Interaction between \textit{Foxc1} and \textit{Fgf8} during Mammalian Jaw Patterning and in the Pathogenesis of Syngnathia

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Abstract

Syngnathia (bony fusion of the upper and lower jaw) is a rare human congenital condition, with fewer than sixty cases reported in the literature. Syngnathia typically presents as part of a complex syndrome comprising widespread oral and maxillofacial anomalies, but it can also occur in isolation. Most cartilage, bone, and connective tissue of the head and face is derived from neural crest cells. Hence, congenital craniofacial anomalies are often attributed to defects in neural crest cell formation, survival, migration, or differentiation. The etiology and pathogenesis of syngnathia however remains unknown. Here, we report that \textit{Foxc1} null embryos display bony syngnathia together with defects in maxillary and mandibular structures, and agenesis of the temporomandibular joint (TMJ). In the absence of \textit{Foxc1}, neural crest cell derived osteogenic patterning is affected, as osteoblasts develop ectopically in the maxillary prominence and fuse with the dentary bone. Furthermore, we observed that the craniofacial musculature is also perturbed in \textit{Foxc1} null mice, which highlights the complex tissue interactions required for proper jaw development. We present evidence that \textit{Foxc1} and \textit{Fgf8} genetically interact and that \textit{Fgf8} dosage is associated with variation in the syngnathic phenotype. Together our data demonstrates that \textit{Foxc1} – \textit{Fgf8} signaling regulates mammalian jaw patterning and provides a mechanistic basis for the pathogenesis of syngnathia. Furthermore, our work provides a framework for understanding jaw patterning and the etiology of other congenital craniofacial anomalies, including temporomandibular joint agenesis.

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Introduction

Jawed vertebrates, or gnathostomes, represent the majority of extant vertebrate species. In fact, more than 99 per cent of the roughly 58,000 living vertebrate species have jaws [1]. A functional, articulating jaw is required for proper nutritional intake, maintenance of oral health, and communication, and its appearance was a turning point in vertebrate evolution. Jaws allowed primitive vertebrates to become effective predators through capturing and processing large, motile prey, and probably account for much of their subsequent success in radiating and adapting to new environments.

The vertebrate jaw consists of separate upper and lower skeletal elements connected by a joint. The mature jaw structures are derived predominantly from the first pharyngeal arch (PA1), an embryonic outgrowth or facial prominence that is composed of (1) a core of mesoderm that will give rise to muscle and vasculature [2–4], (2) a population of neural crest cells that will differentiate into bone, cartilage, and connective tissue [5–8], (3) an endodermal lining, and (4) a covering of ectoderm both of which provide signals that govern proper survival, patterning, and differentiation of each of these cell populations [9–13]. The first pharyngeal arch can be subdivided into discrete upper (maxillary) and lower (mandibular) portions, which contribute to the upper and lower jaw respectively. In addition to these cell populations, the medial (MNP) and lateral (LNP) nasal prominences also make key tissue and signaling contributions to jaw development [14–18]. The jaw is constructed from several distinct and separate skeletal elements derived from these prominences including the maxilla, jugal, squamosal, and dentary bones. The temporomandibular joint (TMJ) is the functional jaw joint in mammals and is essential for jaw articulation. The TMJ is a complex synovial joint and consists of the glenoid fossa of the squamosal bone, the condylar head of the dentary, a fibrocartilaginous disc that is located between these two bones, and muscles and tendons that attach to the joint [19].

Craniofacial anomalies constitute approximately one-third of all congenital defects. Given the anatomical complexity associated with jaw development and function, it is not surprising that jaw malformations occur frequently. Syngnathia which is characterized by fusion of the upper and lower jaw, is a rare disorder for which the genetic or environmental etiology and pathogenesis remains unknown.

\textit{Foxc1} is a member of the forkhead box winged helix transcription factor family distinguished by its highly conserved forkhead DNA binding domain (for reviews, [20,21]). In mice, \textit{Foxc1} has been reported to play roles in meningeal [22,23], calvarial [24–28], ocular [29–31], somitic [32] and renal development [33]. Here we report a novel role for \textit{Foxc1} in
orofacial development. Foxc1 null mutant mouse embryos display bony synagnathia in addition to defects in maxillary and mandibular structures together with agenesis of the TMJ. We present evidence that Foxc1 interacts genetically with Fgf8 to control pattering of the neural crest cell derived jaw and TMJ and furthermore that the variation in the severity of synagnathia is Fgf8 dosage-dependent. Our results therefore demonstrate that Foxc1 plays a critical role in jaw development and disease; provides a mechanistic basis underpinning the pathogenesis of the synagnathia; and establishes a framework for understanding the etiology of other congenital craniofacial anomalies, including temporomandibular joint agenesis.

**Results**

**Foxc1−/− newborn mice display synagnathia**

Foxc1 is initially expressed in the oral ectoderm and cranial mesenchyme in E8.5 embryos (Figure 1A–C). By E9.25, Foxc1 activity has diminished in the oral ectoderm, but continues to be expressed diffusely within the PA1 mesenchyme (Figure 1D, E). At E10.5, Foxc1 is restricted to a discrete caudal-medial domain of the mandibular portion of PA1 (Figure 1F). β-galactosidase expression under the control of the endogenous Foxc1 promoter [26] demarcates a similar spatiotemporal domain of expression in the mandibular mesenchyme (Figure 1G, H). However, the stronger staining intensity may reflect the stability or persistence of lacZ expression over whole mount in situ staining. Nonetheless, this data illustrates the dynamic activity of Foxc1 within the developing PA1 in E8.5–10.5 embryos.

We discovered that Foxc1−/− mutant mice exhibit a bilateral fusion of the upper jaw zygomatic complex to the dentary bone (Figure 2A, B) closely mimicking a condition in humans termed synagnathia. To evaluate the synagnathic phenotype in detail, we dissected the maxillary and mandibular structures from skeletal preparations of Foxc1−/− late gestation to newborn pups (embryonic day 18.5 – postnatal day 0; E18.5-P0 Figure 2). A summary of the skeletal phenotypes is presented in Table 1. Compared to the wild-type controls (Figure 2C), the body of the maxillary bone in Foxc1−/− mutant embryos was reduced in size and the abnormally thickened and shortened zygomatic process of the maxilla was fused to the dentary bone (Figure 2D). Fusion occurred either proximal to the molar alveolar ridge (n = 7/13, Figure 2D) or encompassed the entire alveolar region (n = 6/13, Table 1). Although this phenotype has been previously described as an enlarged zygomatic process of the maxilla [26] or as massively ossified zygomatic and dentary bones [27], our more detailed analyses indicate that Foxc1−/− mutant mice represent a unique previously undescribed model for studying the pathogenesis of synagnathia.

**Temporomandibular joint agenesis in Foxc1−/− embryos**

The articulation of the mammalian jaw occurs at the TMJ located between the condyle of the dentary and the squamosal fossa of the squamosal bone (circle; Figure 2A, C, E). The condyle in Foxc1−/− mutant embryos was hypoplastic and appeared bifurcated (n = 10/13) (arrowhead; Figure 2B, D). Given the degree of fusion, we cannot however, rule out the possibility that this could also represent a duplicated angular process. Nonetheless, the squamosal bone was similarly hypoplastic in Foxc1−/− mutant embryos compared to wild-type controls with no evidence of zygomatic process formation (Figure 2F, G). In E17.5 wild-type embryos, a neural crest cell derived articular disc normally sits between the condyle and squamosal fossa and expresses scleraxis (Scx) [34] (Figure 3A and S1A, B). However, no equivalent domain of Scx positive expression, as evidence of articular disc formation was observed around the hypoplastic condyle in Foxc1−/− embryos (Figure 1C and 3B).

Although the structural features of the TMJ are well documented, the genetic, cellular, and molecular mechanisms involved in TMJ morphogenesis remain poorly understood. To explore the mechanism of the jaw joint anomalies, we analyzed the expression of several genes involved in cartilage and bone formation at E17.5. All markers of proliferating and mature chondrocytes were very similar between wild-type and Foxc1−/− condyles (Figure 3C–H). However, the proportion of ColX positive hypertrophic chondrocytes within the Foxc1−/− condyle appeared to be larger relative to controls, suggesting premature ossification (Figure 3I, J). The expression of ColII, a marker of osteoblasts, remained unchanged in the condyle of mutant embryos (Figure 3K, L), but was not seen across from the condyle indicating that no fossa formed. Collectively, our histological and molecular analyses of the TMJ in Foxc1−/− mutant embryos, illustrate the absence of glenoid fossa and articular joint disc, in addition to an abnormal condyle during TMJ development. This indicates that in addition to the synagnathia described above, a functional TMJ does not form in the absence of Foxc1.

**Abnormalities of the palate in Foxc1−/− embryos**

Skeletal preparations at P0 revealed that in contrast to wild-type littermates, a small region of the posterior palatine bone did not fuse in Foxc1−/− neonates (Figure 3A, C). The soft tissue walls of the buccal cavity were shifted medially in association with the synagnathic jaw, and consequently the palate in Foxc1−/− neonates was slightly more arched than in wild-type (Figure 4C–F). The incisive papilla and rugae were readily identifiable in wild-type and Foxc1−/− palates. Whereas wild-type palates develop 8 rugae in a distinct sequence [35], only 7 rugae form in Foxc1−/− palates. Furthermore, the mutant rugae were not as sharply delineated as in wild-type (Figure 5B, D and S4G, D). Despite abnormalities in the bones of the Foxc1−/− palate, no overt soft tissue clefting was observed in either the primary or secondary palate in Foxc1−/− neonates (Figure 5B, D).
Muscle patterning is abnormal in Foxc1−/− embryos

The pharyngeal arches contribute mesoderm to the tongue and the muscles of mastication – the masseter, temporalis and pterygoid. Given the jaw anomalies evident in Foxc1−/− mutant embryos, we explored whether muscle patterning was also affected. The anterior end of the tongue in Foxc1−/− mutant embryos was abnormally spade shaped and protruded from the oral cavity (Figure S2E, F). Both fungiform and median circumvallate papillae were identified in the Foxc1−/− tongue, and section immunostaining for muscle actin revealed that both intrinsic and extrinsic tongue muscles were well-organized in wild-type and mutant specimens (Figure 4C–F). Thus the tongue muscles form normally in Foxc1−/− mice, although the shape of the tongue may be constricted by the abnormal fusion of bony elements of the jaw.

To examine the patterns of muscle formation further, we performed whole mount immunostaining for neonatal muscle using myosin II in P0 wild-type (n = 8) and Foxc1−/− (n = 4) heads (Figure 4A, B). Compared to wild-type controls the masater and temporalis muscles were reduced in size in the mutants. In histological sections, the temporalis muscle was shifted medially into the region normally occupied by the squamosal bone (Figure S1D–G) and was abnormally associated with the condyle. The lateral and medial pterygoid muscles, which attach to the condyle and angular process respectively were still associated with the correct processes in Foxc1−/− specimens (Figure 4G, H); however, the orientation of both the lateral and medial pterygoid was altered, presumably due to the abnormal position of the bones in the syngnathic Foxc1−/− jaw. The digastric muscle that arises from the second heart field mesoderm was robustly detected in whole mount neonatal muscle myosin II stained heads in both wild-type and mutants (Figure 4I, J). Collectively, these data indicate that abnormal cranial muscle patterning was specific to those derived from PA1, and that these muscles were abnormally shaped, sized, and positioned possibly as a secondary result of altered cranioskeletal patterning.

Ectopic neural crest cell derived osteoblast differentiation in Foxc1−/− embryos

The jaw, TMJ and muscle patterning abnormalities could be due to abnormal development of the neural crest cell derived PA1 mesenchyme. Therefore we examined the formation, migration,
proliferation and differentiation of neural crest cells in *Foxc1*−/− embryos compared to their control littermates. Through *Sox10* in situ hybridization (Figure S3A, B) and *Wnt1-CreZ/EG* lineage tracing experiments (Figure S3C–F), we observed that NCC formation, migration, and contribution to PA1, the nasal prominences, and their derivatives between E9.0-12.5 embryos, were all normal in the absence of *Foxc1*. Similarly, E11.5 *Foxc1*−/− embryos present with a normal pattern of neurofilament immunostaining, suggesting that neural crest cell contribution to cranial ganglia and peripheral nervous system is not dependent on *Foxc1* (Figure S3G, H).

Despite normal neural crest cell colonization, the PA1 in *Foxc1*−/− mutants was smaller than that of wild type littermates as early as E8.75 (Figure 5A, D). From E9.5-11.5, the mutant maxillary and mandibular prominences remained reduced compared to controls and the invagination of oral ectoderm

Figure 2. *Foxc1*−/− neonates exhibit syngnathia and TMJ agenesis. Alizarin red (bone) and alcian blue (cartilage) stained skeletal preparations of *Foxc1*+/+ (A, C, E, F) and *Foxc1*−/− (B, D, G) P0 neonates. (A, B) Intact view of cranial skeleton showing relative position of upper and lower jaw elements. Syngnathia (syn*) is evident in *Foxc1*−/− neonates. Circles highlight the articulating joint. Arrows in A and B highlight lack of ossification of malleus, incus, and stapes in mutant middle ear. (C) Dissected wild-type maxilla (mx) and jugal (jg). (D) The mutant maxilla is fused in the zygomatic region to the dentary (dnt) which displays hypoplastic coronoid (crp), condylar (cdp), and angular (agp) processes compared to controls (E). The *Foxc1*−/− condyle is bifurcated (arrowhead in D). (F, G) The mutant squamosal (sq) and alisphenoid (als) are hypoplastic, and the squamosal lacks a zygomatic process (asterisk). Scale bars: 500 μm Abbreviations: cps, caudal process of squamosal; fmx, frontal process of maxilla; iof, infraorbital foramen; li, lower incisor; rps, retrotympnic process of squamosal; zmx, zygomatic process of maxilla; zps, zygomatic process of squamosal.

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Table 1. Summary of skeletal phenotypes in jaw related structures.

|            | Premaxilla | Nasal Bone | Maxilla | -Body process | -Palatal process | -Frontal process | -Zygomatic process | Jugal | Squamosal Dentary | -Distal region | -Proximal region | Meckel’s cartilage | -Body | -Middle ear | Facial Asymmetry | Boney Palate Fusion |
|------------|------------|------------|---------|---------------|----------------|----------------|-------------------|-------|-----------------|--------------|----------------|----------------|--------|------------|----------------|-----------------|
| Foxc1<sup>-/-</sup> (n = 13) | +          | +          | ↓       | -             | +              | +              | ↓ (12/13)          | ↓     | no              | ↓ (12/13) | ↓              | +              | ↓      | +          | -              | ↓               |
| Fgf8<sup>Null/Neo</sup> (n = 8) | ↓, rounded and fused ventrally | ↓          | ↓       | ↓             | ↓              | ↓              | ↓                 | ↓     | no              | ↓ (7/8) | ↓              | ↓              | ↓      | ↓          | ↓              | ↓               |
| Foxc1<sup>-/-</sup>; Fgf8<sup>Null</sup> (n = 4) | ↓          | ↓          | ↓       | ↓             | ↓              | ↓              | ↓                 | ↓     | no              | ↓ (7/8) | ↓              | ↓              | ↓      | ↓          | ↓              | ↓               |

Legend: +, normal; −, absent; ↓, decreased; multiple arrows for a trait indicate relative severity within the category.

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Figure 3. Molecular analysis of developing TMJ in Foxc1<sup>−/−</sup> embryos. In situ hybridization analysis on serial coronal cryosections in Foxc1<sup>+/+</sup> (A, C, E, G, I, K) and Foxc1<sup>−/−</sup> (B, D, F, H, J, L) mouse heads at E17.5. In (A–L) dashed lines outline the wild-type and bifurcated mutant condyles (cdp and cdp<sup>p</sup>). (A, B) Scol is expressed in the neural crest-derived disc (arrow) and tendon (red arrowheads) of controls. In mutants, Scol is maintained in the tendon (red arrowheads), but no disc is present. (C–F) Sox9 is localized to the proliferating chondrocytes and Acan is localized to the cartilage of the condyle growth plate in wild-type and Foxc1<sup>−/−</sup> condylar processes. (G, H) Ihh is localized to the prehypertrophic chondrocytes of the condylar growth plate. In the absence of Foxc1, the hypertrophic chondrocytes make up a larger proportion of the abnormally bifurcated condyle. (K, L) Coll expression is localized to the osteoblasts of the condyle in control and Foxc1<sup>−/−</sup> embryos; however, the Coll positive glenoid fossa (f) is only observed in control specimens.

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markers known to play key roles in patterning the proximal-distal axis of PA1 [14,37–39]. In E9.5-10.5 wild-type and Foxc1<sup>−/−</sup> mutant embryos, the lateral and medial nasal prominences develop such that morphologically the lambdoidal junction is readily identifiable (Figure 5A). Bmp4, which is a key regulator of distal jaw patterning (Figure S5A–D) and its downstream target Mesp2 (Figure S5E–H) were expressed normally in the distal mandibular ectoderm, the olfactory epithelium, and at the lambdoidal junction in Foxc1<sup>−/−</sup> (n = 4) embryos at E10.5.

Endothelin signaling is also required to pattern the distal elements of the jaw but primarily establishes mandibular identity [40–43]. Hand2, which is a downstream target of endothelin, was expressed normally in the distal PA1 mesenchyme of Foxc1<sup>−/−</sup> (n = 4) embryos (Figure S5I–L). Additionally, Gata3 which is required for endothelin-independent expression of Hand2 in the distal mandibular arch [44], was also properly expressed in both the distal mandibular ectoderm and the lambdoidal junction in E10.5 Foxc1<sup>−/−</sup> embryos (n = 3) (Figure S5M–P). Thus the normal expression of distal jaw signaling factors such as Bmp4, Mesp2, Hand2 and Gata3 is consistent with the normal morphology observed in the distal regions of the dentary and maxilla in Foxc1<sup>−/−</sup> embryos (Figure 2A–D).

Foxf8 expression in PA1 is altered in the absence of Foxc1

Since patterning cues at the distal ends of PA1 were not altered in Foxc1<sup>−/−</sup> embryos, we hypothesized that the ectopic osteoblast differentiation and synangithia reflected altered specification or signaling within the proximal region of PA1. Experimental evidence has implicated Foxf8 as being essential for proximal-distal axis specification and signaling [11,45–49], and it is localized to the oral ectoderm of PA1 near the maxillary-mandibular constriction. Although the oral ectoderm was clearly present, its invagination at the maxillary-mandibular constriction was shallower in E9.0-9.5 Foxc1<sup>−/−</sup> embryos compared to wild-type (Figure 5A). Therefore we examined Foxf8 expression in wild-type and Foxc1<sup>−/−</sup> embryos (Figure 7A; n = 4). In Foxc1<sup>−/−</sup> embryos, Foxf8 expression was expressed at the midbrain-hindbrain boundary and in the frontonasal prominence similar to wild-type controls. However, Foxf8 was reduced in the mandibular oral ectoderm and was absent from the maxillary regions of the oral ectoderm in E8.5-9.5 Foxc1<sup>−/−</sup> embryos.

Therefore we hypothesized that diminished Foxf8 activity correlated with the pathogenesis of synangithia. To determine if alterations of Foxf8 gene dosage could produce a similar syngnathic phenotype, we examined the PA1 derived upper and lower jaw bones in Foxf8<sup><sub>−/−</sub></sup> and Foxf8<sup><sub>−/−</sub></sup>/Neo embryos (Figure 7E, I, M, G, K, O). Indeed E18.5 Foxf8<sup><sub>−/−</sub></sup>/Neo embryos displayed synangithia (7/8) in which the maxilla containing a distinctive frontal process was fused to the malformed dentary, which lacked a condylar process. The site of maxilla-dentary fusion was positioned more distally in Foxf8<sup><sub>−/−</sub></sup>/Neo embryos than in Foxc1<sup><sub>−/−</sub></sup> embryos (Figure 7J, K, N, O). Left-right side differences were often observed in the length of each dentary bone and the location of synangithia along the proximal-distal axis of Foxf8<sup><sub>−/−</sub></sup>/Neo mutants. As each side of the mutant lower jaw was fused at the mandibular symphysis, this produced a distinct asymmetrical shift of the lower jaw (Figure S6G, H). Additional skeletal phenotype details are provided in Table 1, Figure 5 and Figure S6.

These results indicate that the relative levels of Foxf8 in the developing PA1 are key to the pathogenesis of synangithia. Therefore we quantified the comparative levels of Foxf8 in each of the mutant Foxc1 and Foxf8 lines by qPCR (Figure 7B) and observed Foxf8 activity in the oral ectoderm of Foxc1<sup>−/−</sup>/Embryos to be approximately only 80% of wild-type levels. This was a considerable decrease, well below the levels of Foxf8 maintained by Foxf8<sup><sub>−/−</sub></sup> embryos (100% of wild-type), but not quite as severely reduced as in Foxf8<sup><sub>−/−</sub></sup>/Neo embryos (41% of wild type). Collectively these expression data indicate that Foxc1 may be required to maintain proper levels of Foxf8 in the PA1 oral ectoderm.

Genetic interaction of Foxc1 and Foxf8

Since genetically reduced levels of Foxf8 resulted in synangithia and Foxf8 levels were reduced in Foxc1 mutants, we hypothesized that Foxc1 and Foxf8 may genetically interact to influence jaw development. Therefore we generated compound heterozygous Foxc1<sup>−/−</sup>/<sup>−/−</sup>;Foxf8<sup><sub>−/−</sub></sup>/Neo and Foxc1<sup>−/−</sup>/Embryos<sup><sub>−/−</sub></sup>;Foxf8<sup><sub>−/−</sub></sup>/Neo embryos. Four Foxc1<sup>−/−</sup> Foxf8<sup><sub>−/−</sub></sup>/Neo embryos were recovered between E18.5 – P0, which closely resembled Foxc1<sup>−/−</sup> embryos, but the phenotype was much more severe than either Foxc1<sup>−/−</sup> or Foxf8<sup><sub>−/−</sub></sup>/Neo embryos, lacking an oral opening and a visible lower jaw (arrowhead Figure 7H). In skeletal preparations, Foxc1<sup>−/−</sup>;Foxf8<sup><sub>−/−</sub></sup>/Neo embryos showed a more severe phenotype than that of either Foxc1<sup>−/−</sup> or Foxf8<sup><sub>−/−</sub></sup>/Neo embryos (Figure 7I–P). The premaxilla was smaller than controls and the maxilla consisted of a small frontal process and hypoplastic body of the maxilla. The maxilla was fused to a truncated dentary that lacked all proximal processes. The site of fusion was located quite distally along the length of the dentary, nearing the incisor region, however, incisor formation occurred in each of these mutants. Mecckel’s cartilage was present, but the proximal portion that gives rise to the malleus was thin and was associated with a small remnant of the incal cartilage (Figure S6A–C). Similar to Foxc1<sup>−/−</sup> the Foxc1<sup>−/−</sup>;Foxf8<sup><sub>−/−</sub></sup>/Neo mutant middle ear ossicles were still cartilaginous with no evidence of stapes formation at P0, whereas the wild-type malleus and incus were undergoing ossification.

The skeletal phenotypes of the Foxc1<sup>−/−</sup>;Foxf8<sup><sub>−/−</sub></sup> reflected an exacerbated loss of maxillary and TMJ related structures and a distal shift in the location along the proximal-distal axis of the dentary. We also observed facial asymmetry in these embryos similar to that observed in Foxf8<sup><sub>−/−</sub></sup>/Neo embryos (Figure S6G–J). Interestingly, we also occasionally observed synangithia in compound Foxc1<sup>−/−</sup>;Foxf8<sup><sub>−/−</sub></sup> heterozygotes at E18.5 (n = 2/12) (Figure 7L, inset). Abnormalities of the maxilla, dentary, squamosal, and alisphenoid were identical to those seen in Foxc1<sup>−/−</sup> embryos. This lends further evidence for the genetic interaction between Foxc1 and Foxf8. Consistent with this idea, we assessed whether Foxc1 expression was altered by decreased levels of Foxf8. While Foxc1 was properly localized to the PA1 mesenchyme, qPCR analysis indicated a slight decrease in Foxc1 mRNA levels in both E9.5 Foxf8<sup><sub>−/−</sub></sup> (59% of wild type) and Foxf8<sup><sub>−/−</sub></sup>/Neo (70% of wild type).
Figure 4. Abnormalities of PA1 derived muscle in Foxc1<sup>−/−</sup>. (A,B) Whole mount immunostaining for neonatal myosin II in P0 wild-type (A) and Foxc1<sup>−/−</sup> (B) heads. The PA1 masseter (ma) and temporalis (tm) muscles are smaller in the mutant than in wild-type. (C–G) Immunostained paraffin sections of E17.5 Foxc1<sup>+</sup/+ (C, E, G) and Foxc1<sup>−/−</sup> (D, F, H) heads showing muscle actin localization (brown). Sagittal (C, D) and frontal (E, F) sections.
showing organized muscle formation in both wild type and mutant tongues (tg). The palate (pl) is more arched in Foxc1−/− and shows smoother rugae (r) than in Foxc1+/−. (G, H) Frontal sections in the TMJ region. The mutant temporals muscle (tm) is shifted medially, abnormally associates with the bifurcated condyle (cdp), and its fibers are oriented differently than in wild type. The medial (mp) and lateral (lp) pterygoid muscles are appropriately associated with the angular (agg) and condylar processes in Foxc1−/−. The mutant masseter (ma) is reduced to a small component in Foxc1−/− (compare ma regions in F, H to E, G). Ventral view of whole mount myosin II immunostained P0 wild type (I) and Foxc1−/− (J) heads. The second heart field derived anterior digastric muscle (ad) is robustly detected in all specimens. Scale bars: 500 μm. Abbreviations: dnt, dentary; eom, extraocular muscle; f, geniohyoid; g, geniolingual muscle; my, mylohyoid muscle; mx, maxilla; trg, trigeminal ganglion.

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embryos (Figure 7C, D). Thus Fgf8 may be required to maintain proper levels of Foxc1 in the PA1 mesenchyme and conversely Foxc1 may be required to maintain proper levels of Fgf8 in the PA1 oral ectoderm forming a feedback loop critical for jaw development and in the pathogenesis of synangathia and TMJ agenesis.

The proximal Dlx code is disrupted in PA1 of Foxc1−/− embryos

Interestingly Fgf8 is instrumental in activating Dlx genes in neural crest cells thereby triggering the morphogenetic program which specifies regionalized jaw elements [50,51]. Regionalized patterning within PA1 is elaborated through a nested proximal-distal code of Dlx homeobox transcription factors in the mesenchyme [52–57], and the interpretation, pattern, and morphology of the jaw depend on the combinatorial activity of Dlx genes [56]. Therefore we examined the expression of Dlx genes in neural crest derived pharyngeal arch mesenchyme (Figure 8). Dlx2 expression in the maxillary portion of PA1 was reduced or absent in E10.5 Foxc1−/− mutant embryos compared to wild-type controls (n = 4) (Figure 8A–D). Similarly, there was a distinct loss of Dlx5 expression in the mesenchyme at the maxillary-mandibular constriction Foxc1−/− mutant embryos (n = 4) (Figure 8E–H). In contrast, the expression domains of both Dlx3 and Dlx6 appeared to be normal in Foxc1−/− embryos (n = 3) (Figure 8I–P). Therefore, the synangathia and TMJ agenesis observed in Foxc1−/− embryos likely manifests as a result of perturbed Fgf8 signaling in the proximal region of PA1, together with alterations in distinct subdomains of Dlx2 and Dlx5 which collectively lead to regionalized patterning defects in maxillary and mandibular mesenchyme from which the upper and lower jaw elements are derived.

Discussion

Syngathia (fusion of the upper and lower jaw) is a rare human condition with only 56 cases reported to date in the literature (Table S1, and references therein). Syngathia may involve connection between soft tissues (synoechna) or union between bony elements (synostosis). Bony fusion is rare, requires complex surgical repair, and is often present as part of a broader syndrome of congenital malformation. These cases reveal a high degree of variability in the location and extent of jaw fusion and indicate that bony syngathia may be isolated or syndromic. In about 18% of cases, syngathia is associated with known syndromes such as aglossia–adactylia syndrome or hemifacial microsomia. In all the reports of bony syngathia, there is bony fusion between elements of the upper and lower jaw and no known epigenetic, genetic, or molecular etiology.

Previous reports have speculated that congenital bony syngathia is caused by various factors, including abnormal development of the stapedial artery [58]; late gestation trauma or pressure defects in utero [59,60]; and abnormal neural crest cell proliferation and migration [61]. Our review of the case reports suggested that synangathia arises from defects in pharyngeal arch development and that there was likely a molecular or genetic basis for this disorder. In this study we present the first genetic model, a targeted deletion of Foxc1, with which to further understand the etiology and pathogenesis of human syngathia.

Foxc1 knockout mice provide a model for understanding human synangathia

Similar to human bony syngathia, fusion of the zygomatic complex to the dentary bone in Foxc1−/− mutants occurred distal to the temporomandibular joint region. Furthermore, components of the TMJ were also abnormal in the Foxc1−/− model. Defects were observed in the morphology of the condyle and squamosal, in addition to agenesis of the joint disc and glenoid fossa. Our studies also indicate that jaw associated muscles, including the masseter, temporalis, and pterygoids, were deficient and abnormally positioned in the absence of Foxc1. This reduction in size of the muscles of mastication is very similar to the decrease in muscle volume of the masseter, temporalis, and pterygoid reported in human hemifacial microsoma patients [62–65].

Foxc1 plays a role in patterning of maxillary and joint related structures of jaw

Abnormal morphology was observed in Foxc1−/− as early as E8.75 in the form of maxillary and mandibular hypoplasia, with the maxillary prominence more grossly affected. This occurred in the absence of changes in neural crest cell contribution to the arch, or decreases in the mitotic index, or ectopic apoptosis. We did observe a lengthening of the cell cycle in Foxc1−/− cranial mesenchyme, which may contribute to the reduced size of PA1. It is also possible that morphological shape changes contribute to the reduction of the PA1 in Foxc1−/− embryos. Future morphometric analyses may shed further light on the underlying cause of the gross abnormalities of PA1 in Foxc1−/− embryos.

Our data indicate that the epithelial signals associated with the distal regions of PA1 remain intact in the absence of Foxc1. We investigated known growth signaling and transcription factors associated with regionalized patterning of PA1 and subsequent jaw development. Reduction in Dlx2 was restricted to the maxillary prominence mesenchyme and loss of Dlx5 was limited to a discrete region of mesenchyme at the maxillo-mandibular constriction. The loss of Foxc1 has the most dysmorphic effect on the maxilla, jugal, and squamosal indicating a requirement for Foxc1 in maxillary patterning and differentiation, particularly structures of the zygomatic complex. The zygomatic complex is also disrupted when Dlx2 is knocked-out in mouse [53]. In addition, targeted disruption of Dlx5 [54] results in abnormal truncation and arrangement of the condylar and angular processes as well as a deviation or split in Meckel’s cartilages associated with abnormal ossification. These structures are similarly abnormal in Foxc1−/− mutants. Combinatorial heterozygotic loss of Dlx2 and Dlx5 [56], results in abnormalities of maxillary, palate, and dentary structures as well as severe disruption of both the primary (incal-malapel) and secondary (squamosal-condylar) articulations. Interestingly, loss of Dlx2/5 also results in loss of PA1 derived branchiomeric muscle [66] while Foxc1−/− mutants exhibit significant reduction and
abnormality of these muscles. Our data showing discrete changes in the proximal Dlx code and resultant skeletal phenotype in Fgf8Null/Neo mutants is consistent with the previous studies of nested Dlx expression in jaw patterning, and recent studies in mouse indicating that Dlx expression within the arch may be more dynamic than previously appreciated [67]. It appears that the specific levels and domains of Dlx2 and Dlx5 expression that require Foxc1 are critical for patterning elements of the zygomatic complex, maxilla, squamosal and proximal dentary upon which mammalian jaw articulation depends.

**Fgf8 dosage affects severity and phenotypic variability in syngnathia**

Alteration to the Dlx code in the Foxc1−/− PA1 appears to occur downstream of Fgf8. Interestingly the ectodermal domain of Fgf8 overlies the mesenchymal domain of Dlx2 while Bmp4 overlies the epithelial domain [68]. Moreover, the mesenchymal expression of Dlx2 is positively regulated by Fgf8 signaling while the ectodermal activity of Dlx2 is maintained by Bmp4 signaling. Fgf8 therefore is instrumental in activating Dlx genes in neural crest cells and triggering the morphogenetic program, which specifies different jaw elements [50,51]. Consistent with this, we demonstrated that simply reducing the overall dosage of Fgf8 could produce bony syngnathia. Fgf8Null/Neo embryos exhibit jaw fusion similar to that observed in Foxc1−/− embryos. Furthermore, genetically reducing one copy of Fgf8 in combination with the Foxc1 null further exacerbated the extent of jaw fusion and the perturbation of maxillary and dentary elements. Together these data indicate that Foxc1 genetically interacts with Fgf8 to influence jaw patterning, and that overall dosage of Fgf8 affects the severity of the syngnathic phenotype.

The Foxc1−/− syngnathic phenotype is similar to, but less severe than the Fgf8Null/Neo jaw phenotype (see Table 1; Figure 7; Figure S6). However, the combinatorial loss of Foxc1 and Fgf8 displayed the most severe phenotype in the spectrum reported here, which may indicate that Foxc1 and Fgf8 function synergistically in their roles in jaw patterning. Interestingly, as Fgf8 dosage decreased in these mouse mutants, we observed facial asymmetry similar to that reported in human syngnathia [69,70], hemifacial microsomia, and in a high proportion of craniofacial malformations and syndromes [71]. Foxc1 is known to play a key role in establishing left-right asymmetry [72], and conditional loss of Fgf8 in the oral ectoderm of mice [11] and in a hypomorphic zebrafish model [73] result in cranial asymmetry.

**Dynamic expression of Foxc1 in PA1**

Our data indicate that Foxc1 is initially expressed in the oral ectoderm and cranial mesenchyme contributing to the developing PA1 in E8.5 embryos. At this stage, both Foxc1 and Fgf8 are expressed in overlapping domains within the PA1 oral ectoderm (Figures 1A–C and 7A). In Foxc1−/− embryos at E8.5, the domain and levels of Fgf8 are reduced but not absent in the oral ectoderm (Figure 7C). Foxc1 therefore is not required to induce Fgf8 expression in the oral ectoderm, but may be required to maintain proper levels and localization of Fgf8 (Figure 7). Conversely, we showed that Foxc1 activity was reduced in the Fgf8 allelic series of mutants. Taken together this suggests that Fgf8 and Foxc1 potentially form a feedback loop critical for jaw development and in the pathogenesis of syngnathia and TMJ agenesis.

**Models of jaw patterning and evolution**

Given the important role of jaw development in both evolution and disease, many groups have proposed models to address the concept of polarity within the context of pharyngeal arch development and jaw evolution. Two recent models include the dynamic growth zone model [reviewed in (74)] and the hinge and caps model [75,76]. Briefly, the growth zone model posits that dorso (proximal)-ventral (distal) polarity of the PA relies upon Edn1 signaling to establish a ventral/distal zone within the PA. Once established, distal Edn1 and Bmp4 cues regulate nested Dlx gene expression in the PA to establish a combinatorial, dynamic expression code within the arch. The more distal domains contain undifferentiated cell types, while the cells more immediately and proximally positioned within the mandible differentiate, resolving into zones. The intermediate zone, which expresses Bapx1, becomes permissive to forming structures of the jaw joint, and the dorsal/proximal zone is established by jag1b expression.

The hinge and caps model [75,77,78], places articulation, and subsequently the polarity and modularity, of the upper and lower jaws in the context of cranial neural crest competence to respond to localized epithelial signals. The hinge is defined as the epithelial junction of the maxillary and mandibular prominences of the first arch, also known as the maxillo-mandibular constriction. The caps constitute the lamboidal junction and proximal maxillary region for the upper jaw, and the midline of the mandibular prominences for the lower jaw. Proper patterned placement of the hinge at the sight of articulation and balanced patterning at both caps assures that the elements of the upper and lower jaw will develop in register with one another. Modularity and proximodistal polarity is achieved by the integration of hinge and caps signaling, such that the PA is divided into nested, overlapping developmental fields [75].

The data presented in this study aligns with both the growth zone model and the hinge and caps model. Our data indicate that in the Foxc1−/− mutant embryos, distal Edn1 and BMP4 signaling within PA1 are normal, and that Dlx5 and Dlx3 expression within the recently identified [67] intermediate domain of PA1 is maintained. The loss of a discrete domain of Dlx5 expression
Figure 6. Time-course of PA1-derived skeletal abnormalities in Foxc1<sup>−/−</sup> mice. (A, D) Gross view of fixed Foxc1<sup>+/+</sup> (A) and Foxc1<sup>−/−</sup> (D) embryos at E13.5. Cerebral hemispheres are enlarged and the snout is foreshortened in the mutant. (B, E) Whole mount alcian blue staining to detect cartilage differentiation in the wild type (B) and mutant (E) embryos pictured in (A, D). A single, normal Meckel’s cartilage (arrows in B, E) is seen in both control and Foxc1<sup>−/−</sup>. (C, F) Bisected E13.5 heads of Foxc1<sup>+/+</sup> (C) and Foxc1<sup>−/−</sup> (F) embryos stained for endogenous alkaline phosphatase (AP) activity to detect early osteoblast differentiation. In the absence of Foxc1, early osteoblasts of the dentary (dnt) and maxillary (mx) region initially differentiate in a fused, syngnathic pattern (*). (G–L) Whole mount alcian blue (cartilage) and alizarin red (bone) staining of Foxc1<sup>+/+</sup> (G, I, K) and
near the maxillo-mandibular constriction in Foxc1<−/−> embryos is similar to the disruption reported in Edn1<−/−>Foxg1-Cre mutants and provides further evidence of complex control of Dlx expression within specific zones of the developing arch [67]. While it is tempting to speculate that disruption of Foxc1 in our mutants results in a duplication of the condyle or the proximal portion of the dentary rather than a bifurcation, (Figures 3 and 6), we have no molecular data, such as expansion of Dlx3 or Dlx6 into the maxillary region, to support this at this time. Additionally, similar to the reported requirement for ectodermal expression of Edn1 in patterning the intermediate domain, the disruption of Fgβ expression in the oral ectoderm of Foxc1<−/−> embryos may similarly disrupt Dlx2 and Dlx5 in the immediately underlying regions of the PA1 mesenchyme.

With respect to the hinge and caps model, the observed defects in Foxc1<−/−> PA1 morphology and skeletal defects affecting the elements involved in jaw articulation (the squamosal, zygomatic complex, and condyle) indicate disruption may center on the proposed hinge region of PA1. In this manner, the expression of both Foxc1 and Fgβ in the PA1 oral ectoderm at E8.5-9.0, in combination with the apparent decrease in Fgβ expression localized to the Foxc1<−/−> PA1 ectoderm, suggest that Foxc1 may be required to maintain proper hinge associated signals. Localization of Dlx2 and Dlx5 are also primarily altered at the proximal aspect of their expression domains, and the skeletal abnormalities observed are relatively more severe for maxillary jaw elements. Taken together, these may indicate disruption of the symmetric localization of hinge signaling at the maxillo-mandibular constriction in the absence of Foxc1. In agreement with the proposed symmetrical alignment of signals at proximal and distal caps, BMP and Edn signals remain intact in the Foxc1<−/−> embryos and do not display gross evidence of regnathia (Figure 6), suggesting the skeletal elements at the cap retain their alignment. This is in contrast to the observed regnathia when Edn1 is conditionally disrupted [67], which could be interpreted as altering the distal caps signaling while proximal caps signals remain intact.

Our data indicate that Foxc1 is required to regulate Fgβ activity which influences the localization of Dlx2 and Dlx5. In doing so, Foxc1 is a key regulator of jaw patterning cues and the elaboration of proximodistal patterning during jaw development. Consequently, our studies provide a mechanistic basis for understanding the etiology and pathogenesis of the rare condition of syngnathia and TMJ agenesis. Future studies are still needed, however, to refine the precise spatiotemporal requirement for Foxc1 in jaw musculoskeletal development. For example, it is unclear whether the abnormal position and size of jaw musculature in concert with jaw abnormalities reflects a direct requirement for Foxc1 in pharyngeal arch mesoderm development or is an indirect consequence of abnormal patterning of neural crest cell derived mesenchyme.

Previous work has shown that cues from neural crest-derived connective tissue can direct the alignment of mesoderm derived myoblasts [79–80], and furthermore that Dlx expression in NCCs is required for formation of PA1 derived muscles [66]. This implies that Foxc1 may be predominantly required in neural crest cell derived mesenchyme during jaw musculoskeletal development. However, our data cannot separate the roles of Foxc1 in the early cranial mesenchyme, the oral ectoderm, and in the PA1 mesenchymal domain. The recent generation of a conditional Foxc1 allele [30] and increased understanding of the role of Foxc1 within neural crest-derived populations, as well as the relationship between abnormally patterned skeletal elements and their associated muscles, will enhance our collective knowledge of jaw development and inform treatment strategies for human patients with syngnathia and other related craniofacial malformations.

Materials and Methods

Animal husbandry and genotyping

All mice were housed and all experiments were conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee at the Stowers Institute for Medical Research. Foxc1 mice were obtained from Tsutomu Kume and were maintained on a 129S6/SvEv background. Fgβ<Null> and Fgβ<Neo> mice were obtained from Gail Martin and were maintained on a CD1 background. Z/Eg (stock number 003920, TgCAG-Bgeo/GFP/21Lbe/J) and Wnt1-Cre (stock number 003282, Tg[Wnt1-Cre]11Rth Tg[Wnt1-GAL4]11Rth/J) mice were obtained from the Jackson Laboratory and intercrossed with the Foxc1 line to generate both Foxc1;Z/eG and Foxc1; Wnt1-Cre mouse lines. Genotyping of all mouse strains was determined using qPCR with specific probes designed for each strain (Transnetyx, Inc., Cordova, TN, http://www.transnetyx.com). Primer sequences for each assay can be found in Table S2.

Bone and cartilage staining

Early osteoblasts were detected by endogenous alkaline phosphatase activity. At E13.5, heads were bisected, fixed overnight in 4% paraformaldehyde, rinsed in PBS, and then incubated in alkaline phosphatase buffer (100 mM NaCl; 100 mM Tris-HCl, pH 9.5; 50 mM MgCl2; 1% Tween-20). Alkaline phosphatase activity was detected using NBT/BCIP. Whole-mount skeletal preparations were made of embryos and neonates (13.5 dpc-P0) as follows. Embryos E15.0 and older were anesthetized by immersion in cold PBS until no reactive movements were seen, the skin and viscera were removed. All embryos were dehydrated in 95% ethanol, transferred to stain base solution (70% ethanol, 3% acetic acid) for 30 minutes, and then stained in 70% ethanol, 3% acetic acid, 0.02% alcian blue, 0.05% alizarin red for 24–48 hours. Following staining, embryos were rinsed in stain base solution, rinsed in water, incubated in 2.0% potassium hydroxide (KOH) (10 minutes to 6 hours based on embryonic stage), and cleared in a 0.25% KOH-glycerol series.

Histological staining, section and whole-mount in situ hybridization

Unless otherwise noted in text, a minimum of three specimens were examined for each genotype. Heads from E16.5 embryos were fixed in 4% paraformaldehyde (PFA) and embedded in O.C.T. compound. 10 µm sections were stained with hematoxylin and eosin following standard procedures. Section in situ hybridization was performed with digoxigenin-labeled probes as
Figure 7. Foxc1 is required to maintain Fgf8 signaling and genetically interacts with Fgf8. (A) Fgf8 expression is maintained in the frontonasal prominence and midbrain-hindbrain boundary regions of Foxc1−/− embryos, but it is reduced in the PA1 oral ectoderm (red asterisks). (B) Quantification of Fgf8 mRNA in Foxc1, Fgf8Null/+ and Fgf8Null/Neo embryos. (C) Foxc1 is normally localized in Fgf8Null/Neo embryos. (D) Quantification of
Immunohistochemistry and DAPI staining

Newborn (P0) pups were anesthetized by induction of hypothermia followed by decapitation. (Foxc1+/-;Fgf8Null/+ 2/12) in which calvaria had developed normally. This specimen also displayed a syngnathic jaw with TMJ abnormalities grossly identical to Fgf8Null/Foxc1(2, J, N), Fgf8Null/Neo(2, G, O); Foxc1+/-(L, inset), and Foxc1+/-Fgf8Null+(H, L, P) phenotypes. In both gross view (E) and skeletal preparations (E, M, Foxc1+/-), Fgf8Null/+ are indistinguishable from wild-type embryos. (F) Foxc1+/- embryos have shortened frontonasal regions, open eyelids, and shifted external ears, and enlarged, hydrocephalic cerebral hemispheres. (G) Fgf8Null/Neo embryos have a more rounded frontonasal region, small lower jaw, and abnormal, shifted external ears. (H) Compound Foxc1+/-;Fgf8Null+/+ embryos resemble Foxc1+/- specimens, but have more severe frontonasal shortening and no externally visible oral opening/lower jaw (black arrowhead). (J, N) Hypoplastic squamosal (sq), synagathia (syn*), and abnormal condyle formation in the absence of Foxc1. This specimen shows fusion in alveolar region of dentary and absence of the coronoid process (arrow in N). (K, O) Severe hypoplasia and malformation of the squamosal (sq*) was observed in Foxc1+/-;Fgf8Null+/+ specimens. The frontal process of the maxilla with a characteristic infraorbital foramen (iof) formed, and the maxilla fused to the dentary in the alveolar region, more distally than seen in Fgf8Null+/-/2/12. The proximal processes of the dentary are absent (asterisk in O), but distal incisors form. (J, P) In Foxc1+/-;Fgf8Null+/+ embryos, the synagathic phenotype is further exacerbated. No squamosal formed and a small frontal process of the maxilla is attached to the hypoplastic maxilla. This region is fused to the dentary just proximal to the lower incisors resulting in flattening of the normally curved dentary. The proximal dentary is severely truncated and lacks all processes (asterisk in P). (Inset in L) Foxc1+/-;Fgf8Null+/+ compound heterozygote (2/12) in which calvaria had developed normally. This specimen also displayed a syngathic jaw with TMJ abnormalities grossly identical to that of the Foxc1 null. Scale bars: (A, C) 200 μm; (G–H) 1000 μm; (M–P) 500 μm.

β-galactosidase staining

Whole embryos from E8.5-11.5 were collected and fixed for 30 to 60 minutes in 0.2% glutaraldehyde, 5 μM EGTA, 100 μM MgCl2 on ice. Embryos were rinsed and stained according to manufacturer’s protocol (Millipore #BG-6-B, #BG-7-B, #BG-5-C).

Apoptosis and proliferation assays

Embryos (E8.75 - 9.5) were fixed overnight in 4% PFA at 4°C. Embryos were rinsed in PBS and apoptosis was detected in whole embryo samples by TUNEL labeling using the In Situ Cell Death Detection Kit (Roche) for 4 hours at 37°C. Embryos were then counterstained in DAPI, mounted on Vectashield (Vector Labs, H1000), and scanned using a Zeiss LSM5 Upright Pascal Confocal microscope. Projected Z-stacks were flattened and exported to Adobe Photoshop.

Determination of cell cycle length

S-phase and cell cycle length were analyzed by incorporation of IdU-BrdU as previously described [36]. Pregnant females were described in [81]. RNA antisense probes for Sox9, Acan, Ihh, ColX, Col11, and Sca were generously provided by Dr. Clift Tabin. Whole mouse embryos (9.0-10.5 dpc) were fixed overnight in 4% PFA at 4°C, then rinsed in phosphate buffered saline (PBS) with 1% Tween-20 followed by step-wise dehydration to 100% ethanol. Anti-sense digoxigenin-labeled (dig-UTP, Roche) riboprobes were synthesized for Bmp4, Dkk2, Dkk3, Dkk6, Fgf8, Gata3, Hand2, Msx2, and Sox10. Foxc1 probe was generated by RT-PCR amplification of a 471 bp fragment spanning the 3′ end of exon 1 and a portion of the 3′ UTR. Primers: 5′-GTACCTGAAC-CAGGCAGGTG3′, and 5′-AGGAAAAATGGAGGAGG-TT3′. Whole mount in situ hybridizations were performed according to standard protocols [82,83] with minor modifications.
injected intraperitoneally with IdU at 0.1 mg/kg body weight. After 1.5 hours, mice were injected intraperitoneally with 0.1 mg/kg body weight of BrdU. Two hours after IdU injection, mice were euthanized and embryos collected. Cryosections were then prepared and the IdU and BrdU positive cells were detected by immunostaining using mouse anti-BrdU antibody (BD Bioscience, which recognizes both IdU and BrdU) and rat anti-BrdU antibody (Abcam, which recognizes BrdU only). Counts were conducted on 4 non-adjacent sections of the cranial mesenchyme (5–7 s), PA1 mesenchyme (10–13 s), and oral ectoderm for each specimen. Cell cycle length was calculated as described previously [36].

qPCR

The cranial region of E8.5 embryos was isolated by using glass needles to cut transversely just anterior to the heart. For E9.5 embryos, cuts were made posterior and dorsal to PA1 and transversely at the level of the developing eye in order to isolate PA1. For each genotype, 3 pools of total RNA (dissected tissue from 3 embryos per pool) were isolated using the Qiagen RNeasy Mini Kit. Between 250–400 ng of total RNA from each pool was then used to generate random primed single-stranded cDNA (Superscript RTIII First Strand cDNA Synthesis Kit, Invitrogen). Relative levels of mRNA were determined using a qRT-PCR
PowerSYBR (Applied Biosystems) assay with the following primers: Fgf8: 5’AATCCAGCCCAAACTACG3’ and 5’GCTCTGCTCCGTCACTG3’; Foxf1: 5’TTCGTCGTTAGACGACTCG3’ and 5’AGGTATCTTCCGGTCTTTGG3’ and internal control primers for Atp5b, Canx, Gapdh, and Ubc.

Imaging of whole-mount specimens and image processing

Whole mount embryos were photographed using a Leica MZ16 stereo microscope, Nikon Digital Sight DS-Ri1 camera, and Nikon NIS Elements BR 3.2 software, unless otherwise noted. A subset of the images (Figure 1A, B, D, F; Figure 2; Figure 5A–D, M–P; Figure 6A, C, E–F, H–J, L–R; Figure S3A–B, I–J, Figure S7A–C, F,G, I, J) was acquired as a manual series of Z-stacks. These images were further processed using Helicon Focus (Helicon Soft Ltd, http://www.heliconsoft.com) to compile and render the focused regions of the multiple focal planes into a single focus image.

Supporting Information

Figure S1  Histological analysis of TMJ abnormalities in Foxc1−/− embryos. (A) Different cellular zones in the E17.5 wild-type condyle are indicated. (B–G) Representative serial coronal cryosections of E17.5 wild type (B, D, F) and mutant (C, E, G) heads. (B, C) Hematoxylin and eosin stained sections show normal anatomy of the condyle (cdp), glenoid fossa (f), and joint disc (d) separating upper and lower synovial joint cavities in Foxc1+/+ embryos. In Foxc1−/− embryos, the fossa and disc are absent, and the condyle is bifurcated (cdp*). (D–G) To aid visualization muscles are outlined in red, and the trigeminal ganglion (trg), bone, and cartilage elements of the TMJ are outlined in black. (F) Magnification of selected area in (D) showing the Foxc1+/+ TMJ and associated temporals (tm), lateral pterygoid (lp), and medial pterygoid (mp) muscles. The angular process (app) and Meckel’s cartilage (MC) are well formed. (G) Magnification of the selected area in (E) showing the Foxc1−/− TMJ. The bifurcated condyle (cdp*) and MC can clearly be distinguished. The temporals muscle is shifted to occupy the space where the squamosal and fossa are found in the wild-type TMJ. The orientation of the muscles (tm, lp, and mp) is altered in mutants compared to controls. Magnification is 10× (A, B, C); 4× (D, E), and 6× (F, G). Abbreviations: fc, flattened chondrocytes; fl, fibrous cell layer; hc, hypertrophic chondrocytes; pcl, progenitor cell layer; pe, perichondrium; pm, palatal process; ppmx, palatal process of maxilla; pm, palatal process of mandible; pb, processus brevis; sym, symphysis.

Figure S2  Mild palatal defects in absence of Foxc1. (A, C) Ventral view of wild-type (A) and Foxc1−/− (C) P0 skeletal palatal elements. The palatine (pl) and pterygoids (ptg) are small and a small cleft is seen in the palatal process of the palatine (pppl) in the mutant. The mutant basi-sphenoid (bs) remains open at the midline. (B, D) Gross view of Foxc1+/+ (B) and Foxc1−/− (C) palates. No cleft is seen in the soft tissue of the mutant, but the rugae (r) are less sharply delineated and fewer in number than in wild-type, (E, F) Dorsal view of wild-type (E) and mutant (F) tongue. Fungiform (fp) and median circumvallate (mcp) papillae form in the absence of Foxc1. However, the anterior portion of the tongue (atg) is spade shaped, possibly due to constriction of the posterior portion of the tongue by the syngnathic jaw. Scale bars: 500 μm Abbreviations: fp, incisive papilla; ppmx, palatal process of maxilla.

Figure S3  Normal neural crest formation, migration, lineage contribution, and peripheral nervous system patterning in Foxc1−/− embryos. (A, B) Whole-mount in situ hybridization for Sox10 shows normally migrating cranial neural crest cells in Foxc1+/+ (A) and Foxc1−/− (B) embryos at E9.0 (C–F) Whole mount images of freshly dissected Foxc1; Wildtype; Z/EG embryos indicate normal contribution of neural crest derived cells (GFP, green) to cranial regions of both Foxc1+/+ (C, E) and Foxc1−/− (D, F) embryos at E10.5 (C, D) and E12.5 (E, F). (G, H) Whole mount immuno-detection of neurofilament at E11.5 reveals normal formation and patterning of cranial nerves V, VII, VIII, IX, and X in wild type (G) and mutant (H) embryos. Scale bars: (A, B) 200 μm; (C–H) 500 μm. Abbreviations: mabh, mandibular branch of trigeminal nerve; maxb, maxillary branch of trigeminal nerve; op, opthalmic branch of trigeminal nerve. (TIF)

Figure S4  Normal cell proliferation rate and apoptosis in Foxc1−/−; PA1. (A, B) Representative sagittal cryosections through E9.0 Foxc1+/− (A) and Foxc1−/− (B) PA1 immuno-stained for phosphohistone H3 (pH 3) (green) and counterstained with DAPI (blue). For each section, all DAPI stained nuclei were counted as the pH 3 positive nuclei. The white line in each section delimits the region containing the pharyngeal arch in which nuclei were counted. (C) Quantification of mitotic index in Foxc1+/+ and Foxc1−/−; PA1s. No significant difference was found between control and mutant (p = 0.116). Scale bars: 100 μm. (TIF)

Figure S5  Distal patterning cues are normally expressed in Foxc1−/−. Whole-mount in situ hybridization in Foxc1+/+ (A, C, E, G, I, K, M, O) and Foxc1−/− (B, D, F, H, J, L, N, P) E10.5 embryos. (A) Lateral (A, B, E, F, I, J, M, N) and frontal (C, D, G, H, K, L, O, P) views are shown for each probe. (A–D) Bmp4 and its target Msx2 (E–H) are normally expressed in the nasal prominence epithelium, maxillary prominence, and distal mandibular ectoderm in both control and mutant embryos. (I–L) Hand2, a downstream target of endothelin-1 signaling, is normally expressed in the distal PA1 mesenchyme in Foxc1+/+ and Foxc1−/− embryos. (M–P) Gata3, which is required for an endothelin-A receptor independent expression of Hand2, is also normally expressed in the absence of Foxc1. Scale bars: 500 μm. (TIF)

Figure S6  Middle ear, palatal abnormalities, and facial asymmetry in compound Fgf8 and Foxc1;Fgf8−/− mutants. (A–C) Dissected middle ear ossicles from P0 Foxc1+/+(A), Foxc1−/− (B), and Foxc1−/−;Fgf8−/− (C) mice. The wild type malleus (m) and incus (in) show regions of ossification (red), whereas the mutant ossicles are hypoplastic and remain as cartilage. The gonial (gn) ossicles, but is smaller in the mutants. The stapes (s) is formed in wild-type, but is absent in mutants. (D–F) Alcian blue (cartilage) and alizarin red (bone) stained skeletal preparations showing palatal elements of Fgf8+/+ (D), Fgf8−/−/Boxc1 (E), Foxc1−/−;Fgf8−/− (F) embryos at E17.5. (E) In Fgf8−/−/Boxc1 embryos, the palatal process of the maxilla (ppmx) and palatal process of the palate (pppl) are smaller than controls (D). The pterygoids (ptg) and the basi-sphenoid (bs) are misshapen. (F) In Foxc1−/−;Fgf8null/+ embryos, the palatal processes (ppmx, pppl) are not formed. The pterygoids are hypoplastic and only small lateral portions of the basi-sphenoid form. (G) Frontal view of upper and lower jaw elements in Fgf8+/+ (G), Fgf8−/−/Boxc1 (H), Foxc1−/−;Fgf8−/− (I) embryos at E17.5. In both compound mutants, the dosage of Fgf8 is genetically reduced and asymmetry is observed in the skeletal elements of the jaw. (J) Gross frontal view of Foxc1−/−;Fgf8−/−/Boxc1 fetus showing overt facial asymmetry. Scale bars: (A–C) 200 μm; (D–J) 500 μm. Abbreviations: dnt, dentary; fmx, frontal process of the maxilla; mm, manubrium; pb, processus brevis; sym, mandibular synpthesis. (TIF)
Table S1  Case reports of human bony synagnthia. Summary of published cases of synagnthia from 1936–2011 highlighting their characteristic features and isolated versus syndromic occurrence. (DOC)

Table S2  Genotyping primer sequences. Summary of the sequences used for genotyping and RT-PCR. (DOC)

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Author Contributions

Conceived and designed the experiments: KEI PP. Performed the experiments: KEI PP. Analyzed the data: KEI PP TK PAT. Contributed reagents/materials/analysis tools: KEI PP TK PAT. Wrote the paper: KEI PP TK PAT.

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