points to malformed zmx. (D–O) Lateral view of patterns of *Dlx5* (D–F), *Bmp4* (G–I), *Msx1* (J–L), and *Msx2* (M–O) messenger RNA expression in the E10.5 *Six1*^{+/-}*Six2*^{+/-} (D, G, J, M), *Six1*^{-/-} (E, H, K, N), and *Six1*^{-/-}*Six2*^{+/-} (F, I, L, O) embryos. At least 3 embryos of each genotype were analyzed using each probe. Arrowheads point to ectopic expression of *Dlx5* (E, F), *Bmp4* (I), *Msx1* (K, L), and *Msx2* (O) in the maxillary-mandibular junction. Scale bar in D, 200 μ m. jb, jugal bone; zmx, zygomatic process of maxilla.

Six1 and *Six2* Function Is Required in the Neural Crest Lineage for Frontonasal Development

Most of the craniofacial bones affected in the *Six1*^{-/-}*Six2*^{-/-} mutants form from ossification of neural crest-derived mesenchyme (Chai and Maxson 2006). We investigated whether *Six1* is required cell-autonomously in neural crest cells for craniofacial development by analyzing *Six1*^{cl/c}*Wnt1Cre* conditional mutants. All *Six1*^{cl/c}*Wnt1Cre* pups were born with a shortened mandible ($n = 11$) (Fig. 4D, E; compare with control samples in Fig. 4A, B). Notably, the skull, maxillary, and nasal skeletal elements appeared normal in *Six1*^{cl/c}*Wnt1Cre* pups (Fig. 4B, C, E, F). We next analyzed *Six1*^{cl/c}*Six2*^{-/-}*Wnt1Cre* mutant embryos. In contrast to *Six1*^{cl/c}*Wnt1Cre* and *Six2*^{-/-} pups, all *Six1*^{cl/c}*Six2*^{-/-}*Wnt1Cre* mutant pups examined ($n = 8$) showed a midline facial cleft and agenesis of the frontal bones (Fig. 4G–I). In contrast to the *Six1*^{-/-}*Six2*^{-/-} pups (Fig. 2D, D', H, H'), *Six1*^{cl/c}*Six2*^{-/-}*Wnt1Cre* pups had well-formed parietal and mandibular bones comparable to *Six1*^{cl/c}*Wnt1Cre* pups (Fig. 4H) and no maxillomandibular fusion (Fig. 4H, I). Together, these results indicate that *Six1* expression in the neural crest-derived craniofacial mesenchyme is required for mandibular morphogenesis and has a crucial but overlapping function with *Six2* in regulating frontonasal and maxillary development.

We further investigated the cellular basis of midline facial clefting in *Six1*^{-/-}*Six2*^{-/-} embryos. In contrast to *Six1*^{+/-}*Six2*^{+/-} and *Six2*^{-/-} littermates, *Six1*^{-/-}*Six2*^{-/-} embryos showed a large amount of active caspase-3-positive cells in the medial nasal mesenchyme at E10.5 (Fig. 4J–L), indicating that loss of both *Six1* and *Six2* function causes abnormal apoptosis in developing medial nasal processes.

The midline facial defects in *Six1*^{-/-}*Six2*^{-/-} mutant mice are reminiscent of the *Alx1*^{-/-}*Alx4*^{-/-} and *Alx3*^{-/-}*Alx4*^{-/-} mutant mice reported previously (Qu et al.

1999; Beverdam et al. 2001). Through in situ hybridization and RT-qPCR analyses, we found that expression of *Alx1* and *Alx3*, but not that of *Alx4*, was significantly reduced in the developing nasal processes in *Six1^{+/+}Six2^{-/-}* embryos in comparison with the single mutant and control littermates at E10.5 (Fig. 4M–V). These data indicate that *Six1* and *Six2* function in a gene regulatory network with the *Alx* family transcription factors to control frontonasal development.

Discussion

Whereas studies of *Six1* mutant mice have confirmed critical roles for *Six1* in the development of inner ear, kidney, and other organs affected in BOS3 patients (Xu et al. 2003; Bosman et al. 2009), no obvious frontonasal defects have been reported in mice heterozygous or homozygous for *Six2* null alleles. In this report, we show that *Six1* and *Six2* exhibit partly overlapping patterns of expression in the developing frontonasal, maxillary, and mandibular processes (Fig. 1) and that *Six1^{+/+}Six2^{-/-}* double-mutant pups exhibit severe craniofacial defects, including midline facial cleft and agenesis of the frontal and parietal bones. These results identify a crucial but overlapping function of *Six1* and *Six2* in frontonasal development.

Six2 is strongly expressed in the neural crest–derived frontonasal mesenchyme, whereas *Six1* is expressed not only in frontonasal mesenchyme but also in developing nasal and olfactory epithelium during mouse facial development. Our analyses of *Six1^{+/+}Six2^{-/-}Wnt1Cre* mutant mice indicate that a neural crest cell–autonomous function of *Six1* and *Six2* is required for frontonasal development. The midline facial cleft phenotype of *Six1^{+/+}Six2^{-/-}* mutant pups resembles the facial defects in *Alx1^{+/+}Alx4^{-/-}* and *Alx3^{-/-}Alx4^{-/-}* mice (Qu et al. 1999; Beverdam et al. 2001). Both *Alx3^{-/-}Alx4^{-/-}* and *Six1^{+/+}Six2^{-/-}* mutant embryos exhibited increased apoptosis of the developing nasal mesenchyme, whereas *Alx1^{-/-}* embryos showed increased apoptosis of early embryonic forebrain mesenchyme (Zhao et al. 1996; Beverdam et al. 2001; Fig. 4 in this study). Remarkably, expression of both *Alx1* and *Alx3* in the developing nasal processes was significantly reduced in

and *Six1^{+/+}Six2^{-/-}* (L) embryos showing immunofluorescent staining of active caspase-3 (red). White dashes outline the medial nasal processes. Arrows in L point to a large number of active caspase-3–positive cells in the medial nasal mesenchyme in the *Six1^{+/+}Six2^{-/-}* embryo. Scale bar in J, 100 μ m. Multiple serial sections from 3 embryos of each genotype were analyzed. (M–U) Lateral view of patterns of *Alx1* (M–O), *Alx3* (P–R), and *Alx4* (S–U) messenger RNA (mRNA) expression in the E10.5 *Six1^{+/+}Six2^{-/-}* (M, P, S), *Six2^{-/-}* (N, Q, T), and *Six1^{+/+}Six2^{-/-}* (O, R, U) embryos. Arrow points to the lateral nasal process in each sample. At least 2 embryos of each genotype were analyzed using each probe. Scale bar in M, 200 μ m. (V) Quantitative real-time polymerase chain reaction (RT-qPCR) analysis of the levels of expression of *Alx1*, *Alx3*, and *Alx4* mRNAs in the E10.5 *Six1^{+/+}Six2^{-/-}*, *Six2^{-/-}*, and *Six1^{+/+}Six2^{-/-}* embryos ($n = 3$ each). RT-qPCR analyses were performed using total RNAs extracted from microdissected facial tissues from the region of E10.5 embryos as marked by the yellow-dashed lines in panel M. The asterisk indicates significantly different expression levels ($P < 0.05$ by Student's t test), whereas ns indicates no significant difference ($P > 0.05$ by Student's t test). bo, basioccipital; bs, basisphenoid; fb, frontal bone; pb, parietal bone; ps, presphenoid; zmx, zygomatic process of maxilla.

Figure 4. Analyses of craniofacial defects in *Six1^{+/+}Six2^{-/-}Wnt1Cre* embryos and mechanisms underlying frontonasal defects in *Six1^{+/+}Six2^{-/-}* embryos. (A, D, G) Frontal view of E18.5 heads of the *Six1^{+/+}Six2^{-/-}* (A), *Six1^{+/+}Wnt1Cre* (D), and *Six1^{+/+}Six2^{-/-}Wnt1Cre* (G) embryos. (B, E, H) Lateral view of alizarin red- and Alcian blue–stained E18.5 head skeletons of the *Six1^{+/+}Six2^{-/-}* (B), *Six1^{+/+}Wnt1Cre* (E), and *Six1^{+/+}Six2^{-/-}Wnt1Cre* (H) embryos. (C, F, I) Ventral view of E18.5 head skeletons of the *Six1^{+/+}Six2^{-/-}* (C), *Six1^{+/+}Wnt1Cre* (F), and *Six1^{+/+}Six2^{-/-}Wnt1Cre* (I) embryos. Asterisk in G indicates midline facial cleft in the *Six1^{+/+}Six2^{-/-}Wnt1Cre* embryo. The thick arrow in H points to the abnormal shape of the nasal region, whereas the thin arrow points to the deficiency in frontal bones in the *Six1^{+/+}Six2^{-/-}Wnt1Cre* mutant. The arrow in I points to the midline facial cleft in *Six1^{+/+}Six2^{-/-}Wnt1Cre* mutant. The asterisk in the ps*/bs* and zmx* labels in Panel I points to malformation of those structures. Scale bar in A and B, 1 mm. (J–L) Representative frontal sections of E10.5 *Six1^{+/+}Six2^{-/-}* (J), *Six2^{-/-}* (K),

Six1^{-/-}*Six2*^{-/-} embryos (Fig. 4M–V). These data suggest that *Six1* and *Six2* regulate frontonasal development at least in part through regulating expression of the *Alx* family genes and that the pathogenic mechanism underlying frontonasal dysplasia in patients with deletion of *SIX2* is related to that in patients with loss of *ALX* gene function.

In addition to validating a crucial role of *Six2* in frontonasal development, we found that *Six2* complements *Six1* function in patterning the jaws. Partial transformation of the maxillary zygomatic bone to a mandibular condyle-like structure has only been reported in 1 of 3 previously characterized *Six1* null mutant mouse lines (Laclef et al. 2003; Guo et al. 2011; Tavares et al. 2017). In this report, we found that 39.3% of mice homozygous for the *Six1*^{Cre/hpAP} allele (Guo et al. 2011) in the ICR background exhibited the zygomatic bone defect. The lower frequency of maxillary-to-mandibular transformation in this line of *Six1* mutant mice than that observed by Tavares et al. (2017) in the *Six1*^{tm1Kwk} mutant line (Ozaki et al. 2004) could be due to differences in the gene-targeted alleles and/or genetic background modification. We found that inactivating 1 allele of *Six2* increased the frequency of the maxillary defect in the *Six1*^{-/-} mice in the ICR background to 88.9%. Moreover, the *Six1*^{-/-}*Six2*^{-/-} double homozygous mutant pups showed much more severe maxillary and mandibular defects than in *Six1*^{-/-} and *Six1*^{-/-}*Six2*^{+/-} mutants. These data indicate that *Six2* complements *Six1* function in maxillary and mandibular development. Furthermore, we found that the *Six1*^{-/-}*Six2*^{+/-} mutant embryos had ectopic expression of *Bmp4*, *Msx1*, and *Msx2* in the proximal maxillary processes and that inactivating 1 allele of *Bmp4* significantly rescued maxillary bone formation in both *Six1*^{-/-} and *Six1*^{-/-}*Six2*^{+/-} mutants. Thus, in addition to the previously identified role of *Six1* in regulating Edn1-EDNRA signaling (Tavares et al. 2017), our results indicate that *Six1* and *Six2* act together to pattern the mammalian jaws through regulating BMP signaling as well. Related to this finding, Garcez et al. (2014) showed previously that double-stranded RNA-mediated silencing of *Six1*, *Six2*, and *Six4* mRNAs in the cranial neural crest cells abolished formation of craniofacial skeletal structures in chick embryos and that overexpression of *Noggin*, a BMP antagonist, in the cranial neural crest cells nearly completely restored facial skeleton formation in *Six1/2/4* triple-knockdown chick embryos. Together, these results indicate that *Six1* and *Six2* play an evolutionarily conserved role in patterning the facial skeleton through controlling BMP signaling.

Author Contributions

Z. Liu and C. Li contributed to design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; J. Xu and H. Liu contributed to data acquisition, critically revised the manuscript; Y. Lan and X. Wang contributed to design, data analysis, interpretation, and critically revised the manuscript; X. Li and P. Maire contributed to design, critically revised the manuscript; R. Jiang contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript.

All authors gave final approval and agree to be accountable for all aspects of the work.

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