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“Delineating the roles of *Grhl2* in craniofacial development through tissue-specific conditional deletion and epistasis approaches in mouse”

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Keywords: Grainyhead-like, transcription factor, craniofacial development, micrognathia, endoderm, lung development

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/dvdy.322](https://doi.org/10.1002/dvdy.322)

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Abstract

Background: The highly-conserved *Grainyhead-like* (*Grhl*) family of transcription factors play critical roles in the development of the neural tube and craniofacial skeleton. In particular, deletion of family member *Grainyhead-like 2* (*Grhl2*) leads to mid-gestational embryonic lethality, maxillary clefting, abdominoschisis, and both cranial and caudal neural tube closure defects. These highly pleiotropic and systemic defects suggest that *Grhl2* plays numerous critical developmental roles to ensure correct morphogenesis and patterning.

Results: Here, using four separate *Cre-lox* conditional deletion models, as well as one genetic epistasis approach (*Grhl2*^{+/-};*Edn1*^{+/-} double heterozygous mice) we have investigated tissue-specific roles of *Grhl2* in embryonic development, with a particular focus on the craniofacial skeleton. We find that loss of *Grhl2* in the pharyngeal epithelium (using the *Shh*^{Cre} driver) leads to low-penetrance micrognathia, whereas deletion of *Grhl2* within the ectoderm of the pharynx (*Nestin*^{Cre}) leads to small, albeit significant, differences in the proximal-distal elongation of both the maxilla and mandible. Loss of *Grhl2* in endoderm (*Sox17-2a*^{iCre}) resulted in noticeable lung defects and a single instance of secondary palatal clefting, although formation of other endoderm-derived organs such as the stomach, bladder and intestines was not affected. Lastly, deletion of *Grhl2* in cells of the neural crest (*Wnt1*^{Cre}) did not lead to any discernible defects in craniofacial development, and similarly, our epistasis approach did not detect any phenotypic consequences of loss of a single allele of both *Grhl2* and *Edn1*.

Conclusion: Taken together, our study identifies a pharyngeal-epithelium intrinsic, non cell-autonomous role for *Grhl2* in the patterning and formation of the craniofacial skeleton, as well as an endoderm-specific role for *Grhl2* in the formation and establishment of the mammalian lung.

Introduction

Craniofacial development in vertebrates occurs through the establishment of facial and pharyngeal domains, followed by the influx and integration of neural crest cells (NCCs) to populate these domains and ultimately differentiate into the bones and nerves of the head and face. When this process fails, it results in congenital anomalies of the craniofacial structures, recognized clinically under the broad term of craniofacial defects (CFD). CFD are widely prevalent, as they are observed in approximately three-quarters of human birth defects¹, and affect 0.1-0.3% of all births. As a significant proportion of CFD are due (at least in part) to genetic mutations, and therefore, understanding the nature of normal genetic regulation of craniofacial development is critical for the establishment of preventative strategies to limit disease burden severity.

CFD that affect the maxilla and mandible (upper and lower jaws respectively) can arise either through a defect in NCCs or a disruption in the formation or maintenance of the pharyngeal arch microenvironment. NCC defects (termed neurocristopathies) comprise cell-intrinsic disorders that largely affect craniofacial morphology, such as Treacher-Collins, CHARGE, Di George and Goldenhaar Syndromes². Disorders affecting the upper jaw and face typically involve failure of prominence fusion, resulting in anomalies such as facial (Tessier) clefts or a non-fused (cleft) hard palate, with or without a cleft lip. Such disorders tend to be a defect of prominence growth or fusion defects due to aberrantly-formed or maintained epithelia^{3,4}. Lower-jaw defects, such as Pierre-Robin Sequence, retrognathia or micrognathia are often due to either mechanical hindrance of embryonic growth within the womb, or a failure of neural crest survival and/or production following migration into the mandibular prominence^{5,6}. Therefore, CFD may arise from a disruption of normal cellular patterning, migration, maintenance and survival, affecting cells derived from all three germinal layers (endoderm, ectoderm and mesoderm), as well as the neural crest.

A novel set of candidate genes recently implicated in craniofacial development are the highly-conserved *Grainyhead-like* (*Grhl*) family of transcription factors. Comprising three orthologues in most vertebrate species (*Grhl1-3*), these genes regulate neural tube closure, midbrain/hindbrain patterning and morphogenesis, planar cell polarity signalling, wound repair and epidermal barrier formation⁷⁻¹⁰. Recent work has also shown the requirement for *Grhl3* in formation, growth and/or fusion of the craniofacial skeleton in mouse¹¹ and human^{4,12}, indicating that this gene is a critical vertebrate regulator of craniofacial development¹³.

Likewise, *Grhl2* is also critical for craniofacial development, as *Grhl2* loss leads to exencephaly, maxillary and facial clefting and impaired development of the pharyngeal arches¹⁴⁻¹⁹, and loss of the zebrafish orthologue *grhl3* (that is expressed within the pharyngeal arches in a pattern that recapitulates craniofacial expression of mammalian *Grhl2*) leads to hypoplasia of the lower jaw⁶. However, mouse models that lack *Grhl2* (*Grhl2*^{-/-} embryos) are embryonic lethal by E11.5^{14,15}, and although “escapers” are occasionally seen at E18.5, these present with severe pleiotropic developmental defects¹⁷, making it difficult to ascertain the tissue-specific roles of *Grhl2* in regulating development.

Here we report conditional (*Cre-lox*) deletion and genetic epistasis approach strategies to precisely delineate tissue-specific requirements for *Grhl2* in embryonic development, with a particular focus on the formation of the craniofacial region. Additionally, in numerous instances, our *Cre*-deletion approaches allowed us to identify and characterise tissue-specific defects that occurred outside the craniofacial region (particularly the lung), thereby characterising further novel roles for *Grhl2* in embryogenesis.

Results:

Tissue-specific loss of *Grhl2* in the pharyngeal epithelium (*Shh^{Cre}-Grhl2^{CKO}*) results in a low-penetrance hypomorphic mandible phenotype

We had previously reported that deletion of *Grhl2* in epithelium of the developing respiratory system using the *Shh^{Cre}* driver led to substantial defects in lung and tracheal development²⁰. As *Shh* is also expressed within the epithelium of the first pharyngeal arch, from which the maxilla and mandible develop²¹, we used the same model to determine whether conditional deletion of *Grhl2* within the pharynx also led to craniofacial defects. Observed genotypic frequencies of *Shh^{Cre}-Grhl2^{CKO}* embryos/pups did not differ from expected Mendelian ratios ($p=0.84$ for E14.5-E16.5 and $p=0.39$ for E18.5-P1 by χ -square test). Although the majority of *Shh^{Cre}-Grhl2^{CKO}* pups were phenotypically normal, we noticed that several of these presented with micrognathia at P0 (Fig. 1A-G; $n=3/33$; 9.1%). Brightfield microscopy revealed a significantly underdeveloped lower jaw (Fig. 1A,D), and skeletal stains to highlight jaw architecture in these *Shh^{Cre}-Grhl2^{CKO}* pups revealed severe hypomorphism of the mandible compared to littermate controls (Fig. 1B-G), although a mandibular bone was present and patterned correctly. Next, we quantitated the lower jaw lengths in the remaining 30/33 *Shh^{Cre}-Grhl2^{CKO}* embryos that did not present with micrognathia to determine whether these mice displayed an intermediate jaw length between that of the *Shh^{Cre}-Grhl2^{CKO}* pups with micrognathia and controls. We found that these embryos did not display any significant differences in lower jaw length to that of controls (Fig. 1H), indicating that micrognathia was an ‘all or nothing’ phenotype following conditional deletion of *Grhl2* within the pharyngeal epithelium.

The hypomorphic lower jaw observed in *Shh^{Cre}-Grhl2^{CKO}* mice was similar to the phenotype displayed by *grhl3* loss in zebrafish⁶, a craniofacial orthologue of murine *Grhl2*, whereby

down-regulation of *grhl3* signalling led to hypoplasia, albeit not mis-patterning, of the lower jaw. Lower jaw hypomorphism in the zebrafish model was due partly to impaired proliferation of cranial neural crest cells (CNCCs) within the pharyngeal arches. Therefore, we examined cell proliferation within the maxilla and mandible of *Shh^{Cre}-Grhl2^{CKO}* embryos at E14.5 (Fig. 2A-F), reasoning that any defects in proliferation would be more apparent at this timepoint rather than at E18.5 or P0, by which time the lower jaw had largely formed. Unlike the zebrafish model, however, we found no significant differences in proliferation within the epithelia of either the maxilla or mandible (Fig. 2G; $p=0.48$ for maxillary epithelium; $p=0.23$ for mandible; $p=0.28$ for mandibular epithelium), indicating that *Shh^{Cre}*-mediated deletion of *Grhl2* does not typically or consistently impair proliferation within the craniofacial epithelia in non-micrognathic embryos.

Oral epithelium-specific loss of *Grhl2* during craniofacial development

The soluble growth factor *branchless/FGF8* is an important pro-survival signal for CNCCs during craniofacial development^{22,23} and had previously been implicated as an upstream regulator of *grh/Grhl* in both *Drosophila*²⁴ and zebrafish⁸. As conditional deletion of FGF8 in the pharyngeal ectoderm using *Nes^{Cre}* leads to a hypomorphic lower jaw phenotype similar to that observed in a proportion of our *Shh^{Cre}-Grhl2^{CKO}* embryos²⁵, we hypothesised that tissue-specific deletion of *Grhl2* in the pharyngeal ectoderm may also result in lower jaw hypomorphism. Moreover, we had previously also characterised a neural-specific role for the *Grhl2* family-member *Grhl3* in the control of locomotor and anxiety behaviour²⁶, and although *Grhl2* expression is not detectable in brain tissue by autoradiographic in situ hybridisation^{26,27}, nonetheless, the *Nes^{Cre}*-mediated deletion strategy allowed us to investigate whether *Grhl2* may also play a role in the formation of the brain.

In order to investigate the consequences of *Grhl2*-loss on both neural and pharyngeal ectoderm development, we generated 11(4 female, 7 male) *Nes^{Cre}-Grhl2^{CKO}* mice alongside 8 (4 female, 4 male) *Nes^{Cre};Grhl2^{fllox/+}* controls, and observed these mice from birth until 12-18 months of age (between 311 and 597 days), in the manner we have described previously for *Nes^{Cre}-Grhl3^{CKO}* mice²⁶. Genotyping to determine *Cre*-mediated excision of the floxed region of the *Grhl2* allele in neural stem/progenitor cells indicated a very high-efficiency of deletion (Fig. 3A-B). We found no substantial differences in adult brain formation movement or behaviour in *Nes^{Cre}-Grhl2^{CKO}* mice (data not shown); moreover, neither males nor females displayed any overall differences in size or weight, nor any specific differences in the size or weight of the brain. The average weight of *Nes^{Cre}-Grhl2^{CKO}* mice was 43.8±9.5g SD, compared to 44.5±12.1g SD for *Nes^{Cre};Grhl2^{fllox/+}* controls; $p=0.89$ by Student's t-test. These data indicate that deletion of *Grhl2* in *Nestin*-positive cells did not impact on gross development, motor function or body weight.

In order to determine specific effects of *Grhl2*-loss on jaw development during embryogenesis, skeletal stains were performed on control and *Nes^{Cre}-Grhl2^{CKO}* embryos at E18.5, and we measured the upper jaw, mandibular and skull lengths, as well as the mandibular and skull widths (Fig. 4A-D') in order to determine whether deletion of *Grhl2* in pharyngeal ectoderm-derived epithelia led to defects in craniofacial patterning, morphogenesis and development. Qualitatively, we observed no gross morphological differences in *Nes^{Cre}-Grhl2^{CKO}* embryos relative to controls, however quantitation of jaw and skull lengths revealed a small, albeit statistically-significant reduction in the lengths of the maxilla, mandible and skull in *Nes^{Cre}-Grhl2^{CKO}* embryos (Fig. 4E). No significant differences were observed in either mandibular or skull width. These data suggest that unlike the phenotype observed in *Shh^{Cre}-Grhl2^{CKO}* embryos, *Grhl2* has a role in the pharyngeal ectoderm that impacts on anterior-posterior extension of the mandible during craniofacial development.

Previous work had showed that within the craniofacial primordia, *Grhl2* expression is exclusively epithelial during embryogenesis, being expressed in both ectoderm and endoderm-derived epithelial compartments²⁷. We had also reported previously¹⁹, and confirm here through craniofacial measurements (Fig. 5A) that deletion of *Grhl2* in the neural crest, using *Wnt*^{Cre28-30}, does not lead to any qualitative or quantitative craniofacial defects, indicating that anomalies that do arise within the craniofacial region in embryos with abrogated *Grhl2* expression are due to defects within the pharyngeal microenvironment, rather than neural crest cell-intrinsic defects. Lower jaw hypomorphism caused by loss of *grhl3* in zebrafish can be phenotypically rescued by injection of mRNA encoding the direct *grhl3*-target gene *edn1*, which restores the normal proliferative capacity of CNCCs within the pharyngeal micro-environment⁶. Moreover, sub-phenotypic co-knockdown of *grhl3* and *edn1* together, achieved by co-injecting morpholinos targeting both genes at doses that did not lead to a phenotype when a morpholino targeting either gene was injected singly, led to lower jaw hypoplasia in zebrafish embryos⁶. We therefore sought to determine whether *Edn1* may be epistatic to *Grhl2* in the context of craniofacial patterning and development in mice through inter-crossing *Grhl2*^{+/-} mice with mice lacking an allele of *Edn1* (*Edn1*^{+/-} mice). Of note, complete *Edn1* loss in mice leads to total loss of lower jaw formation³¹, as it does in zebrafish, although like *Grhl2*^{+/-} mice, adult *Edn1*^{+/-} mice are phenotypically normal. Within the present study, however, our experiments examining *Grhl2*^{+/-};*Edn1*^{+/-} double heterozygous mice indicated no evidence of abnormal lower (or upper) jaw development or mandibular extension in these embryos (Fig. 5B), suggesting that heterozygosity for both these genes within a single mouse embryo does not impact on craniofacial development.

Endoderm-specific deletion of *Grhl2* during craniofacial development

As *Shh* drives recombination in epithelia derived from both endodermal and ectodermal germ layers, we sought to dissociate just the endoderm-specific role of *Grhl2* through the use of the *Sox17-2a^{iCre}* model, which strongly and exclusively drives deletion within cells of the endodermal lineage from as early as embryonic day 6 (E6.0)³². Moreover, our work in zebrafish had shown that within the craniofacial microenvironment, *grhl3* was expressed strongly within the medial endoderm, rather than ectoderm, at the onset of craniofacial development⁶. Breeding analysis indicated no differences in expected Mendelian ratios of *Sox17-2a^{iCre}-Grhl2^{cKO}* embryos relative to other genotypes at E18.5 (Fig. 6A-B), although intriguingly, our initial experiments in *Sox17-2a^{iCre}-Grhl2^{cKO}* embryos identified a single instance of an E18.5 embryo (n=1/18) with a fully penetrant cleft lip and palate (Fig. 7A-B). Although this is a low-penetrance phenotype, palatal clefting had never previously been described in *Sox17-2a^{iCre}* mice, although this phenotype is highly consistent with facial and palatal clefting described in mouse embryos lacking *Grhl2*^{15,17,19,33}. No other craniofacial defects were observed in any other *Sox17-2a^{iCre}-Grhl2^{cKO}* embryos at E18.5 or P0, and the one pup presenting with cleft palate similarly did not present with any other craniofacial anomalies.

Within the respiratory system, we noticed non-cell autonomous defects within the mesoderm-derived trachea, namely a significant decrease in tracheal width coupled with shorter, disjointed and aberrantly shaped tracheal cartilage rings (Fig. 7C-D; n=6), consistent with tracheal morphology we had reported previously following *Shh^{Cre}*-mediated deletion of *Grhl2*²⁰. Measurements of the tracheal width (diameter) confirmed statistically significant stenosis in the *Sox17-2a^{iCre}-Grhl2^{cKO}* versus control littermates (Fig. 7E; Control 555.9µm ± 15.5µm SEM; *Sox17-2a^{iCre}-Grhl2^{cKO}* 421.3µm ± 6.4µm SEM), although tracheal length was unchanged (Fig. 7F; Control 2005µm ± 29.1µm SEM; *Sox17-2a^{iCre}-Grhl2^{cKO}*: 1870.16 ±

138.1µm SEM). Within the lungs, we firstly noted an apparent loss of blood flow in *Sox17-2a^{iCre}-Grhl2^{CKO}* lungs (Fig. 8A-B), a phenotype consistent with that described previously in the hearts of *Grhl2^{-/-}* embryos¹⁷, and indicative of either vascularisation or circulatory defects and/or anemia. Next, through histological observation noted that blood vessel formation appeared to be unaffected and no apparent gross-defects were visible in the morphology of *Sox17-2a^{iCre}-Grhl2^{CKO}* lungs at E18.5 (Fig. 8C-D), although there were substantial defects in the ability of lungs to inflate at P0 (Fig. ; Fig. 8E-F). Clustering and bunching of alveolar wall cells was observed, and these defects were incompatible with life, leading to rapid post-natal lethality (within a few hours) at P0 in *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos (n=20) relative to *Sox17-2a^{iCre}-Grhl2^{HET}* (n=15), *Grhl2^{+/-}* (n=23) or *Grhl2^{+/+}* (n=27) control pups (n=65 total). These data were again consistent with the phenotypes we reported previously in *Shh^{Cre}-Grhl2^{CKO}* embryos²⁰.

We had also previously described that loss of *Grhl2* in *Shh^{Cre}-Grhl2^{CKO}* mice led to significant increases in the both the number of proliferating cells and *Sox9⁺* progenitor cells within the lung²⁰. To that end, we investigated whether similar increases in cell proliferation existed within the lungs of *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos, and confirmed that these mice indeed showed increases in both cellular proliferation (Fig. 9A-C) and the number of progenitor cells (Fig. 9D-F) within the lungs at E18.5. Taken together, these data indicate that a substantial degree of *Grhl2*-mediated control of tracheal and lung development takes place within the endoderm-derived epithelial tissue compartment.

Lastly, we determined whether *Grhl2* was important in regulating the development and formation of other organs that were comprised of endoderm-derived tissue, such as intestine, thymus, heart, bladder and liver. Genotyping to determine *Cre*-mediated excision of the floxed region of the *Grhl2* allele indicated substantial recombination in all tissues tested (Fig. 9G), although the ultimate efficiency of endodermal-deletion is diluted by the presence of

many non-endodermal cell types within each organ examined. As *Grhl2* had previously been implicated in the formation and disease progression of the gastrointestinal (GI) tract, we first examined the morphology, cellular proliferation and Sox9⁺ progenitor cell differentiation within the small intestine. Unlike the lung, when we examined the ultrastructural organisation and cell production within the villi, epithelia and mucosa of the small intestine, we found no significant differences in either morphology or cell number (Fig. 9H-M). Likewise, we found no qualitative differences in morphology of the thymus, heart, bladder, liver, stomach and large intestine (Fig. 10A-L). Although one *Sox17-2a^{iCre}-Grhl2^{CKO}* embryo did present with a large focus of acute hepatocellular necrosis characterised by nuclear pyknosis and cytoplasmic shrinkage, hypereosinophilia and an amorphous appearance (likely as a result of mortality), no other significant phenotypic features were detected in any of the endoderm-derived organs in *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos. These data indicate that *Grhl2* is not a major regulator of endodermal differentiation and tissue development.

Discussion

The objective of this study was to explore the tissue-specific roles played by *Grhl2* during embryogenesis, particularly the craniofacial region. We utilised multiple conditional deletion and epistasis models to explore the role of *Grhl2* in the neural crest (*Wnt1^{Cre}*), pharyngeal epithelium (*Shh^{Cre}*), pharyngeal ectoderm (*Nes^{Cre}*), endoderm-derived organs (*Sox17-2a^{iCre}*) and *Grhl2^{+/-};Edn1^{+/-}* double-heterozygotes to examine potential epistasis in the context of lower-jaw development. Overall, our data support a conserved role for *Grhl2* in the formation and extension of the lower jaw, re-affirm a role for *Grhl2* in the pharyngeal epithelium (particularly endoderm) in the context of palatal clefting and lung morphogenesis, although

do not support a global role for *Grhl2* in the developmental regulation of endoderm-derived organ morphogenesis.

A cell-intrinsic role for *Grhl2* in the cranial neural crest had been suggested previously by in situ hybridisation analysis in zebrafish⁸, and also by expression data showing that *Grhl2* is localised in regions lateral to the neural tube prior to fusion (consistent with NCC localisation)¹⁷. However, data presented within this study do not provide any evidence for a cell-intrinsic role for *Grhl2* within the cranial neural crest, as *Wnt^{Cre}-Grhl2^{CKO}* mice displayed no CFD phenotypes or even minor differences in elongation or development of the major bones of the craniofacial skeleton, consistent with our previous experiments that showed *Wnt^{Cre}-Grhl2^{CKO}* embryos did not present with palatal clefts¹⁹. Although we cannot rule out *Grhl2* expression in NCCs that contribute to other organs, such as the heart, smooth muscle, peripheral nervous system, chondrocytes or osteoblasts, nonetheless, we can rule out *Grhl2* as a major cell-autonomous driver of cranial neural crest cell-mediated control of craniofacial development.

The pharyngeal epithelium is a critical component of craniofacial development, providing numerous important patterning and pro-survival signals to cranial neural crest cells^{5,21,22,34,35}. *Grhl2* is expressed in both endoderm and ectoderm-derived epithelium of the pharynx²⁷, particularly surrounding the maxillary and mandibular processes of the first pharyngeal arch, as is the zebrafish *Grhl2* orthologue responsible for regulating lower jaw development, *grhl3*. Moreover, morpholino-mediated knockdown of *grhl3* results in lower jaw hypomorphism⁶, although a role for *Grhl2* in mandibular development in mammals had not previously been described. Therefore we hypothesised that tissue-specific loss of *Grhl2* in the pharyngeal epithelium would lead to impaired craniofacial development. Previous findings have implicated *Shh* as an important regulator of the pharyngeal epithelium^{21,35,36}, whereas *Nes^{Cre}*

drives deletion in the ectodermal compartment²⁵, and *Sox17-2a*^{iCre} within the endoderm³². Therefore, we utilised all three of these models to refine the specific roles of *Grhl2* in the pharyngeal epithelium.

Pertaining to the mandible, we found that ~9% of *Shh*^{Cre}-*Grhl2*^{cKO} embryos presented with severe micrognathia, indicative of a near complete failure of mandibular elongation, although a hypoplastic mandible was formed. These experiments suggest that *Grhl2* is required for morphogenesis, albeit not patterning, of the mandible. However, we did not note intermediate mandibular length phenotypes in the remaining ~91% of *Shh*^{Cre}-*Grhl2*^{cKO} embryos, suggesting either that *Grhl2*-deletion may be overcome through other means in *Grhl2*-deficient embryos, or more likely, that stochastic effects of temporal expression of *Shh* and *Grhl2* may lead to more efficient recombination of the floxed *Grhl2* allele in some embryos relative to others. As expression of both *Grhl2* and *Shh* in the pharyngeal epithelium during craniofacial development first occurs at relatively similar time points, at approximately E6.5-E8.5^{27,36} it is possible that in a minority of *Shh*^{Cre}-*Grhl2*^{cKO} embryos, *Shh* may be expressed substantially earlier than *Grhl2*, leading to severe defects. Additionally, although initially co-expressed within the entire epithelial domain, ultimately *Grhl2* expression is more widespread in the pharyngeal epithelium compared to *Shh*, whose expression becomes restricted to a ventral sub-region of PA1^{36,37}. Therefore, earlier epithelial-specific *Cre*-drivers may need to be utilised, in addition to *Shh*^{Cre}, to effectively drive *Grhl2* deletion within the mandibular epithelium.

To differentiate between the disparate germ layer sources of oral epithelium, we dissected the contributions of both oral ectoderm and endoderm-derived tissue. *Nes*^{Cre}-*Grhl2*^{cKO} embryos presented with small, albeit statistically significant differences in both skull and jaw length

(but not width). Interestingly, skull-length (but not width) abnormalities have been reported previously following *Nes^{Cre}* mediated deletion of *FGFR2*³⁸, indicating that perhaps Nestin may also drive deletion in non-neural and non-pharyngeal ectoderm tissues as well, possibly skin. We had previously reported a non-cell-autonomous role for *Grhl3* in premature skull suture fusion (analogous to the human condition craniosynostosis)¹¹, likely due to defects in the overlying epidermis during development, and we hypothesise that the defects in skull length within the present study could perhaps similarly be influenced by loss of *Grhl2* within the overlying developing skin..

The *Nes^{Cre}* model had previously been used to conditionally delete *FGF8* in the pharyngeal ectoderm, resulting in a striking lower-jaw defect in *Nes^{Cre}-FGF8^{CKO}* embryos²⁵. Considering *FGF8* is part of a pro-survival signalling pathway upstream of *Edn1* during PA1 morphogenesis²⁵, we also examined *Grhl2^{+/-};Edn1^{+/-}* double-heterozygotes to determine whether these embryos presented evidence of functional interaction in the context of possible haplo-insufficiency of the *Grhl2-Edn1* pathway, that we had previously reported within lower jaw development of zebrafish⁶. Although we did not detect epistasis between these genes in our model, it is instructive that homozygous deletion of either *Grhl2* or *Edn1* leads to fully-penetrant defects in craniofacial development^{15,17,31}. Therefore, more sensitive approaches may be required to further titrate the gene dosage of this pathway. We had previously shown that deleting a critical *Grhl2*-enhancer element (*mm1286*) on a *Grhl2^{+/-}* background led to a partially penetrant cleft-palate phenotype³³, and therefore one option may be to cross *Edn1^{+/-}* mice with these mice to remove an *Edn1* allele in this “sensitised” model system. Moreover, this model could also be used to test the role of other critical craniofacial patterning genes that may interact with *Grhl2*, such as *FGF8*⁸ or *IRF6*^{3,4,39}.

Lastly, although *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos did not present with appreciable differences in lower jaw morphogenesis, we did note a single instance of palatal clefting in these embryos. We believe this is due to the fact that whereas *Shh* is expressed in specific regions of the endodermal and ectodermal derived oral epithelial layer of the developing face³⁷, *Sox17* activity is limited to the oral epithelia of endodermal origin in the posterior region the oral cavity near the pharynx. However, the *Sox17-2a^{iCre}-Grhl2^{CKO}* low-penetrance phenotype is still of potential interest, as *Grhl2*-loss is known to lead to maxillary clefting and midline defects, including cleft palate^{19,33}. It is unclear at this stage as to why the phenotype is so lowly-penetrant, however it is intriguing that the endoderm-derived component of the maxillary prominence is the only region (outside the respiratory system) where we noted a phenotype following conditional deletion of *Grhl2* within the endoderm.

Whereas the defects we observed in lung phenocopied those reported previously in *Shh^{Cre}-Grhl2^{CKO}* embryos²⁰, the lack of phenotype in other endoderm-derived organs was unexpected. *Grhl2* is strongly expressed within the gastro-intestinal tract^{14,18,27} and had previously been implicated as a key driver of proliferation in colorectal cancer cell lines⁴⁰. Importantly, loss of the essential adherens junctions component E-cadherin (a direct regulatory target of *Grhl2* within the intestine)¹⁴, and subsequent destabilisation of the adherens junctions has been linked to the development of chronic inflammatory bowel disease (IBD), such as ulcerative colitis and Crohn's disease⁴¹. Moreover, increased *Grhl2* expression in the GI tract had been reported in murine models of non-alcoholic fatty liver fibrosis and intestinal mucosal barrier dysfunction and shRNA-mediated *in vivo* silencing of *Grhl2* ameliorated both these conditions, indicating a role for *Grhl2* in GI pathology⁴². *Grhl2* had also been previously shown to act as both a pro-proliferative factor in hepatocellular carcinoma⁴³ and also a hepatoprotective agent in ethanol-induced liver disease⁴⁴, suggesting

that the one embryo we observed with hepatocellular necrosis could potentially indicate a further low-penetrance phenotype in *Sox17-2a^{iCre}-Grhl2^{cKO}* embryos. It may be, therefore, that *Sox17-2a^{iCre}-Grhl2^{cKO}* embryos do not show GI phenotypes under conditions where the mothers are housed in clean, specific-pathogen free animal facilities, but may yet do so under conditions of stress, disease, toxin administration or neoplasia.

Ultimately, although our data somewhat delineate certain tissue-specific roles for *Grhl2* in craniofacial development, the early embryonic expression of *Grhl2* within the pharyngeal arches (by E8.5) suggests that *Cre*-mediated conditional deletion approaches may not always achieve sufficient deletion early enough in development to effect a phenotype, and the relative dearth of early-expressing *Cre*-driver models in the literature is one hindrance to these studies.. The *Cre*-driver mice we used in the present study have all been robustly validated in many previous publications, to efficiently drive deletion in our tissues of interest, and had not been reported to present with phenotypes due to off-target effects or *Cre*-mediated toxicity. Additionally, our previous work showed that conditional deletion of the floxed *Grhl2* allele at the 2-cell stage of development using the *B6-actin^{cre}* driver²⁰ fully recapitulated the neural tube, craniofacial and embryonic lethality of *Grhl2^{-/-}* mice¹⁵, validating the robustness of the phenotypes we report here. Ultimately, selection of *Cre*-recombinase mouse models that are driven by genes expressed early in embryogenesis, prior to the onset of *Grhl2* expression, will be essential for future studies to determine tissue-specific roles of *Grhl2* in embryonic development.

Experimental Procedures:

Ethics declaration

All mice were housed under controlled temperatures ($21^{\circ}\text{C} \pm 1^{\circ}\text{C}$) on a 12-hour light/dark cycle. All procedures were in accordance with projects no. E/1200/2012/M and AEC16-72 approved by the Alfred Medical Research and Education Precinct (AMREP) and La Trobe University (LTU) Animal Ethics Committees respectively.

Generation of experimental animals

The previously described *Grhl2*^{fl^{ox}/fl^{ox}} mice²⁰ were used to achieve conditional deletion of *Grhl2*. *Cre*-driver mice were used to effect deletion within the neural crest (*Wnt1*^{Cre})²⁸, pharyngeal epithelium (*Shh*^{Cre})⁴⁵, all endodermal tissue (*Sox17-2a*^{iCre})³² or pharyngeal ectoderm (*Nestin*^{Cre})²⁵. In all cases, the individual *Cre*-driver mice were first crossed with *Grhl2*^{+/-} mice¹⁵ in order to establish a mouse line that was positive for the *Cre* transgene and heterozygous for *Grhl2* (i.e. *Cre*⁺;*Grhl2*^{+/-} mice), as *Cre*-mediated deletion functions most effectively when only one floxed allele is present²⁰. Breeding colonies were established to facilitate the crosses of type “*Grhl2*^{fl^{ox}/fl^{ox}} x *Grhl2*^{+/-};*driverCre*⁺”. Embryos with tissue specific deletion (conditional knockout; cKO) therefore were of the genotype *driverCre*⁺-*Grhl2*^{Δ/-}, with “Δ” indicating the deleted allele, and for clarity, are referred to as “*driverCre*-*Grhl2*^{cKO}” within this manuscript. Control animals were typically of the genotype “*driverCre*-*Grhl2*^{fl/+}” (i.e. a conditional heterozygote) and for clarity are referred to as “control” unless otherwise indicated in the text. *Endothelin1*^{+/-} mice³¹ were crossed with *Grhl2*^{+/-} mice for the epistasis experiments.

ki-67 staining for detection of proliferation

All steps were conducted at RT unless otherwise stated. After dewaxing and antigen retrieval, slides were immersed in 250ml 3% H₂O₂ in PBS for 10 min to quench endogenous peroxidases. The slides were then washed for 2 x 5 minutes in PBS, before being blocked with 10% normal goat serum (NGS) in PBS for 30 minutes. The blocking solution was removed and the slides were incubated with rabbit anti-mouse ki-67 primary antibody (1:100 in 10% NGS in PBS; Abcam) for 45 minutes. Next, slides were washed for 3 x 5 min in PBS and subsequently incubated with biotinylated goat anti-rabbit secondary antibody (1:200 in 10% NGS in PBS; Vector Labs) for 30 minutes. The slides were washed for 3 x 5 min in PBS, incubated with Avidin/Biotinylated enzyme Complex (ABC; Vector Labs) solution for 30 min and washed again for 3 x 5 min in PBS. The slides were then immersed in Diaminobenzidine (DAB; Vector Labs) substrate for 2 minutes, washed for 2 x 2 minutes in H₂O, counterstained with haematoxylin for 3 seconds, rinsed for 30 seconds in H₂O, blued in Scott's tapwater for ~10 seconds, washed for 3 x 2 minutes in H₂O, dehydrated in ethanol, cleared in Xylene and cover-slipped in DePeX (Ajax Finechem).

Automated quantitation of ki-67 staining

A macro allowing for the automated quantitation of ki-67 staining was developed using the Fiji imaging software⁴⁶. The area(s) of interest for analysis were outlined using a freehand tracing tool, and images were separated into haematoxylin and DAB channels using the Colour Deconvolution module. A rolling ball background subtraction (25px) was performed on each channel to allow for accurate and even detection of positive cells. Positive haematoxylin and DAB nuclei were counted in each channel using the Find Maxima command (with noise tolerance set to 25).

Skeletal staining

Heads removed from E14.5-E18.5 mouse embryos were fixed in 80-90% ethanol for 168 hours and dehydrated in 96% ethanol for 72 hours. Next, the heads were incubated in Alcian Blue solution for 72 hours to stain cartilage. Samples were rehydrated by sequential immersion in 70% Ethanol (2-3 hours), 40% ethanol (2-3 hours), 15% ethanol (2-3 hours) and then H₂O for 24-48 hours. Skin and viscera were removed by immersing the samples in a 1% Potassium Hydroxide (KOH) solution in H₂O for 24-48 hours. Next, samples were immersed in Alizarin Red for 4 hours to stain bone, followed by 3 x 2-hour washes in 1% KOH. Samples were prepared for storage by sequential immersion in increasing concentrations of glycerol in 1% KOH (20% glycerol in 1% KOH, 50% glycerol in 1% KOH, and 80% glycerol in 1% KOH) with approx. 24 hours required at each concentration.

Histological analyses

All ultrastructural and histological analyses of *Sox17-2a^{iCre}-Grhl2^{CKO}* (n=3) and control embryonic tissue was conducted by the Histopathology and Organ Pathology department of the Australian Phenomics Network at the University of Melbourne. Tissue samples were fixed in 4% PFA for 48 hours, before being stored and delivered in 70% Ethanol, prior to processing, paraffin-embedding and sectioning using standard histological procedures. Sagittal sections (5µm thick; 200µm intervals) were taken from the midline, lateral through the body, and rostral sections through the heads. Samples were stained in hematoxylin and eosin prior to microscopic analysis using standard light microscopy techniques.

Statistical analysis

Unless otherwise stated, all experiments were conducted on a minimum of three animals per genotype. Where applicable, results are expressed as means ± standard error of the mean (SEM). For statistical analysis, the 2-tailed student's t-test method was employed unless

otherwise stated, where $p < 0.05$ was considered statistically significant. Automated analysis of variance (ANOVA) was performed using the SPSS Statistics software package (IBM).

Acknowledgments

The authors would like to thank Mr. Cameron Nowell (Monash University) for assistance with developing macros for automated Ki67⁺ cell counting. This study also utilised the Australian Phenomics Network Histopathology and Organ Pathology Service, University of Melbourne. This work was supported by grants from the Australian National Health and Medical Research Council (NHMRC, GNT1063837), Australian Research Council (ARC) Discovery Early Career Researcher Award (DECRA, DE140100500), Australian Cranio-Maxillo Facial Foundation (Craniofacial Australia), and La Trobe University ([#2000003053](#)). The authors declare no competing financial or other conflicts of interest.

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Figure legends:

Figure 1: *Shh*^{Cre}-mediated deletion of *Grhl2* in pharyngeal epithelium leads to low-penetrance micrognathia Control pups at P1 show a clear lower jaw in brightfield imaging (A), and the mandible is clearly visible in lateral (B) and ventral (C) views following Alizarin-red staining of the facial and skull bones (arrowheads A-C). In contrast, the lower jaw in micrognathic *Shh*^{Cre}-*Grhl2*^{cKO} is significantly regressed in brightfield images (D), appears to be largely absent in later views following Alizarin red staining (E), and is shown to be substantially underdeveloped in ventral views (F; arrowheads D-F). Dashed lines in (C) and (F) indicate lower jaw length measurements taken. Scatterplot analysis and quantitation of micrognathia incidence (G) shows that this defect occurred in 3/33 (9.1%) of *Shh*^{Cre}-*Grhl2*^{cKO} mice (*** asterisks indicate mandible lengths that are greater than 3 S.D. below mean mandibular length). However, *Shh*^{Cre}-*Grhl2*^{cKO} embryos without visible micrognathia presented with no significant differences in mandibular length (H).

Figure 2: Non-micrognathic *Shh*^{Cre}-*Grhl2*^{cKO} embryos displayed no qualitative or quantitative differences in proliferation in lower jaw and oral epithelium at E14.5 (A-B) No obvious differences in ki-67 expression are visible in whole sagittal sections of the head in control (A) compared to *Shh*^{Cre}-*Grhl2*^{cKO} embryos (B) at E14.5. Similarly, no qualitative differences are apparent in sagittal sections of the maxilla (C-D) and mandible (E-F) in control (C,E) embryos relative to *Shh*^{Cre}-*Grhl2*^{cKO} embryos (D,F). Automated morphometric quantitation confirms there is no significant difference in the proportion of ki67+ cells in the maxillary epithelium, whole mandible or mandibular epithelium in control embryos relative to *Shh*^{Cre}-*Grhl2*^{cKO} embryos (G; n=3 embryos per genotype).

Figure 3: Genotyping strategy and confirmation of successful Cre-mediated deletion. (A) Two separate primers sets were used to firstly identify mice with a *Grhl2*-KO allele (mBOM11; LacZrv; 237bp), and between deleted (Δ) and non-deleted (floxed) *Grhl2*^{lox} alleles (BOM Fw, BOM Rv, BOM Rv delta: non-recombined allele yielding a 258bp fragment, recombined allele yielding a 576bp fragment by agarose gel electrophoresis). (B) Genotyping to determine *Grhl2* floxed-allele excision (in neural stem cells) shows complete excision in cells derived from *Nestin*^{Cre}-positive *Grhl2*^{lox/-} mice (lanes 1-4; i.e. *Nestin*^{Cre+};*Grhl2*^{Δ/-}), but not in cells from *Nestin*^{Cre}-negative *Grhl2*^{lox/-} mice (lane 5; i.e. *Nestin*^{Cre-};*Grhl2*^{lox/-}), confirming robust recombination.

Figure 4: *Nestin^{Cre}-Grhl2^{CKO}* embryos present with significant quantitative differences in skull and jaw length, albeit not width. (A-B) Schematic diagram showing measurements taken. **(C-D')** Alcian Blue (cartilage) and Alizarin Red (bone) staining reveals no discernible differences between *Nestin^{Cre}-Grhl2^{CKO}* pups at P0 **(C-C')** relative to controls **(D-D')** in either lateral views of the head and upper skeleton **(C-D)** or ventral views of the skull **(C'-D')**. **(E)** Quantitation of the mandibular, upper jaw and skull length in *Nestin^{Cre}-Grhl2^{CKO}* pups (n=3) revealed small, albeit statistically significant differences relative to control pups (n=5). Quantitation of skull and mandibular width revealed no significant differences in *Nestin^{Cre}-Grhl2^{CKO}* pups. (Error bars indicate standard deviation; *p<0.05 by Student's t-test).

Figure 5: Craniofacial architecture is not disrupted in *Wnt^{Cre}-Grhl2^{CKO}* or *Grhl2^{+/-};Edn1^{+/-}* embryos (A) Quantitation of the mandibular, upper jaw and skull length and skull and mandibular widths in *Wnt^{Cre}-Grhl2^{CKO}* pups (n=16) revealed no significant differences relative to control pups (n=12; p>0.05 by Student's t-test). **(B)** Similarly, quantitation of these skull and jaw lengths and widths in *Grhl2^{+/-};Edn1^{+/-}* embryos (n=16) also revealed no significant differences relative to control *Grhl2^{+/+};Edn1^{+/+}* embryos (n=7).

Figure 6: Conditional deletion of *Grhl2* in the endoderm does not lead to embryonic lethality. (A) Breeding strategy to generate *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos. Mice homozygous for *Grhl2* alleles flanked by *LoxP* sequences (Flox, fl) were crossed with mice heterozygous for mutant *Grhl2* and the *Cre* transgene driven by the *Sox17* promoter. **(B)** Incidence of expected genotypes showed no significant differences from expected Mendelian ratios at either E18.5 or P0.

Figure 7: Deletion of *Grhl2* in the endoderm (*Sox17-2a^{iCre}-Grhl2^{CKO}*) results in low-penetrance cleft palate, tracheal cartilage malformation and decreased trachea width. Frontal **(A)** and ventral **(B)** views of a single *Sox17-2a^{iCre}-Grhl2^{CKO}* embryo at E18.5 showing unilateral left-sided cleft lip and cleft secondary palate **(B)**. Alcian Blue staining of cleared tracheal samples from E18.5 *Control* **(C)** and *Sox17-2a^{iCre}-Grhl2^{CKO}* **(D)** show malformed, incomplete and irregular cartilaginous rings in *Sox17-2a^{iCre}-Grhl2^{CKO}* (n=6). **(E-F)** Quantitation confirms that average width of the *Sox17-2a^{iCre}-Grhl2^{CKO}* tracheas (421.3µm ± 6.4 SEM; n=5) is significantly reduced relative to control littermates (555.9µm ± 15.5 SEM, n=5; *p<0.01 by Student's t-test), although average tracheal length is unaltered in *Sox17-2a^{iCre}-Grhl2^{CKO}* tracheas (1870.2µm ± 138.1 SEM, n=5) relative to control embryos (2005µm ± 6.4 SEM; n=5). Scale bar = 500µm.

Figure 8: *Sox17^{Cre}-Grhl2^{CKO}* mice present with lung blood-flow and inflation defects. (A-B) E18.5 control embryos show evidence of extensive blood flow (arrows; **A**), which is absent in *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos (**B**). Transverse H+E sections of both control (**C**) and *Sox17-2a^{iCre}-Grhl2^{CKO}* (**D**) lungs at E18.5 *in situ* do not present with any apparent morphological or cellular differences. However, comparable transverse H+E sections at P0 show that control lungs (**E**) have inflated normally with characteristic thin, single-cell thickness alveolar epithelial cell walls surrounding air pockets (inset, **E**). In contrast, *Sox17-2a^{iCre}-Grhl2^{CKO}* lung epithelial cells (**F**) retain a disorganised and clumped appearance, without forming single-cell thickness alveolar walls (inset, **F**) littermates. Scale bar = 500µm. RL: Right lung, E: Esophagus, H: Heart.

Figure 9: *Sox17-2a^{iCre}-Grhl2^{CKO}* lungs, but not small intestine, present with increased proliferation and Sox9+ stem cell populations at E18.5 (A-B) Representative images of ki-67 expression (brown) in transverse sections of control (**A**) and *Sox17-2a^{iCre}-Grhl2^{CKO}* (**B**) lung. (**C**) Quantitative analysis of the proportion of ki-67+ cells shows a significant increase in proliferation in *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos than control littermates (n=3 per genotype, p=0.011 by Student's t-test), and blood vessels have formed normally. AS: Airspace, V: Blood Vessel. (**D-E**) Representative images of Sox9 expression (green) in transverse sections of control (**D**) and *Sox17-2a^{iCre}-Grhl2^{CKO}* (**E**) lung. (**F**) Quantitative analysis of the proportion of Sox9+ cells shows a significant increase in the number of stem/progenitor cells present in *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos than control littermates (n=3 per genotype, p=0.0008 by Student's t-test). (**G**) Representative PCR of tissues extracted from control (*Sox17-2a^{iCre}-Grhl2^{Flox/-}*) and *Sox17-2a^{iCre}-Grhl2^{CKO}* (*Sox17-2a^{iCre};^{Grhl2}^{Δ/-}*) littermates. Genotyping indicates substantial recombination in all *Sox17-2a^{iCre}-Grhl2^{CKO}* tissues examined, save for tail tissue utilised as a negative control, as it does not contain endoderm-derived cells. Full recombination is not expected, or seen, as all organs examined contain large proportions of non-endodermal cell types. Quantitative analyses of proliferation (**H-J**) and Sox9 expression (**K-M**) in the small intestine of control (**G****H** **K**) or *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos (**I**, **L**) do not show any significant differences in either the number of proliferating cells in the villi, epithelial lumen or mucosa (**J**), nor overall numbers of stem/progenitor cells in the intestine (**M**; n=3 embryos per genotype). E: Epithelia, M: Mucosa, V: Villi.

Figure 10: Histopathological examination did not reveal any qualitative defects in organs comprising endoderm-derived cells in *Sox17-2a^{iCre}-Grhl2^{CKO}* embryos. Examination of transverse sections taken through the thymus (**A-B**), heart (**C-D**), bladder (**E-F**), liver (**G-H**), Stomach (**I-J**) and intestine (**K-L**) of control (**A, C, E, G, I, K, M**) and *Sox17-2a^{iCre}-Grhl2^{CKO}* (**B,D,F,H,J,L,N**) embryos.

Fig. 1

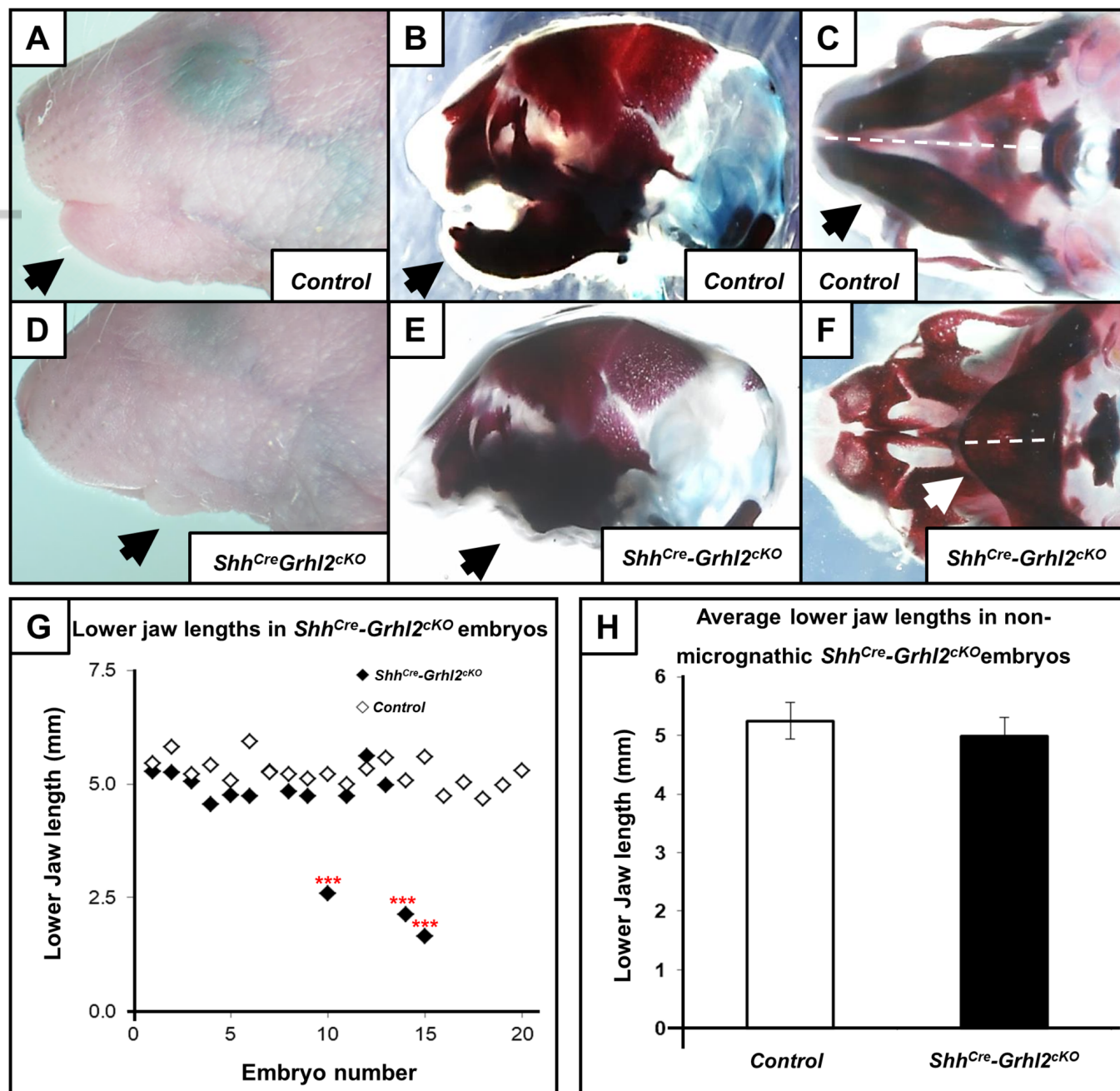


Fig. 2

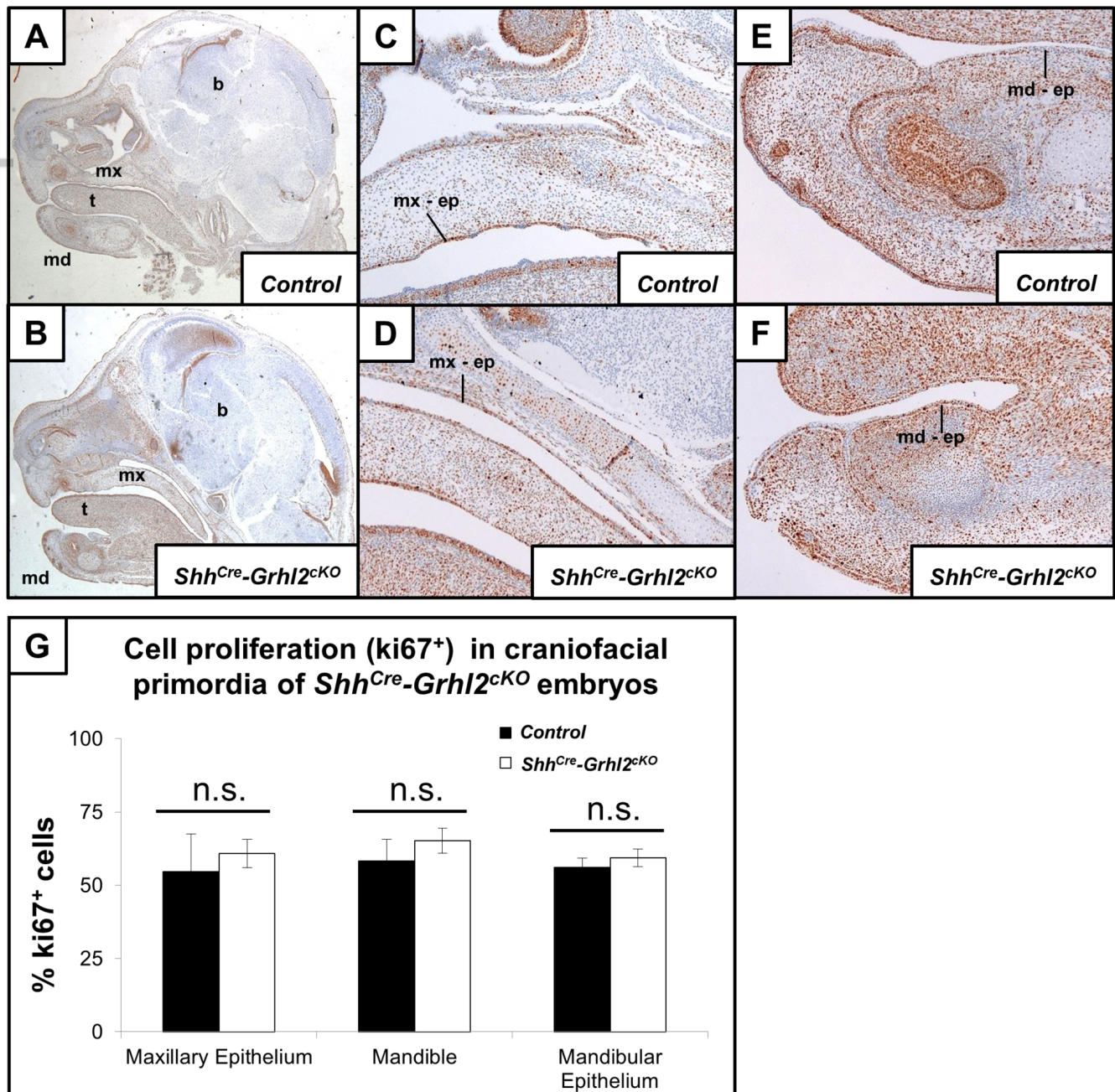


Fig. 3

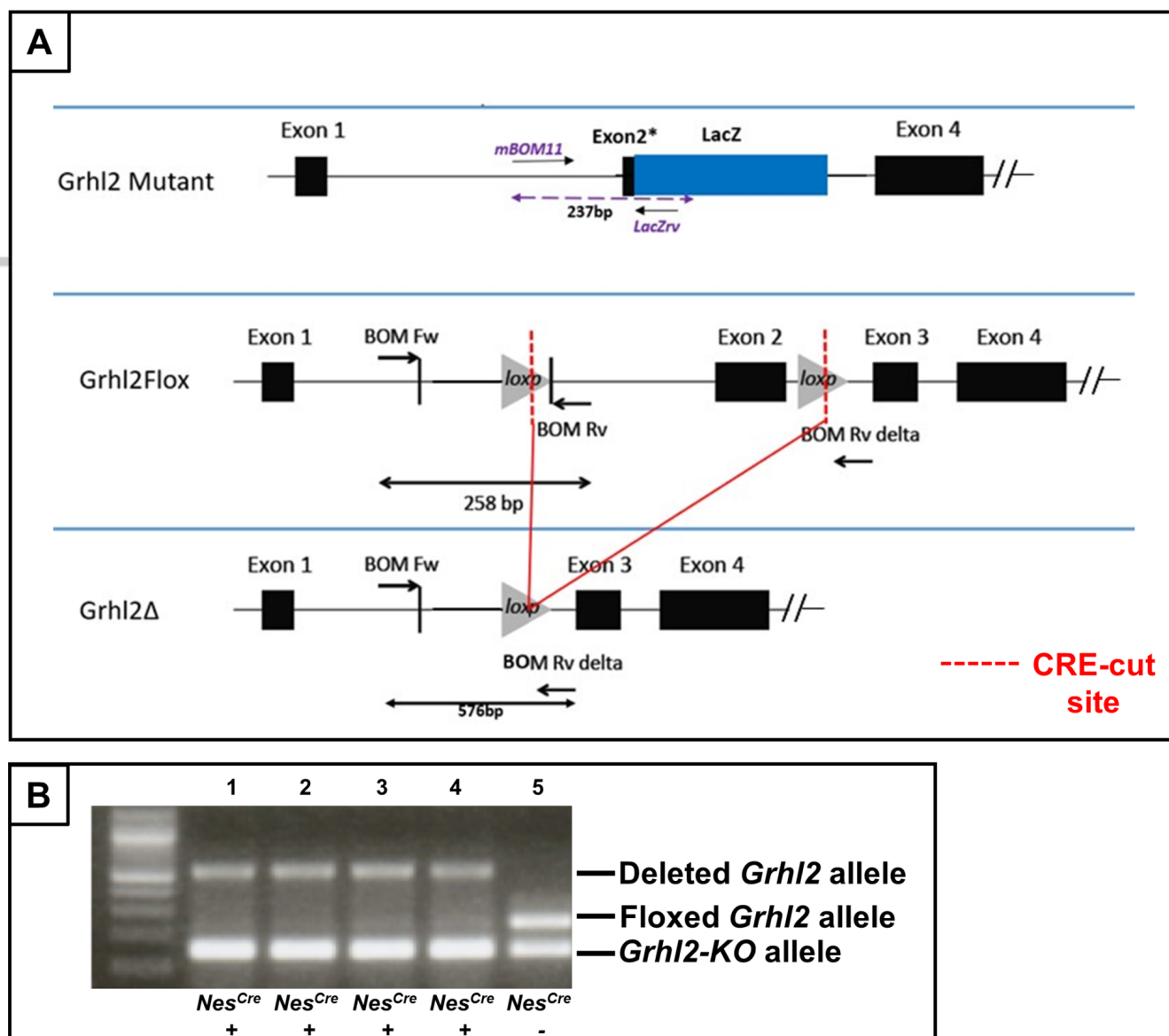


Fig. 4

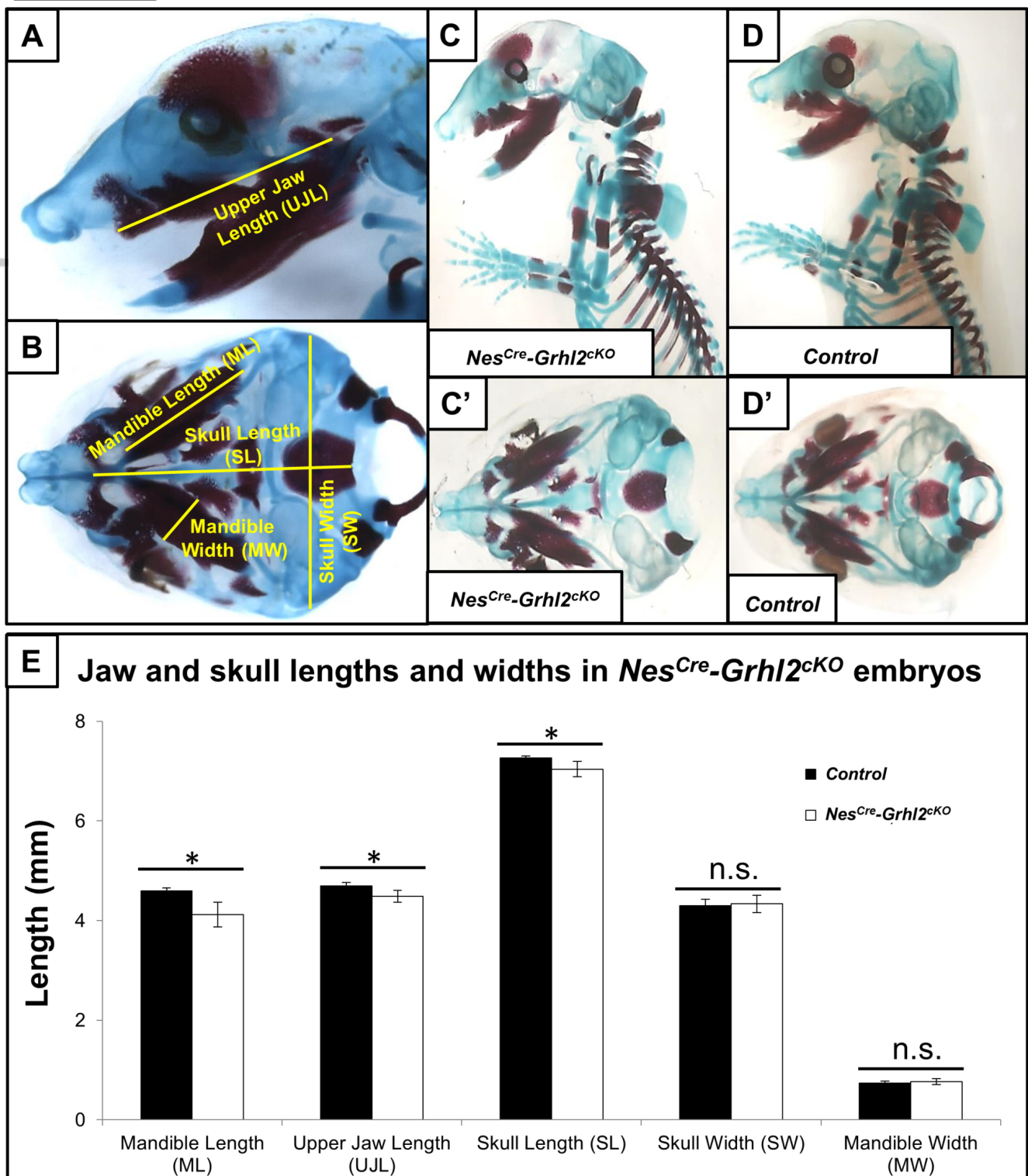


Fig. 5

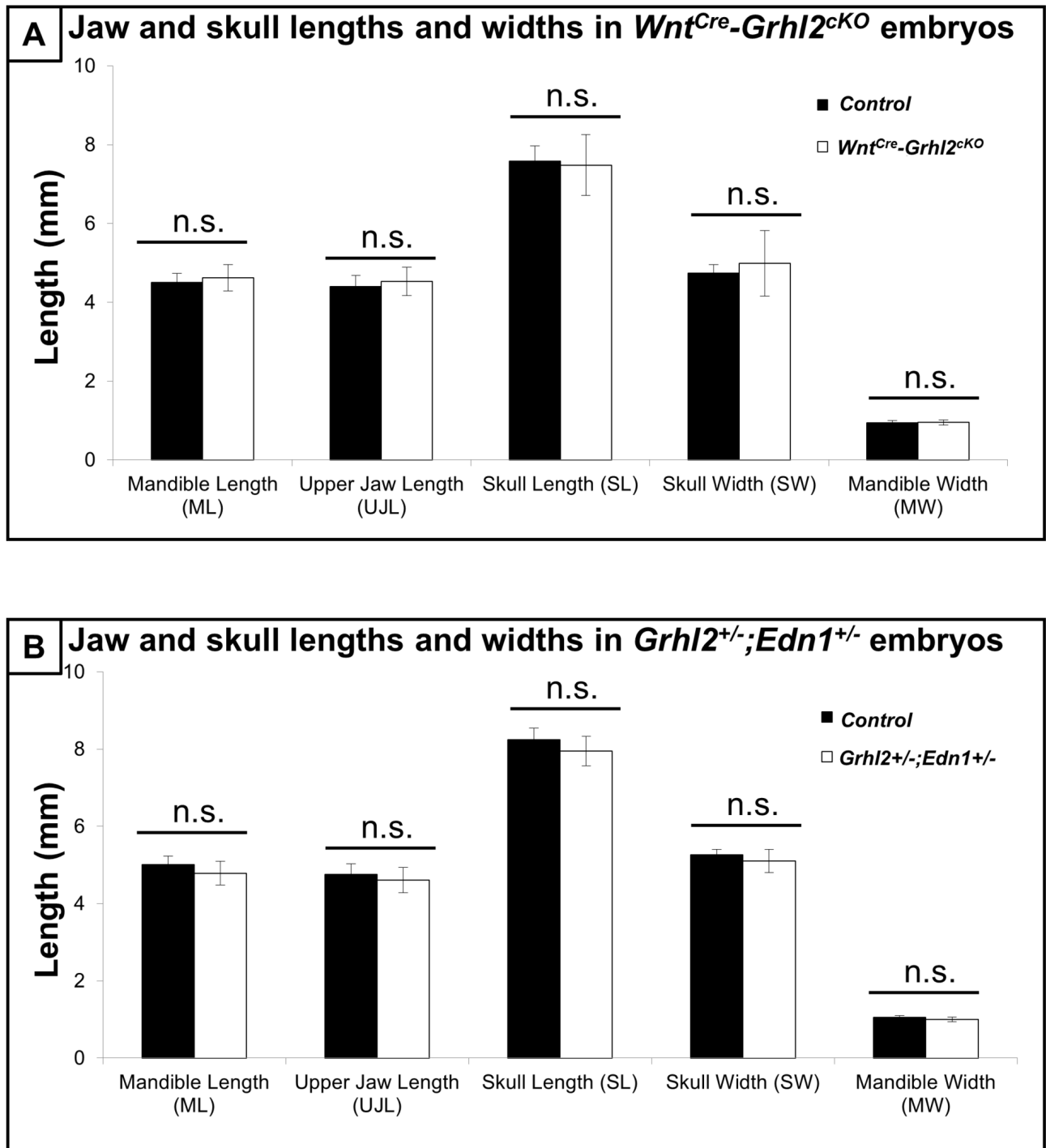
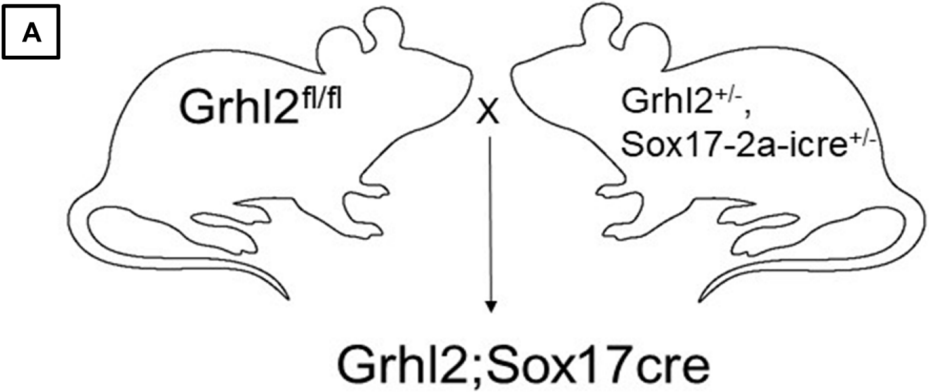


Fig. 6



B

Sox17-2a- <i>iCre⁺</i> ; <i>Grhl2cKO</i>		Sox17-2a- <i>iCre⁺</i> ; <i>Grhl2cHET</i>		Sox17-2a- <i>iCre⁺</i> ; <i>Grhl2-HET</i>		Sox17-2a- <i>iCre⁻</i> - <i>Grhl2-WT</i>	
$\Delta/-$; cre/+		$\Delta/+$; cre/+		fl/-; +/+		fl/+; +/+	
E18.5		Obs(n)	Exp(n)	Obs(%)	Exp(%)	χ^2	
Grhl2 :	cKO	$\Delta/-$; cre/+	18	19.5	23.08	25	0.6852
	cHET	$\Delta/+$; cre/+	17	19.5	21.79	25	
	HET	fl/-; +/+	24	19.5	30.77	25	
	WT	fl/+; +/+	19	19.5	24.36	25	
		78					
P0		Obs(n)	Exp(n)	Obs(%)	Exp(%)	χ^2	
Grhl2 :	cKO	$\Delta/-$; cre/+	20	21.25	23.53	25	0.3066
	cHET	$\Delta/+$; cre/+	15	21.25	17.65	25	
	HET	fl/-; +/+	23	21.25	27.06	25	
	WT	fl/+; +/+	27	21.25	31.76	25	
		85					

Fig. 7

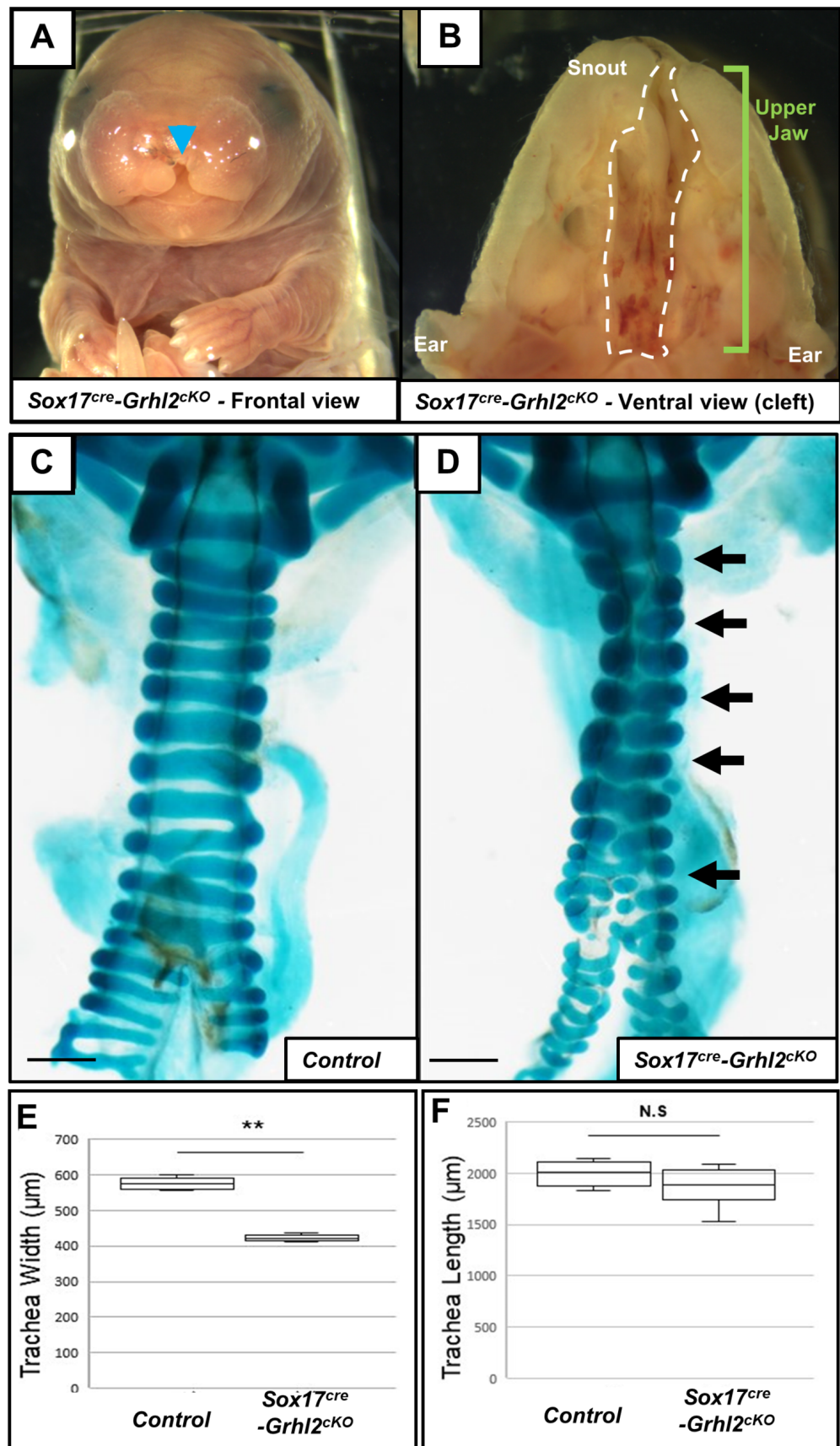


Fig. 8

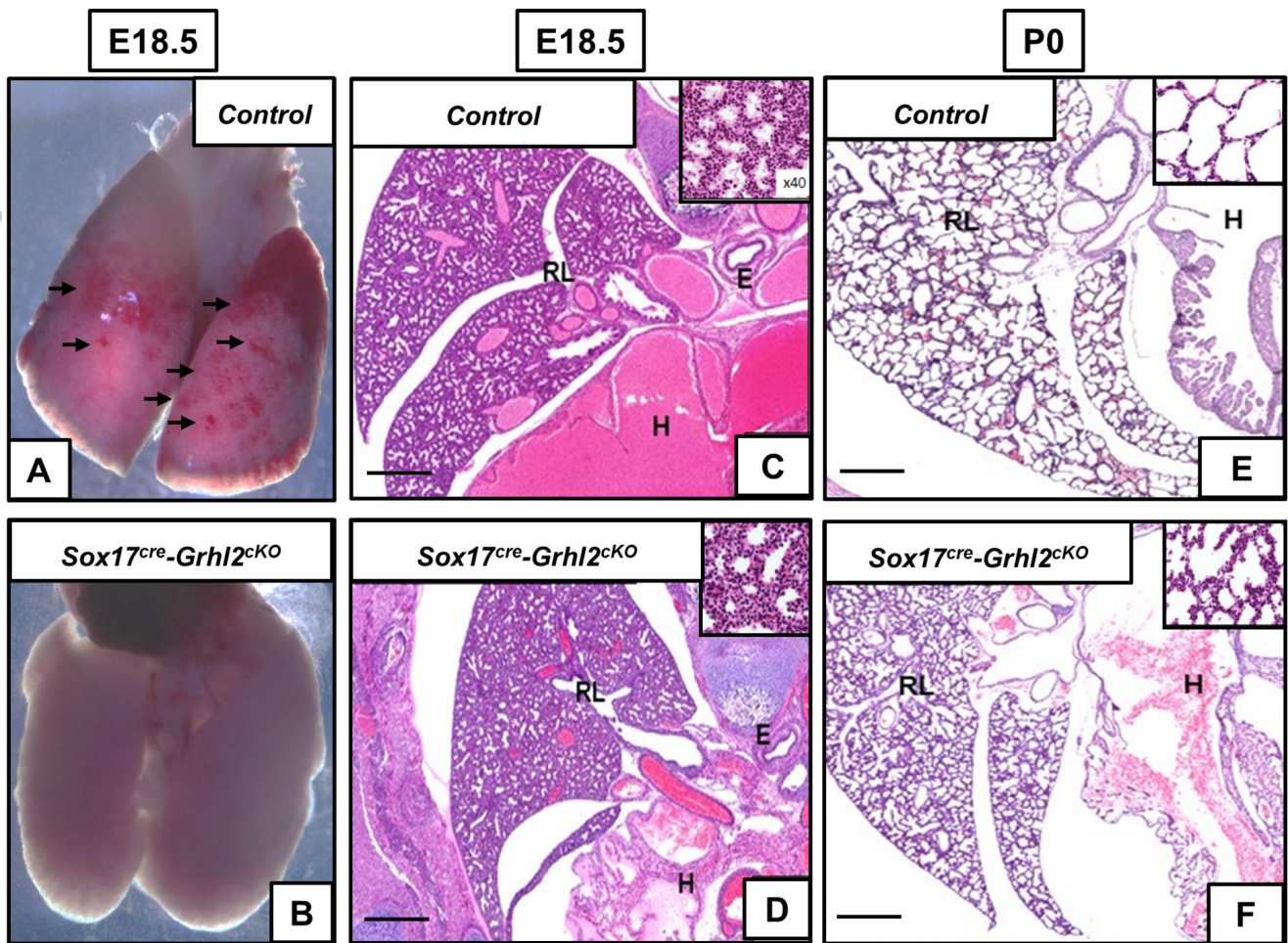


Fig. 9

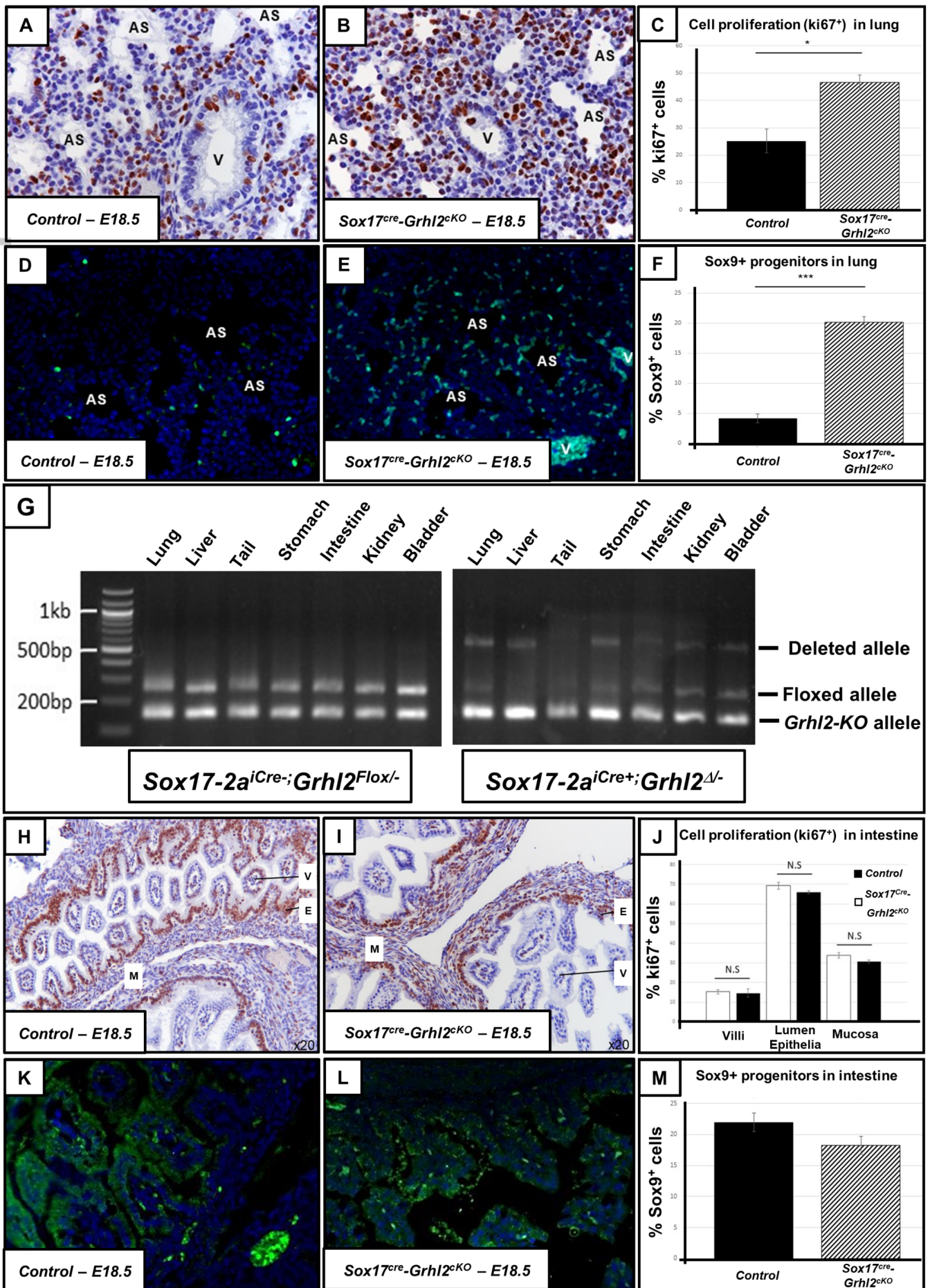


Fig. 10

