A screen of mutants generated and imaged by the International Mouse Phenotyping Consortium identifies 39 novel genes regulating the development of the secondary palate.

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Abstract

The International Mouse Phenotyping Consortium (IMPC) has generated thousands of knockout mouse lines, of which a large proportion is embryonic or early neonatal lethal. The IMPC has generated and imaged embryos from lethal lines and made the three-dimensional image data sets publicly available. In this study, we used this resource to screen homozygous null mutants for defects in the development of the secondary palate. Altogether, we analyzed optical sections from 3216 embryos isolated at embryonic day (E) 15.5 and E18.5 from 478 homozygous mutant lines. Through this analysis, we discovered 39 novel genes important for palatal development. These studies provide new insights into the molecular regulation of palatogenesis and craniofacial disease and offer a useful resource for future exploration.

Introduction

Cleft palate with or without a cleft lip (known as orofacial clefts) is the most prevalent craniofacial birth defect, affecting approximately 1 in 800 babies each year (Rahimov et al., 2012). There is a significant difference in the incidence of orofacial clefting among different ethnic groups: the greatest incidence was found among Native Americans at 3.6 per 1000 births, followed by Japanese and Chinese populations, with a prevalence of 2.1 and 1.7 per 1000 births, respectively, while the lowest incidence is among African-Americans, at approximately 0.3 per 1000 births (Wojcicki et al., 2016). Children born with this disorder have difficulties with feeding and speech when untreated. In developed countries, orofacial clefts are surgically treated early in childhood to close the clefts (Wojcicki et al., 2016). Despite the treatment, some affected individuals may still experience negative psychological, psychosocial, and financial hardships (Wehby and Cassell, 2010).

Palate formation proceeds in multiple phases (Hammond and Dixon, 2022; Som and Naidich, 2013). During the early stages of palate development, multi-potent neural crest (NC) cells migrate to populate the first branchial arch, which subsequently gives rise to the maxillary and mandibular prominences. The secondary palate, the tissue that separates the nasal and oral cavities, forms when the paired outgrowths from the maxillary processes fuse at the midline and then with the primary palate, which is derived from the frontonasal prominence. In humans, palatal development takes place between weeks 4 and 10 of gestation (Mossey et al., 2009; Som and Naidich, 2013). Due to the distinct developmental processes underlying the formation of the primary and secondary palate, several types of clefting are observed in patients and vertebrate model organisms: isolated cleft lip, isolated cleft palate, or clefting can be present both in the lip and palate (Mossey et al., 2009; Selleri and Rijli, 2023).

Mice are an established model for studying palate formation and clefting due to similarities in palate development between mice and humans (Selleri and Rijli, 2023). In mice, the growth of paired palatal shelves from the maxillary processes can be observed at embryonic day (E) 11.5. These processes undergo a dramatic increase in size through the proliferation of NC-derived mesenchyme and initially move downwards to be positioned on either side of the tongue by E13.5. The paired palatal shelves then elevate to become positioned horizontally above the tongue by E14.5. The fusion of palatal shelves at the midline begins soon afterward and results in the formation of a continuous shelf of the secondary palate. The fusion of the

secondary palatal shelves is completed by E15.5, and the fusion of the primary and secondary palatal shelves is typically completed by E17.5 (Bush and Jiang, 2012).

The causes of cleft lip and palate in the human population are not completely understood. Epidemiological studies, genetic analyses, and modeling of lip and palate clefting in mice indicated that both environmental factors and genetic mutations are at play (Hammond and Dixon, 2022; Mossey et al., 2009; Selleri and Rijli, 2023). Among common mechanisms mediating palatal development in humans and mice are those regulated by Sonic Hedgehog (Shh) signaling (Cohen, 2010; Mansilla et al., 2006). Modeling studies in mice indicated that precise levels of Shh gene expression in the NC-derived cells are essential for proper palate development, and both downregulation and upregulation of Shh in the NC-derived cells cause clefting and other defects in facial development (Jeong et al., 2004; Li et al., 2017). Additional players important for palate development include genes encoding secreted growth factors such as BMPs, Wnts, and Fgfs, and transcriptional regulators such as the Pbx family of transcription factors and *Snail1* (Ferretti et al., 2011; Losa et al., 2018; Welsh et al., 2018). However, much still needs to be learned. Therefore, it is critical to identify additional genes and their roles in palate development to broaden our understanding of mechanisms leading to the clefting of the lip and palate and to create more effective prognostic, diagnostic, and treatment options. To discover additional genes regulating the formation of the secondary palate, we examined images of mouse mutants generated and imaged by the International Mouse Phenotyping Consortium (IMPC) using micro-computed tomography (micro-CT) and looked for the presence of clefting in the secondary palate at E15.5 and E18.5 in coronal optical sections.

Materials and Methods

Image Analysis: To identify novel genes regulating the development of the secondary palate, we screened E15.5 and E18.5 homozygous-null mutant embryos generated and imaged by the IMPC: https://www.mousephenotype.org). Coronal optical sections of micro-CT images were analyzed using the tools embedded within the IMPC website in order to identify embryos with defective palates. Embryos at E18.5 were preferred for analysis due to the palate being fully fused at this stage in wild-type mouse embryos. E15.5 embryos were also analyzed. In E15.5 controls, the anterior-most and the posterior palate was often not yet fused. Therefore, cleft

palate was only marked in those E15.5 mutants in which the palatal shelves were not fused throughout the entire length of the palate.

Statistical Analysis: According to the Center for Disease Control, the incidence of cleft palate in the human population is 1 in 1700 (Mai et al., 2019). Of 100 E18.5 wild-type embryos examined in this study, only one had a cleft palate (wild-type embryo ID: X63574-04). Using the binomial probability formula, we obtained a value of p=0.0554948102 (rounded up to 0.06 or 6%), which is the incidence of cleft palate expected solely by chance. Among the identified novel mutants with cleft palate, homozygous-null mutants in the *Spop* gene exhibited the smallest penetrance of cleft palate; only 1 of 11 *Spop*-null embryos had cleft palate. The binomial probability of observing 1 cleft palate out of 11 individuals is 0.007, indicating that the presence of a cleft palate was unlikely by chance alone, even at such low penetrance. In fact, the cutoff would be 1 cleft palate in 80 embryos (p=0.047).

Identification of novel cleft palate genes: To find which of the cleft palate mutations were novel, we determined Mammalian Phenotype ontology terms associated with each gene on our list (Supplemental Table 1). To do so, we queried the list of genes in Supplemental Table 1 against mammalian phenotype terms (https://www.informatics.jax.org/batch). In addition, we compared the genes on our list with those in a cleft gene database (https://bioinfo.uth.edu). Genes not previously identified as playing a role in palatal development were considered novel (Supplemental Table 1, red ink).

Pathway enrichment analyses: We used Metascape (Zhou et al., 2019) to identify enriched biological processes and pathways (Supplemental Table 2).

Results and discussion

We examined the palatal morphology using coronal optical sections in 3216 embryos from 478 homozygous mutant lines that were generated by the IMPC and imaged using micro-CT by the IMPC (Table 3). Out of 478 lines, 61 lines displayed cleft palate (Table 1). To determine which lines represented novel cleft palate genes, we used gene ontology terms and the Mouse Genome Informatics (MGI) along with the cleft palate database

(https://bioinfo.uth.edu/CleftGeneDB/). These analyses indicated that of 61 genes, mutations in 39 were not previously associated with cleft palate; therefore, these 39 genes were marked as novel cleft palate genes (Supplemental Table 1, marked in red ink, and Figures 1-2). 38 of the 39 genes were not previously associated with cleft palate phenotypes according to both the MGI and the CleftGeneDB databases. The *Col27a1* gene was associated with the cleft palate MP term in the MGI database but not in the CleftGeneDB database. Mutant *Col27a1* alleles were generated independently of the IMPC; however, palate development was not examined in these mice (Plumb et al., 2011). And even though mutations in *Col27a1* were found in some patients with Steel syndrome whose symptoms included cleft palate (Girisha et al., 2022), no studies directly linked mutations in *Col27a1* to cleft palate. Therefore, we considered *Col27a1* a novel cleft palate gene. A gene that was initially considered a novel cleft palate gene but ultimately not considered novel in our analysis was Bmi1. Bmi1 was not associated with cleft palate MP terms in the MGI database, but it was present in the CleftGeneDB. Upon further literature review, we found a statement, albeit without supporting data, that 2 of 6 Bmi1-null embryos had cleft palate (Akasaka et al., 2001), and therefore, we excluded Bmi1 from our list of novel cleft palate genes. Using the list of novel cleft palate genes as an input, we performed pathway enrichment analysis using Metascape (Zhou et al., 2019), which utilizes ontology resources including *Kegg Pathway, Reactome Gene Sets, GO Biological Processes, Canonical Pathways, CORUM,* and *Wikipathways* in order to discover enriched pathways regulated by cleft palate genes identified in our screen.

Homozygous mutants in *Arhgef18, Memo1,* and *Tctn3* exhibited a nearly 100% incidence of cleft palate at E18.5 (Fig. 1, Supplemental Table 1). Arhgef18 is RhoA guanine nucleotide exchange factor (GEF), also known as p114RhoGEF, that regulates RhoA activation and actomyosin contractility in multiple contexts (Beal et al., 2021; Nakajima and Tanoue, 2011; Tsuji et al., 2010; Xu et al., 2013). Arhgef18, thus, may be important for the fusion of the palatal shelves. As the two palatal shelves contact each other, the ectodermal epithelial cells, a.k.a. medial edge epithelial layers (MEE) that border the palatal mesenchyme, intercalate to form the midline epithelial seam (MES) (Lan et al., 2015). Activation of Rho and actomyosin contractility are essential for this intercalation and cell extrusion to form a continuous palatal tissue (Kim et al., 2015; Rosenblatt et al., 2001), and Arhgef18 could be an essential activator of RhoA in this process. Future conditional mutagenesis studies are necessary to test this hypothesis and to

determine the precise tissues in which Arhgef18 functions during palatal development. This is especially important since recent studies found mutations in Arhgef18 in patients with sporadic cleft palate (El-Sibai et al., 2021).

Interestingly, Memo1 also regulates RhoA signaling, cytoskeletal dynamics, and cell migration (Marone et al., 2004; Zaoui et al., 2008), and thus, could regulate palatal fusion by mediating cell intercalation and extrusion behaviors leading to MES formation and dissolution. The identification of *Arhgef18* and *Memo1* as important regulators of palatal development provides novel molecular players to explore in the future and gain additional insights into the regulation of MES formation and dissolution, which are relatively understudied but essential processes for palatal morphogenesis.

A homozygous null mutation in *Tctn3* (tectonic family member 3) also resulted in penetrant cleft palate (Fig. 1 and Supplemental Table 1). In humans, mutations in *Tctn*3 are associated with the Mohr-Majewski Syndrome, and cleft palate was found in one case (Thomas et al., 2012). However, palatal development was not examined in Tctn3-null mutants generated prior to the IMPC screen (Wang et al., 2017); therefore, it was not clear whether Tctn3 was required for the development of the palate. Tctn3 is a membrane protein that localizes to the primary cilium and is essential for the activation of the Shh signaling (Reiter and Skarnes, 2006; Wang et al., 2017). Ablation of Tctn3 resulted in defective ciliogenesis (Wang et al., 2017). Interestingly, ciliopathies (defects resulting in dysfunctional primary cilia) are common causes of craniofacial defects and underly a number of craniofacial syndromes, such as Joubert syndrome [OMIM 213300] and Meckel-Gruber syndrome [OMIM 249000], Ellis-van Creveld syndrome [OMIM 225500], Oral–facial–digital syndromes types IX [OMIM 258865] and XIV (Adly et al., 2014; Thauvin-Robinet et al., 2014), and Weyers acrofacial dysostosis [OMIM 193530] (Moore, 2022; Reynolds et al., 2020). Consistent with this, our gene set enrichment analysis (GSEA) showed that “Genes related to primary cilium” category was a statistically enriched category in our dataset (q=0.04624) and included the following genes: *Tctn3, Cep97, Inpp5e, Fuz*, and *Clasp1* (Supplemental Table 2), among which *Tctn3, Cep97*, and *Clasp1* are novel cleft palate genes identified in our screen (Figs. 1-2). Cep97 localizes at the centrioles and regulates cilia assembly (Spektor et al., 2007). Clasp1 is a plus-end microtubule-binding protein regulating the microtubule dynamics (Akhmanova et al., 2001; Maiato et al., 2005), an essential process for the

biogenesis of cilia (Malicki and Johnson, 2017). Thus, our screen identified two additional cilia genes (*Cep97* and *Clasp1*) with roles in microtubule dynamics and palate development.

Perhaps not surprisingly, "Embryonic organ development" and "Skeletal system development" were the top two enriched categories (q=2.477E-4) of our GSEA. Among the 13 genes that fell into these 2 categories, 2 genes, *Celf1 and Col27a1*, were not previously known to regulate palate development. Our phenotyping analyses indicated that 1 of 6 homozygous *Celf1*-null mutants imaged by the IMPC exhibited a cleft palate (Fig. 1). The binomial probability of cleft palate in this strain is $p<0.004$, which indicates that cleft palate did not occur simply by chance. *Celf1* (CUGBP Elav-like family member 1) is an RNA-binding protein regulating diverse aspects of mRNA biology, including mRNA alternative splicing, stability, and miRNA processing (Vlasova-St Louis et al., 2013). *Celf1* knockout animals were previously generated on a mixed C57Bl6-129Sv background, and although ~50% of the knockout animals did not survive past day 10 after birth, palatal development was not examined (Kress et al., 2007). Celf1 protein binds GU-rich sequences in introns as well as in 5'- and 3'-UTRs of mRNAs. The role of Celf1 in palate development could be related to its ability to promote epithelial-to-mesenchymal transition (EMT) due to its ability to bind to and increase translation of mRNAs encoding EMT regulators (Chaudhury et al., 2016). Among these regulators is *Snail1*, which is important for palate development (Losa et al., 2018; Murray et al., 2007). Thus, *Celf1* could be another novel player in regulating EMT during the dissolution of the MES.

Col27a1 (a.k.a. type XXVII collagen) is an evolutionarily conserved member of extracellular matrix proteins that belongs to a family of fibrillar collagens (Boot-Handford et al., 2003). The high conservation in vertebrates, the unusual domain composition relative to other fibrillar collagens, and the developmentally restricted expression of Col27a1 suggested that this protein plays important developmental roles (Plumb et al., 2007). Mutations in the *Col27a1* genes have been associated with Steel syndrome, a rare autosomal recessive disease of the skeleton (Girisha et al., 2022; Gonzaga-Jauregui et al., 2020; Kotabagi et al., 2017). Among these patients, a pair of twin brothers was also diagnosed with cleft palate (Girisha et al., 2022). However, no other patients with *Col27a1* mutations or mouse mutants in *Col27a1* had palatal clefting (Plumb et al., 2011). Although only one of eight *Col27a1*-null mutants exhibited cleft palate (**Fig. 2**), this incidence is significant, $p<0.005$, excluding that cleft palate occurred purely by chance in this

strain. However, the low incidence of cleft palate in *Col27a1*-null mutants may complicate further studies to understand the role of this protein in palatal shelf elevation or fusion.

"Melanoma" pathway (*CDK4, FGF9, FGF10, FGF19*) was also a statistically significant enriched category of genes (q=0.02398) in our dataset. Melanocytes are derived from the neural crest and melanomas are highly proliferative metastatic tumors (Mort et al., 2015). This is consistent with the literature indicating that control of cell proliferation is essential during palate development (Hammond and Dixon, 2022). Among genes in this category, *Cdk4* is a novel regulator of palatal development, with two of eight *Cdk4*-null mutants displaying cleft palate in our screen (Fig. 2).

Since the neural crest gives rise to the palatal mesenchyme and craniofacial bones and cartilage, it was consistent that pathways related to neural crest development were significantly enriched in our dataset. These pathways were: "Ear development (q = 0.03899)", "sensory organ development (q = 0.005662)", "digestive system development (q=0.021777)", and "roof of mouth development (q = 0.03882)". The enrichment in the "Ear Development" pathway was especially intriguing as it contained the greatest number of novel cleft palate genes identified in our screen (Supplemental Table 2). 22 genes from our screen comprised this category, and 8 of them had not been associated with palate development before our work. These genes are *MAF, CDK4, RUNX1T1, PODXL, COL27A1, CELF1, TMEM115, UGT1A6*. *Maf* encodes a group of transcription factors that can act as transcriptional activators or repressors. Of the four MAFs (*MAFA, MAFB, c-MAF, NRL*), *MAFB* is essential for the formation of inner ears, among its other functions, such as the development of pancreatic endocrine cells (Takahashi, 2021). *Runx1t1* encodes a transcriptional corepressor implicated in the positive regulation of angiogenesis (Liao et al., 2017). In addition, Runx1t1 is commonly mutated in acute myeloid leukemia through its fusion with Runx1 (Grinev et al., 2021). Curiously, there was a case study reporting a mutation in Runx1t1 in a patient with a protruding ear (Huynh et al., 2012). The enrichment in the "Ear development" category in our dataset suggests that screening for ear anomalies and hearing loss may be reasonable in patients with cleft palate.

The statistically significant enrichment in the "Kidney development" category (q=0.03548) was surprising mainly because the set of genes in this category did not include cilia genes (*AP2B1, CDKN1C, EDNRA, FGF10, PODXL, PYGO2*), since defects in cilia development contribute both to craniofacial and kidney disorders (McConnachie et al., 2021; Moore, 2022).

Published studies indicated that *Pygo2*, a component of canonical Wnt signaling required for the development of both the palate and the kidney (Schwab et al., 2007), although the mechanism by which palate development is regulated by *Pygo2* mutants is still unknown. The enrichment in this category suggests the existence of common regulatory mechanisms mediating the development of the kidney and the palate in addition to those regulated by cilia and suggests that patients presenting with cleft palate may need to be followed up for anomalies in other organ systems, such as the sensory nervous system, the digestive system, and the ear, as well as in a seemingly unexpected organ, such as the kidney. Analyses of additional IMPC mutants in the future will provide important insights into the mechanisms of palatal development and identify genes and additional pathways important in this process and disease. Our screen of IMPC mutants identified 39 novel cleft palate genes, providing fertile grounds for more mechanistic studies in the future. Another value of our analyses lies in identifying additional and unanticipated organs/pathways that could be affected alongside the cleft palate in patients, allowing a more thorough clinical follow-up and treatment of all associated symptoms.

Figure Legends:

Figure 1. E18.5 mutants with cleft palate. Snapshots of coronal optical sections from micro-CT images were taken at eye level. Eyes were marked by arrowheads. Long arrows point at palatal shelves. Short arrows point at the tongue. Supplemental Table 4 contains additional information about the displayed embryos. All scale bars are 1 mm.

Figure 2. E15.5 mutants with cleft palate. Snapshots of coronal optical sections from micro-CT images were taken at eye level. Eyes were marked by arrowheads. Long arrows point at palatal shelves. Short arrows point at the tongue. Supplemental Table 5 contains additional information about the displayed embryos. All scale bars are 1 mm.

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Figure 1

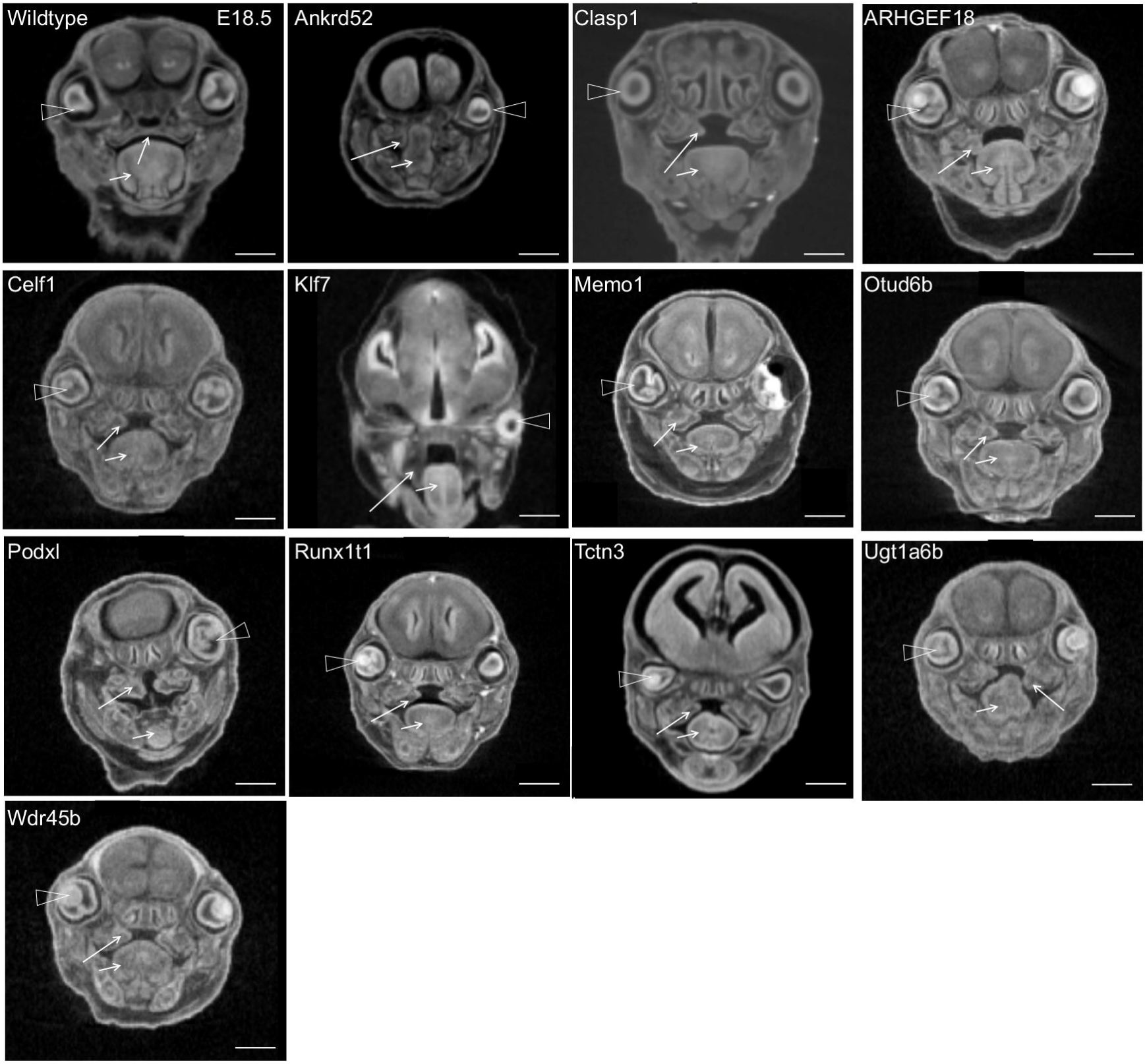
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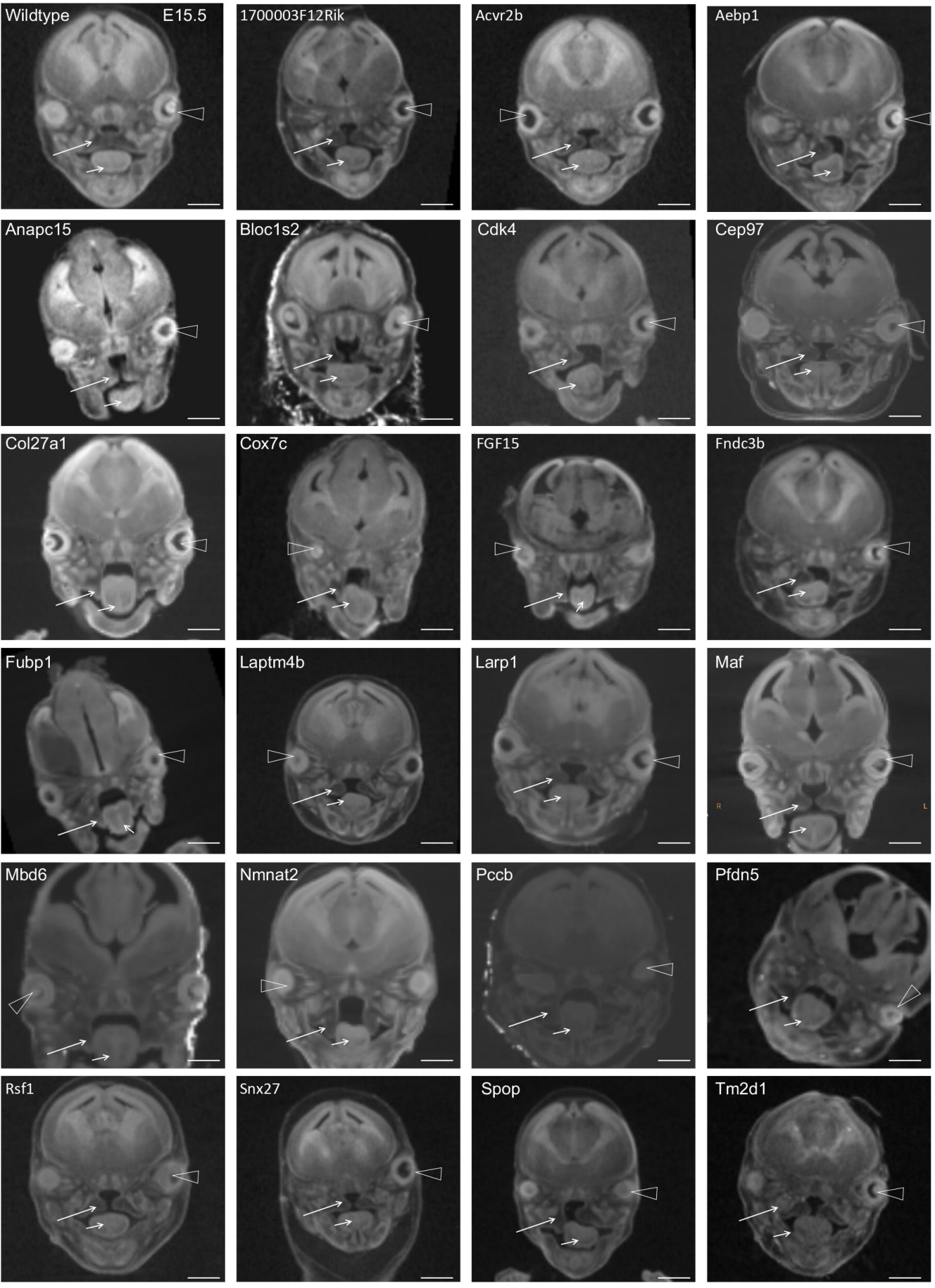
Figure 2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