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Short Communication

Foxi1 promotes late-stage pharyngeal pouch morphogenesis through ectodermal Wnt4a activation

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ABSTRACT

The pharyngeal pouches are a series of epithelial outgrowths of the foregut endoderm. Pharyngeal pouches segment precursors of the vertebrate face into pharyngeal arches and pattern the facial skeleton. These pouches fail to develop normally in zebrafish *foxi1* mutants, yet the role Foxi1 plays in pouch development remains to be determined. Here we show that ectodermal Foxi1 acts downstream of Fgf8a during the late stage of pouch development to promote rearrangement of pouch-forming cells into bilayers. During this phase, *foxi1* and *wnt4a* are coexpressed in the facial ectoderm and their expression is expanded in *fgf8a* mutants. *foxi1* expression is unaffected in *wnt4a* mutants; conversely, ectodermal *wnt4a* expression is abolished in *foxi1* mutants. Consistent with this, *foxi1* mutant pouch and facial skeletal defects resemble those of *wnt4a* mutants. These findings suggest that ectodermal Foxi1 mediates late-stage pouch morphogenesis through *wnt4a* expression. We therefore propose that Fox1 activation of Wnt4a in the ectoderm signals the epithelial stabilization of pouch-forming cells during late-stage of pouch morphogenesis.

1. Introduction

Vertebrate craniofacial development relies on precise spatiotemporal interactions and signals between cranial-neural-crest-derived pharyngeal arches, their mesodermal cores, and surrounding ectodermal and endodermal epithelia (Couly et al., 2002; Crump et al., 2004; Piotrowski et al., 2003). The endodermal epithelia exist as segmented pharyngeal pouches, required for patterning and morphogenesis of the craniofacial skeleton. Later, these pouches go on to generate important organs including the Eustachian tube, thymus, parathyroid, and gills in the face and neck (Gordon et al., 2001; Graham et al., 2005; Grevellec and Tucker, 2010; Proctor, 1967; Schwend and Ahlgren, 2009).

Pouch morphogenesis occurs in stages in which pouch-forming cells undergo dynamic epithelial transitions, remodeling, and changes in cell shape and neighbor relationships (Choe et al., 2013). Morphogenesis takes place in two stages which are controlled by multiple ectodermal, mesodermal, and endodermal signaling pathways. During the early stage, endodermal pouch-forming cells lose their columnar morphology, become multilayered, and migrate collectively toward facial ectoderm (Choe et al., 2013). Tbx1-dependent Wnt11r expression from the adjacent mesoderm is responsible for cellular shape changes while mesodermal Tbx1 activation of Fgf8a guides the collective migration of these cells (Choe et al., 2013; Choe and Crump, 2014).

As pouches grow toward the facial ectoderm, ectodermal Wnt4a is required for the junctional localization of Alcama in endodermal pouch-forming cells (Choe et al., 2013). During late-stage pouch morphogenesis, Alcama drives the rearrangement of migrating endodermal cells into the formation of bilayered pouches (Choe et al., 2013). At the end of the late stage, Wnt4a and EphrinB activation of Pak2a maintains the integrity of bilayered mature pouches by further increasing intercellular adhesion (Choe and Crump, 2015). While the mesodermal Tbx1-Wnt11r-Fgf8a pathway that controls early-stage pouch morphogenesis is well understood, genetic control of late-stage, ectodermal Wnt4a expression remains poorly understood (Choe and Crump, 2014). Here, we report that ectodermal *wnt4a* expression is positively regulated by Foxi1, while both *wnt4a* and *foxi1* expression are restricted by Fgf8a in the ectoderm.

Zebrafish *foxi1* mutants and mouse *foxi3* (a *foxi1* functional homologue) mutants display craniofacial defects in otic placode, facial skeleton, and pharyngeal pouch development (Edlund et al., 2014; Nissen et al., 2003; Solomon et al., 2003). Perturbations in survival and proliferation of cranial-neural-crest-derived ectomesenchymes in the pharyngeal arches result in facial skeletal defects (Edlund et al., 2014;

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Nissen et al., 2003; Solomon et al., 2003). In zebrafish the survival and proliferation deficits of these ectomesenchymes were partially rescued by activating Fgf3/8 signaling in the facial ectoderm at 20 h post-fertilization (hpf), yet they could not rescued by activating Fgf3/8 signaling after 22 hpf when most pouches have developed in zebrafish (Edlund et al., 2014). Since it has been demonstrated that pouches play a role in ectomesenchymal survival (Brito et al., 2006), the facial skeletal defects seen in *foxi1* mutants could also be attributed to defective pouch formation. *foxi1* is expressed in the pharyngeal endoderm as well as in the facial ectoderm, but not in the ectomesenchymes of the arches during pharyngeal arch development (Nissen et al., 2003; Solomon et al., 2003). Previously, it was proposed that a Fgf3- and Foxi1-dependent regulation of Pax8 may pattern the pharyngeal endoderm autonomously to form pouches, as Foxi1 regulates Pax8 expression in the pouches through endodermal Fgf3 expression (Nissen et al., 2003). However, the role that ectodermal Foxi1 plays in remodeling endoderm into pouches has yet to be analyzed. Here, we show that ectodermal Foxi1 promotes rearrangements of pouch-forming cells into bilayered pouches during late-stage pouch morphogenesis through ectodermal Wnt4a activation.

2. Results

2.1. Foxi1 is required for remodeling of pouch-forming cells during late-stage pouch morphogenesis

In order to understand how Foxi1 controls pouch morphogenesis at the cellular level, we first reanalyzed *foxi1* mutants using Alcama immunohistochemistry to label pouches. While five bilayered mature pouches were present at 34 hpf in wild-type siblings (Fig. 1A), fewer abnormally shaped pouches formed in *foxi1* mutants whose anterior pouches were typically missing (Fig. 1B, M). Upon close inspection these pouch-forming cells were inappropriately multilayered compared to wild-type pouches (Fig. 1B, F), which suggests that pouch morphogenesis fails to progress to the late stage when pouches become bilayered. Previously, we reported that pouch patterning mutants can be divided into two groups: one which includes *tbx1* and *wnt11r* mutants that display early-stage defects including a delay or failure of pouch outgrowth, and a second group including *wnt4a* and *ephrinb2a* mutants, that display late-state defects such as multilayered, immature or missing pouches (Choe et al., 2013; Choe and Crump, 2014, 2015). Even though we cannot completely rule out that the absence of the anterior two pouches in *foxi1* mutants could be a consequence of early defects in pouch outgrowth, the failure of pouch-forming cells to mature into a bilayer in mutants suggests that Foxi1 is required for late-stage pouch morphogenesis.

2.2. Foxi1 regulates late-stage pouch morphogenesis through ectodermal Wnt4a

We examined the effect of the *foxi1* mutation on late-stage pouch development. It has been proposed that Foxi1 modulates downstream cellular responses to Fgf3 signaling during pouch development based on a loss of *fgf3* expression in *foxi1* mutant pouches (Nissen et al., 2003). Consistent with this model, we predicted that similar pouch defects would be found in both *foxi1* and *fgf3* mutants. We tested this possibility by first confirming the reduction in *fgf3* expression seen in *foxi1* mutant pouches. In wild-type embryos, *fgf3* is expressed in pouches, the strongest expression in nascent pouches (David et al., 2002). As pouches formed we noticed an anterior-to-posterior wave of increasing *fgf3* expression with the most robust expression found in the last three pouches (Fig. 2A). In *foxi1* mutants, we observed a similar *fgf3* expression pattern in the posteriormost two to three pouches, but we found that *fgf3* expression in the nascent pouches was weaker relative to wild-type expression, confirming the positive regulation of Fgf3 by Foxi1 in the endoderm (Fig. 2B). We next analyzed whether Foxi1 regulated late-stage pouch morphogenesis through Fgf3 signaling. As Fgf3 has been implicated in pouch development by controlling pouch cell migration (Crump et al., 2004; Herzog et al., 2004), we compared *fgf3* mutant pouches with *foxi1* mutant pouches expecting to see similar, multilayered immature pouches in both. In *fgf3* mutants, the anterior two pouches formed, while the posterior pouch-forming cells failed to migrate out (Fig. 1C, G, M). These *fgf3* mutant phenotypes differ markedly from *foxi1* mutant phenotypes and suggest that posterior pouch morphogenesis fails to initiate in *fgf3* mutant pharyngeal endoderm. We also analyzed ceratobranchial (CB) cartilages whose development is contingent upon appropriate pouch formation in 5 dpf-old *fgf3* mutants. In wild-type embryos, five CB cartilages form from the posterior pharyngeal arches that are segmented by posterior pouches (Fig. 1I, M). In *fgf3* mutants, vestiges of the anterior CB cartilages form, yet the posterior CB cartilages form as one fused cartilage, consistent with the absence of posterior pouches (Fig. 1K, M). The defects in outgrowth of pouch-forming cells and the fused giant CB cartilages seen in *fgf3* mutants strongly suggest that pouch formation fails to initiate during early-stage pouch morphogenesis, which is in contrast to the defects expected in *foxi1* mutants. We also noted that the CB cartilage defects found in *foxi1* mutants differed from those of *fgf3* mutants. Instead of a single, fused CB, we found a reduction in the number of normally shaped CBs in *foxi1* mutants (Fig. 1J, M). Though we cannot completely rule out the possibility that Fgf3 signaling acts with Foxi1 to control late-stage pouch morphogenesis, we are impeded from testing this as the *fgf3* mutant pouches fail to progress beyond the early stage. Therefore, we are unable to find any evidence suggesting that Foxi1 regulates late-stage pouch morphogenesis through Fgf3.

We found that the multilayered pouch phenotypes and the missing CB cartilage phenotypes seen in *foxi1* mutant pouches were more severe yet reminiscent of the *wnt4a* mutant phenotypes (Fig. 1D, H, L, M; Choe et al., 2013). These common phenotypes between *foxi1* and *wnt4a* mutants suggest that Foxi1 and Wnt4a are genetically linked to control late-stage pouch morphogenesis. We found these common phenotypes intriguing and examined the expression of *foxi1* and *wnt4a* during late-stage pouch morphogenesis. While *wnt4a* is expressed segmentally in the facial ectoderm (Fig. 2D; Choe et al., 2013), *foxi1* is expressed in the ectoderm as well as pouch endoderm during wild-type pouch morphogenesis (Fig. 2F; Nissen et al., 2003; Solomon et al., 2003). We found that *wnt4a* and *foxi1* were co-expressed in the ectoderm of wild-type embryos (Fig. 2C), while *wnt4a* expression was significantly reduced in *foxi1* mutants (Fig. 2D, E). However, the endodermal and ectodermal *foxi1* expression was unaffected in *wnt4a* mutants (Fig. 2F, G). This epistatic analysis indicates that Foxi1 acts upstream of Wnt4a to positively regulate its ectodermal expression during pouch development. We therefore propose that Foxi1 controls late-stage pouch morphogenesis through ectodermal Wnt4a, organizing pouch-forming cells into bilayered pouches.

2.3. Fgf8a acts as a negative regulator of ectodermal foxi1 expression during the pouch morphogenesis

In order to further understand the genetic hierarchies at play during pouch morphogenesis, we analyzed *foxi1* expression in *fgf3*, *tbx1*, and *fgf8a* mutants that showed pouch defects (Crump et al., 2004; Herzog et al., 2004; Piotrowski et al., 2003; Piotrowski and Nusslein-Volhard, 2000). Both endodermal and ectodermal *foxi1* expression were unaffected in *fgf3* mutants compared to wild-type embryos (Fig. 3A, B, E, F, I, J). In *tbx1* mutants, the endodermal *foxi1* expression was significantly reduced, whereas the ectodermal expression was expanded, suggesting that Tbx1 regulates *foxi1* expression positively and negatively in the endoderm and ectoderm, respectively (Fig. 3C, G, K). Interestingly, in *fgf8a* mutants, the ectodermal expression of *foxi1* was dramatically expanded, yet normal *foxi1* expression was seen in the disorganized endoderm (Fig. 3D, H, L).

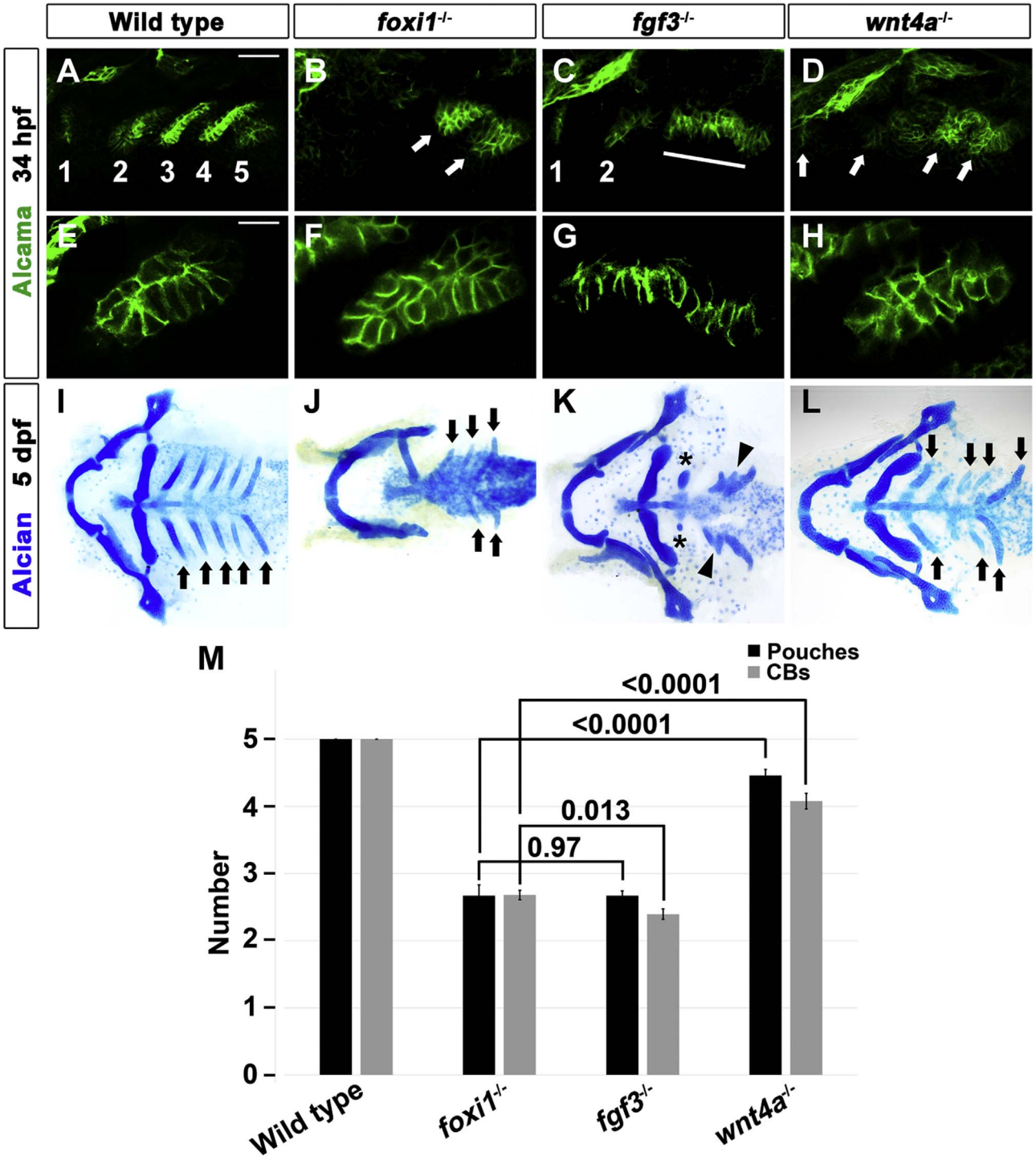

Fig. 1. Requirement for Foxi1 in the late-stage of pouch development. (A-H) Alcama immunohistochemistry (green) showed five pouches (1–5 in A) in wild-type embryos at 34 hpf. *foxi1* mutants had posterior pouches shaped abnormally (arrows in B), whereas *fgf3* mutants had two anterior pouches (1 and 2 in C) with a cell mass that failed to migrate out to form posterior pouches (line in C). *wnt4a* mutants displayed four abnormally shaped pouches (arrows in D). Scale bar: 40 μm (A-D). (E) Alcama immunohistochemistry (green) showed a normal bilayered pouch morphology in wild-type embryos. (F and H) In *foxi1* and *wnt4a* mutants, an aberrant, similar multilayered pouch morphology was found. (G) In *fgf3* mutants, posterior pouch-forming cells failed to migrate out. Scale bar: 20 μm (E-H). (I-L) Ventral views of facial cartilages. In wild-type embryos, a bilateral set of five CBs (arrows in I) formed. Fewer CBs were observed in *foxi1* and *wnt4a* mutants, whereas fusions of CBs (arrowheads in K) were observed with reduced anterior CBs (asterisks in K) in *fgf3* mutants. (J, L) Normally shaped CBs were marked with arrows in *foxi1* and *wnt4a* mutants. (M) Quantification of pouch and CB defects in wild-type embryos and mutants. Data represent mean ± s.e.m. *P* values are shown.

However, the mesodermal *fgf8a* expression during pouch formation was fairly normal in *foxi1* mutants, indicating that Fgf8a acts upstream of ectodermal Foxi1 (Fig. 2H, I). Taken together with the finding that Tbx1 positively regulates Fgf8a in the mesoderm (Choe and Crump, 2014), a mesodermal Tbx1-Fgf8a pathway, therefore, acts to repress ectodermal *foxi1* expression. Since ectodermal *wnt4a* expression was regulated positively by *foxi1*, we also examined if this was also the case in *fgf8a* mutants where *foxi1* expression was expanded. Indeed, the distinct *wnt4a* expression domains that were observed in wild-type ectoderm were upregulated and expanded ectopically throughout the facial ectoderm in *fgf8a* mutants (Fig. 3M, N). Because there is down regulation of ectodermal *wnt4a* expression in *foxi1* mutants, the

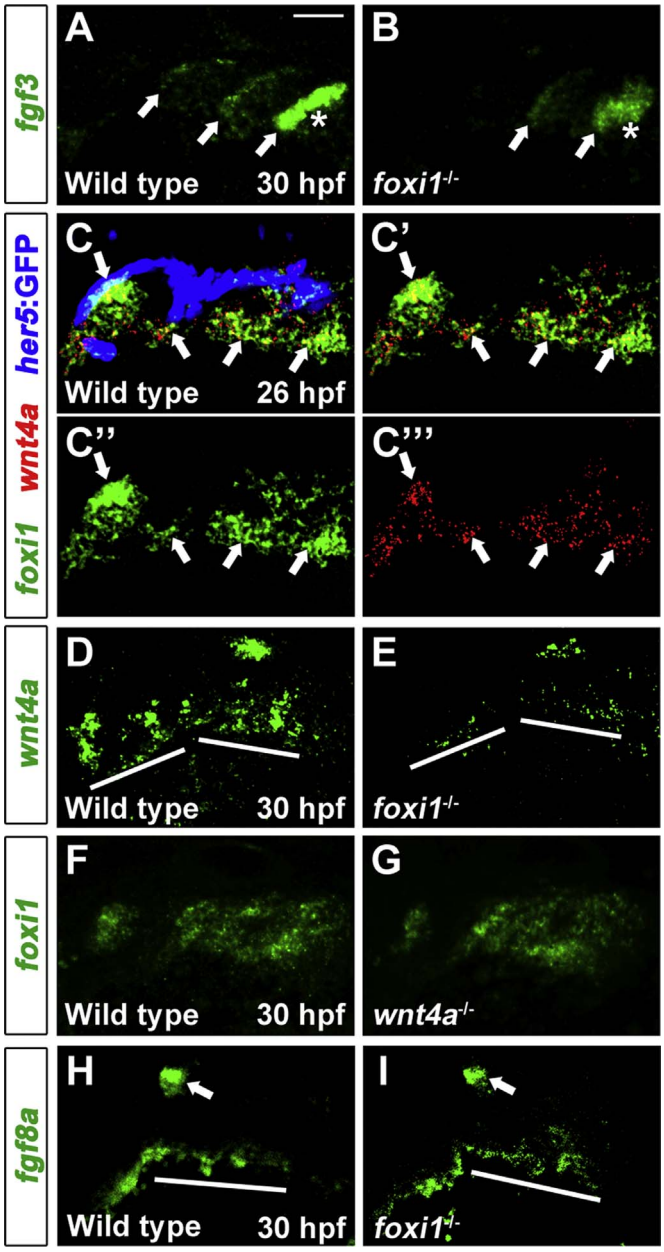

Fig. 2. Foxi1 requirement in the ectodermal Wnt4a expression during pouch formation. (A, B) Fluorescent in situ hybridization showed *fgf3* expression (green) in the posteriormost three pouches (arrows in A), with increased expression in the newly forming pouch (asterisk in A) in wild-type embryos. While *fgf3* expression was observed in the posteriormost two pouches (arrows in B), the intensity in the newly forming pouch (asterisk in B) was weaker than that of wild-type embryos (compare to asterisk in A). (C) Double fluorescent in situ hybridization showed colocalization of *wnt4a* (red) with *foxi1* (green, arrows in C) but not with *her5*-positive pouch endoderm labeled by GFP immunohistochemistry (blue). (C') Green and red channels. (C") Green channel only. (C‴) Red channel only. (D, E) Fluorescent in situ hybridization for *wnt4a* (green). In the facial ectoderm, *foxi1* mutants had significantly reduced *wnt4a* staining, compared with wild-type embryos (lines in D and E). (F, G) Fluorescent in situ hybridization for *foxi1* (green). Similar *foxi1* expression in the pharyngeal regions was observed in *wnt4a* mutants (G), compared with wild-type embryos (F). (H, I) Fluorescent in situ hybridization for *fgf8a* (green). (H) In wild-type embryos, *fgf8a* was expressed in mesoderm (line) adjacent to outgrowing pouches as well as in the otic vesicle (arrow). (I) In *foxi1* mutants, *fgf8a* expression was observed in the mesoderm (line) as well as in the otic vesicle (arrow), even though *fgf8a* expressing mesoderm was disorganized compared to wild-type mesoderm. Scale bar: 40 μm.

coincident expansion of *foxi1* and *wnt4a* expression in the ectoderm of *fgf8a* mutants strongly suggests that ectodermal Foxi1 regulates ectodermal Wnt4a expression positively. Thus, we propose that ectodermal Foxi1 expression is restricted by Fgf8a and triggers remodeling of pouch-forming cells into the bilayered pouches through the function of ectodermal Wnt4a.

3. Discussion

In this study, we identified a requirement for ectodermal Foxi1 during late-stage pharyngeal pouch morphogenesis in zebrafish. Foxi1 expression in distinct regions of the facial ectoderm is established, in part, through repression by a mesodermal Tbx1-Fgf8a pathway (Fig. 4). Foxi1 activates segmental Wnt4a expression in the ectoderm, which signals pouch-forming cells to drive cellular rearrangements into bilayered pouches (Fig. 4).

During craniofacial development, *foxi1* is required for endodermal pouch and ectodermal otic vesicle development consistent with its expression in the pharyngeal endoderm and facial ectoderm (Nissen et al., 2003; Solomon et al., 2003). The roles of Foxi1 in the development of pouches and the otic vesicle have been studied tissue-autonomously. Thus, it was proposed that endodermal Foxi1 controls pouch development through endodermal Fgf3 (Nissen et al., 2003). Yet, even though we could confirm the presence of Foxi1-dependent Fgf3 signaling in pharyngeal endoderm, we could not find evidence for its role in late-stage pouch morphogenesis specifically. On the contrary, our analysis implies that Fgf3 controls early-stage pouch morphogenesis, precluding us from studying the late stage. We also noted that endodermal Foxi1 is positively regulated by Tbx1, yet we have previously shown that endodermal Tbx1, specifically, is not crucial for the development of pouches (Choe and Crump, 2014). These findings indicate that the functions of Fgf3 and Tbx1 at specific points in pouch development require further experimentation.

We propose that the ectodermal rather than endodermal Foxi1 controls late-stage pouch morphogenesis and this role is dependent upon ectodermal Wnt4a. While ectodermal Foxi1 has been proposed to control otic vesicle development (Nissen et al., 2003), its role for endodermal pouch development remained unclear. The findings that Foxi1 activates Wnt4a, both are enhanced in *fgf8a* mutants, and the similarities between *wnt4a* and *foxi1* mutant pouches and CB cartilages support our model that ectodermal Foxi1 promotes late-stage pouch morphogenesis through Wnt4a (Fig. 4). Moreover, our discovery that Fgf8a is a negative regulator of ectodermal Foxi1 expression suggests that early-stage and late-stage pouch morphogenesis is precisely regulated by Fgf8a, especially in light of our previous findings that Tbx1 and Fgf8a guide the migration of pouch-forming cells toward the ectoderm (Choe and Crump, 2014). Fgf8a repression of Foxi1 and Wnt4a may serve to prevent unnecessary, early stabilization of pouch epithelia that could inhibit cell migration (Fig. 4).

The gene regulatory network that is responsible for activating Foxi1 in the ectoderm remains to be determined. Recently, it was reported that Pax1 is required to activate Tbx1 and Fgf3 for pouch development in Medaka (Okada et al., 2016). Tbx1 and Fgf3 play essential roles in pouch morphogenesis (Herzog et al., 2004; Piotrowski et al., 2003; Piotrowski and Nusslein-Volhard, 2000), and are key upstream regulators of gene regulatory networks implicated in pouch development in zebrafish (Choe and Crump, 2014; unpublished data). Since *pax1* is expressed in all tissues in the head during craniofacial development in zebrafish (Qiu et al., 2016), it will be interesting to examine if Pax1 is required to activate Fgf3, Tbx1, and Foxi1 in the pharyngeal endoderm, mesoderm, and facial ectoderm, respectively, and serves as the master regulator of a gene regulatory network controlling pouch morphogenesis.

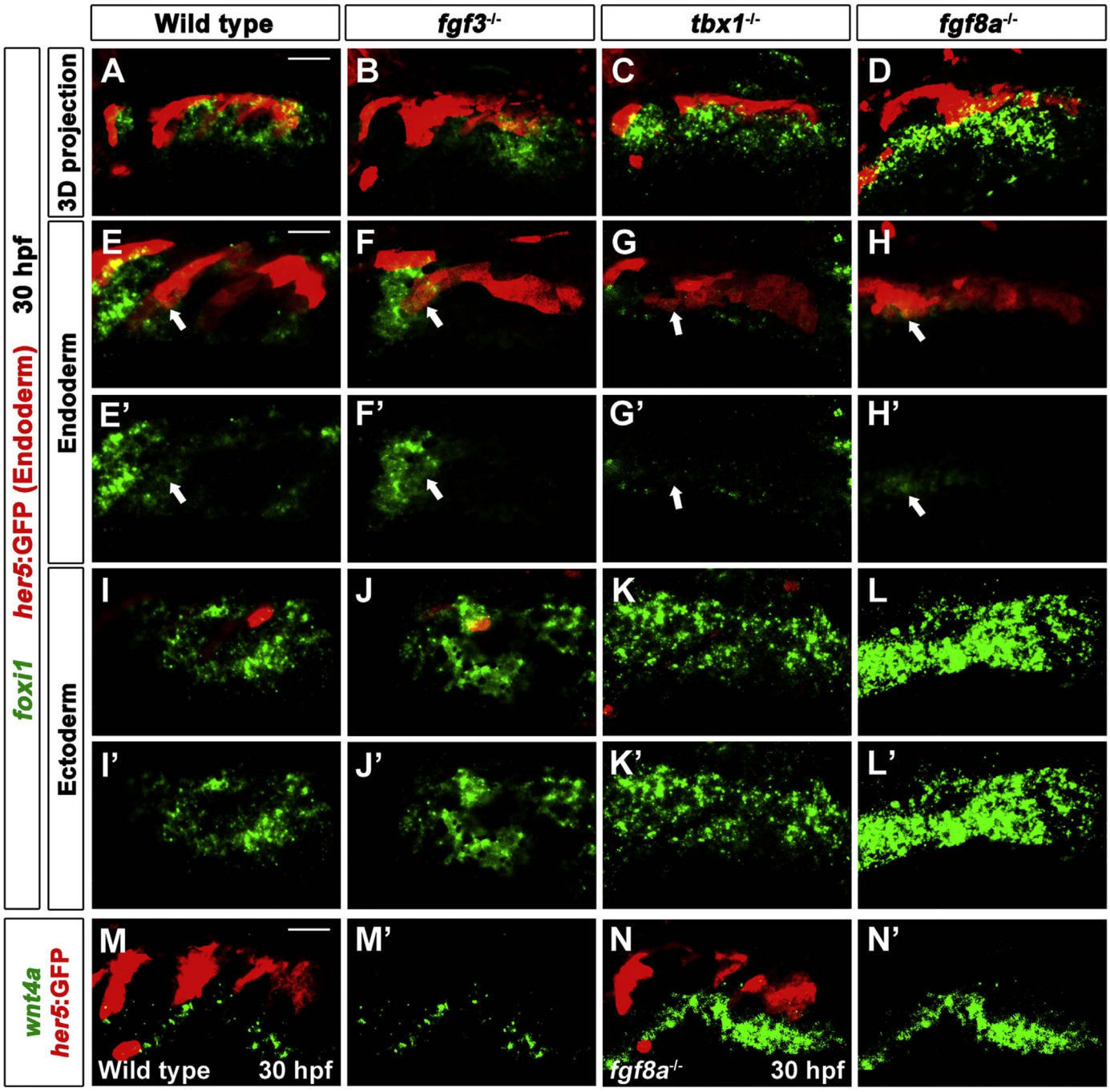

Fig. 3. Requirements for Tbx1 and Fgf8a in repression of the ectodermal Foxi1 and Wnt4a. (A-L) Fluorescent in situ hybridization for *foxi1* (green) and GFP immunohistochemistry to detect *her5*:GFP-positive endoderm (red) at 30 hpf. (A, B) In wild-type embryos and *fgf3* mutants, *foxi1* expression was observed in *her5*:GFP-positive endoderm (arrows in E and F) as well as in facial ectoderm (I and J). (C) In *tbx1* mutants, *foxi1* expression was significantly decreased in the *her5*:GFP-positive endoderm (arrow in G), whereas it was expanded ectopically in the facial ectoderm (K). (D) In *fgf8a* mutants, *foxi1* expression was detected in the *her5*:GFP-positive endoderm (arrow in H) and *foxi1* expression in the facial ectoderm was expanded ectopically and increased in intensity (also see L). (E-G) Higher magnification image of wild- type embryos and mutants focusing on the *her5*:GFP-positive endoderm. (I-L) Higher magnification image of wild-type embryos and mutants focusing on the facial ectoderm. (M, N) Fluorescent in situ hybridization for *wnt4a* (green) and GFP immunohistochemistry to visualize *her5*:GFP-positive pouches (red) at 30 hpf. *wnt4a* expression was observed in ectodermal patches in wild-type embryos (M), whereas it became stronger and was expanded ectopically in the facial ectoderm of *fgf8a* mutants (N). (E′-N′) Green channel only. Scale bars: 40 μm (A-D, M, and N), 20 μm (E-L).

4. Materials and methods

4.1. Zebrafish lines

All animal work was approved by Gyeongsang National University and the University of Southern California Institutional Animal Care and Use Committee (protocol #11995). Published lines include *foxi1*^{em1} (*hearsay*) (Solomon et al., 2003), *tbx1*^{tu285} (*van gogh*) (Piotrowski et al., 2003), *fgf3*^{t24152} (*lim-absent*) (Herzog et al., 2004), *fgf8a*^{ti282a} (*acerebellar*) (Reifers et al., 1998), *wnt4a*^{fh295} (Choe et al., 2013), and *Tg(~3.4her5: EGFP)*^{ne1911} (Tallafuss and Bally-Cuif, 2003). Genotyping of *foxi1*^{em1}, *tbx1*^{tu285}, *fgf3*^{t24152}, and *wnt4a*^{fh295} was as described previously (Choe et al., 2013; Choe and Crump, 2014; Herzog et al., 2004; Solomon et al., 2003). *fgf8a*^{ti282a} mutant embryos were scored by loss of cerebellum (Reifers et al., 1998).

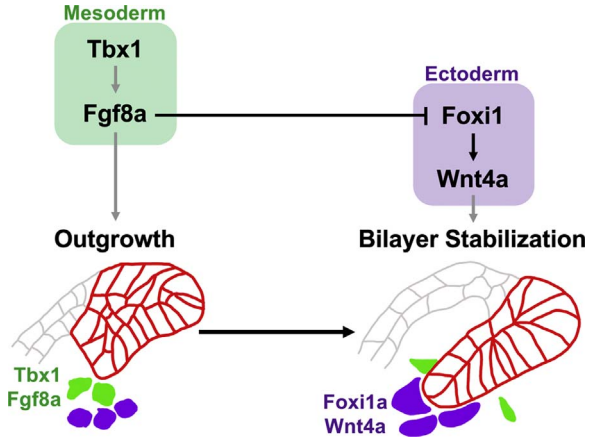

Fig. 4. A model of Foxi1 function in the late-stage of pouch morphogenesis. Ectodermal Foxi1 and Wnt4a (violet square) function downstream of mesodermal Tbx1 and Fgf8a (green square) to mediate the rearrangement and stabilization of pouch-forming cells into bilayered, mature pouch epithelium during late-stage pouch morphogenesis. Mesodermal cells expressing Tbx1 and Fgf8a (green cells) guide pouch outgrowth while repressing Foxi1 and Wnt4a expression in the ectoderm (violet cells). As pouch-forming cells (red outlines) migrate toward ectoderm, Foxi1 is derepressed in the ectoderm and activates Wnt4a expression, which signals pouch-forming cells to rearrange into a bilayered mature pouch.

4.2. Staining

Fluorescent in situ hybridizations, immunohistochemistry for Alcama/ZN8 (Zebrafish International Resource Center, 1:400), and Alcian Blue staining were performed as described previously (Crump et al., 2004; Zuniga et al., 2011). The in situ probes were generated as previously described *fgf3* (Choe and Crump, 2014), *fgf8a* (Choe and Crump, 2014), and *wnt4a* (Choe et al., 2013). The *foxi1* probe was generated by using PCR (5′-GAGCACCAACCCTTACCT-3′ and 5′-ATTTCACAGACATCGCGT-3′) and cloned into the pGEM®-T Easy Vector (Promega), and digoxigenin-labeled RNAs were synthesized using T7 RNA polymerase (Sigma-Aldrich).

4.3. Imaging, scoring, and statistics

Fluorescent images of Alcama-stained or in situ hybridized embryos were acquired on a Zeiss LSM5 confocal microscope using ZEN software. Images of dissected craniofacial cartilages were taken on a Leica DM2500 upright microscope using Leica software. To quantify pouch defects in embryos, normal pouches were scored as 1, (greater than 50%) reduced or misshapen pouches as 0.5, and a completely absent pouches as 0. Wild-type embryos invariably had five pouches per side at 34 hpf. To quantify skeletal cartilages, we scored fusions of greater than one CBs as 1.5, normal CBs as 1.0, reduced CBs as 0.5 and absent CBs as 0. Wild-type embryos also invariably had five CBs per side at 5 dpf. Comparisons between the means were made by ANOVA followed by the Tukey-Kramer multiple comparison test. See Supplementary material Table S1 for numbers of animals employed.

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Competing interests

The authors declare that they have no conflict of interest.

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Author contributions

C.P.C. designed the experiments. S.J., J.O, and C.P.C. performed the experiments. S.J., F.S., and C.P.C. interpreted results and wrote the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2018.06.011.

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