Developmental Biology 424 (2017) 189–197

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Original research article

Chordin and *dickkopf-1b* are essential for the formation of head structures through activation of the FGF signaling pathway in zebrafish

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ARTICLE INFO

Keywords:
chordin
dickkopf-1b
FGF signaling
Head induction
The Spemann organizer

ABSTRACT

The ability of the Spemann organizer to induce dorsal axis formation is dependent on downstream factors of the maternal Wnt/β-catenin signaling pathway. The fibroblast growth factor (FGF) signaling pathway has been identified as one of the downstream components of the maternal Wnt/β-catenin signaling pathway. The ability of the FGF signaling pathway to induce the formation of a dorsal axis with a complete head structure requires *chordin* (*chd*) expression; however, the molecular mechanisms involved in this developmental process, due to activation of FGF signaling, remain unclear. In this study, we showed that activation of the FGF signaling pathway induced the formation of complete head structures through the expression of *chd* and *dickkopf-1b* (*dkk1b*). Using the organizer-deficient maternal mutant, *ichabod*, we identified *dkk1b* as a novel downstream factor in the FGF signaling pathway. We also demonstrate that *dkk1b* expression is necessary, after activation of the FGF signaling pathway, to induce neuroectoderm patterning along the anteroposterior (AP) axis and for formation of complete head structures. Co-injection of *chd* and *dkk1b* mRNA resulted in the formation of a dorsal axis with a complete head structure in *ichabod* embryos, confirming the role of these factors in this developmental process. Unexpectedly, we found that *chd* induced *dkk1b* expression in *ichabod* embryos at the shield stage. However, *chd* failed to maintain *dkk1b* expression levels in cells of the shield and, subsequently, in the cells of the prechordal plate after mid-gastrula stage. In contrast, activation of the FGF signaling pathway maintained the *dkk1b* expression from the beginning of gastrulation to early somitogenesis. In conclusion, activation of the FGF signaling pathway induces the formation of a dorsal axis with a complete head structure through the expression of *chd* and subsequent maintenance of *dkk1b* expression levels.

1. Introduction

The Spemann organizer plays a crucial role in the development of the vertebrate dorsal axis. In *Xenopus*, the dorsal blastopore lip acts as the organizer, and induces the formation of a secondary dorsal axis with a complete head structure when the lip is transplanted in the ventral side of an embryo (reviewed in De Robertis and Kuroda (2004)). In zebrafish, the embryonic shield is a tissue equivalent to the *Xenopus* embryo dorsal blastopore lip. Transplantation of the embryonic shield into the ventral side of a zebrafish embryo also results in the formation of a secondary dorsal axis with a complete head structure (Saude et al., 2000).

The maternal Wnt/β-catenin signaling pathway is required for the formation of the organizer; it also has the ability to induce dorsal axis formation. In fact, ectopic activation of the maternal Wnt/β-catenin signaling pathway induces the formation of a secondary dorsal axis with a complete head structure in both zebrafish (Kelly et al., 1995) and *Xenopus* (Funayama et al., 1995). On the other hand, genetic ablation of the dorsal organizer, due to maternal Wnt/β-catenin signaling deficiencies, results in failure to form a dorsal axis in zebrafish (Kelly et al., 2000). The maternal Wnt/β-catenin signaling pathway promotes β-catenin stabilization and subsequent nuclear localization to the dorsal side of zebrafish and *Xenopus* embryos (Schneider et al., 1996).

The fibroblast growth factor (FGF) signaling pathway has been identified as a contributing factor in dorsal axis formation mediated by the maternal Wnt/β-catenin signaling pathway. Zebrafish and *Xenopus* embryos injected with mRNA encoding XFD, the dominant negative FGF receptor, showed severe defects in the trunk and tail structures (Amaya et al., 1991; Griffin et al., 1995), and injection of FGF8 RNA into zebrafish embryos induced the formation of a partial secondary dorsal axis with an incomplete head structure (Fürthauer et al., 1997). These results suggest a role for the FGF signaling pathway in organizer functions.

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http://dx.doi.org/10.1016/j.ydbio.2017.02.018
Received 25 July 2016; Received in revised form 16 February 2017; Accepted 28 February 2017
Available online 01 March 2017

Additionally, the *chordin* (*chd*) gene has been investigated as one of the components involved in dorsal axis formation. Gene expression analyses of the zebrafish *chd* mutant, *chordino*, reveal that *chd* is required for neuroectoderm and anterior notochord cell differentiation (Hammerschmidt et al., 1996). *Xenopus* organizer grafts treated with morpholino oligonucleotides (MOs) against *chd* failed to induce the formation of a secondary dorsal axis when transplanted in the ventral side of *Xenopus* embryos, suggesting that the ability of the organizer to induce the formation of a secondary axis is dependent on *chd* expression (Oelgeschlager et al., 2003). Moreover, ectopic *chd* expression induces the formation of a partial secondary dorsal axis in zebrafish, supporting the role of *chd* in organizer functions (Fox and Bruce, 2009). Secretion of Chd from the organizer acts as an antagonist for the bone morphogenetic protein (BMP), thus regulating its activity in *Xenopus* (Piccolo et al., 1996). BMP, acting as a morphogen, provides different dorsoventral cell-specific fates depending on BMP activity levels (Hashiguchi and Mullins, 2013; Hawley et al., 1995). Chd maintains low BMP activity levels in the dorsal side, inducing the formation of dorsal tissues, such as neuroectoderm (Hashiguchi and Mullins, 2013; Tuazon and Mullins, 2015; Varga et al., 2007).

We have used the *ichabod* embryo, which is deficient in the maternal Wnt/β-catenin signaling, to investigate the epistatic relationship between various downstream factors, such as FGF and *chd*, of the maternal Wnt/β-catenin signaling pathway (Maegawa et al., 2006). The previous studies demonstrated that the maternal Wnt/β-catenin signaling pathway activates the two zygotic genes, *bozozok* (*boz*), encoding a homeodomain protein, and *squint* (*sqt*), encoding a nodal-related protein (Kelly et al., 2000; Maegawa et al., 2006), to induce a dorsal axis in zebrafish. Moreover, activation of the FGF signaling pathway downstream of *boz* and *sqt* is required for β-catenin-mediated induction of a dorsal axis, and that activation of the FGF signaling pathway in *ichabod* embryos induces the formation of a dorsal axis with a complete head structure (Maegawa et al., 2006). In addition, activation of the FGF signaling pathway induces *chd* expression, a factor necessary for FGF-mediated dorsal axis formation (Maegawa et al., 2006). We concluded that β-catenin-mediated induction of a dorsal axis acts through a pathway consisting of β-catenin→Squint/Bozozok→FGF→Chordin. However, it remains unknown which downstream factors of the FGF signaling pathway, in addition to *chd,* have a role in the formation of a dorsal axis with a complete head structure.

We focused on the *dickkopf-1* (*dkk1*) gene as a novel downstream factor of the FGF signaling pathway. Dkk1 proteins function as a head inducer during dorsal axis development (Glinka et al., 1998) by antagonizing zygotic Wnt signaling through interaction with the Wnt coreceptor, LRP5/6, during gastrulation (Bafico et al., 2001). Activation of the zygotic Wnt signaling pathway triggers a cell differentiation pattern along the anteroposterior (AP) axis in a concentration-dependent manner (Itoh and Sokol, 1997; Kiecker and Niehrs, 2001). Overexpression of *dkk1* in *Xenopus* embryos promotes anterior neural cell fates instead of posterior neural cell fates, a process determined by zygotic Wnt signaling (Glinka et al., 1998; Kazanskaya et al., 2000). *Dkk1b*, the zebrafish homolog of the *Xenopus dkk1* gene, is expressed in the axial mesoderm during gastrulation and promotes anterior cell fates (Hashimoto et al., 2000; Shinya et al., 2000). Overexpression of *dkk1b* leads to the formation of an enlarged head instead of the trunk and tail structures, whereas *dkk1b* morphants show a headless phenotype (Caneparo et al., 2007; Seiliez et al., 2006; Shinya et al., 2000). These studies led us to hypothesize that FGF-mediated head induction in *ichabod* embryos would involve *dkk1b*.

We here report that activation of the FGF signaling pathway induces *dkk1b*, as well as *chd* expression, resulting in the formation of complete head structures. We first examined whether injection of *chd* mRNA was able to induce the formation of a dorsal axis with a complete head structure in *ichabod* embryos. As expected, injection of *chd* mRNA induced the formation of a dorsal axis in *ichabod* embryos, but only with a partial head structure. Next, we identified *dkk1b* as a novel downstream factor of the FGF signaling pathway in *ichabod* embryos. FGF-induced *dkk1b* expression resulted in the formation of a complete head structure and proper neuroectoderm patterning along the whole AP axis in *ichabod* embryos. Furthermore, co-injection of *chd* and *dkk1b* mRNA also induced the formation of a dorsal axis with a complete head structure in *ichabod* embryos. Unexpectedly, we found that injection of *chd* mRNA, alone, induced the expression of *dkk1b* in *ichabod* embryos at the shield stage, but failed to maintain *dkk1b* expression levels in cells of the shield and, subsequently, in the prechordal plate after mid-gastrula stage. In contrast, activation of the FGF signaling pathway was able to maintain *dkk1b* expression levels from the beginning of gastrulation to early somitogenesis. We conclude that activation of the FGF signaling pathway induces the formation of a dorsal axis with a complete head structure through expression of *chd* and subsequent maintenance of *dkk1b* expression.

2. Material and methods

2.1. Zebrafish

Zebrafish were maintained under the conditions described in (Aoyama et al., 2015). We obtained homozygous *ichabod* female and male fish by injecting β-catenin mRNA into *ichabod* embryos to rescue the ventralized phenotype. *Ichabod* embryos in the present study were obtained by breeding *ichabod* homozygous females with *ichabod* heterozygous or homozygous males. The clutches of *ichabod* embryos that predominantly developed the most ventralzed phenotype class (class 1: Fig. 2A, (Kelly et al., 2000)) were used in this study. AB embryos were used as a wild-type strain and were obtained by breeding AB strain males and females.

2.2. mRNA synthesis and morpholino antisense oligonucleotides

Capped mRNAs were synthesized using the mMESSAGE mMACHINE kit (Thermo Fisher Scientific, Waltham, USA) following the manufacturer's protocol. Morpholino antisense oligonucleotides were purchased from Gene Tools, LLC (Philomath, USA): to target the *chd* gene (MO*chd*): 5′-ATCCACAGCAGCCCCTCCATCATCC-3′ (Nasevicius and Ekker, 2000); to target the *dkk1b* gene (MO*dkk1b*): 5′-TAGAGAGCATGGCGATGTGCATCAT-3′ (Caneparo et al., 2007)). Both MO solutions contained phenol red to visualize injected solution in embryos.

2.3. Injection of mRNA and phenotypic observation of zebrafish embryos

We used the following mRNA concentrations for our study: *chd* (5 ng/μl), *dkk1b* (2.5 ng/μl), *chd* + *dkk1b* (5 ng/μl *chd*, 2.5 ng/μl *dkk1b*), *β-catenin-1* (50 ng/μl), *fgf8* (0.1 ng/μl), XFD (200 ng/μl), and d50 (200 ng/μl). The concentration of each MO solution was as follows: MO*chd* (1 mg/ml) and MO*dkk1b* (1 mg/ml). Approximately 1 nl of the mRNA or MO solutions was injected in *ichabod* embryos. Each mRNA solution was injected in a single cell at a 4- or 8-cell stage. Each MO solution was injected at 1-cell stage. The phenotypes of the injected embryos were observed at 24 h post fertilization (hpf). The phenotypes of *ichabod* embryos were classified according to previous studies (Kelly et al., 2000; Maegawa et al., 2006). Images of the embryos were captured by a SZX16 stereo microscope (Olympus, Tokyo, Japan) coupled to a CCD digital camera MicroPublisher 5.0 (QImaging, Surrey, Canada).

2.4. Whole mount in situ hybridization

Each RNA probe was synthesized to detect *chd*, *dkk1b*, *otx2*, or *hoxb1b* endogenous mRNA following previously established protocols (Hashimoto et al., 2000; Maegawa et al., 2006). Whole mount *in situ*

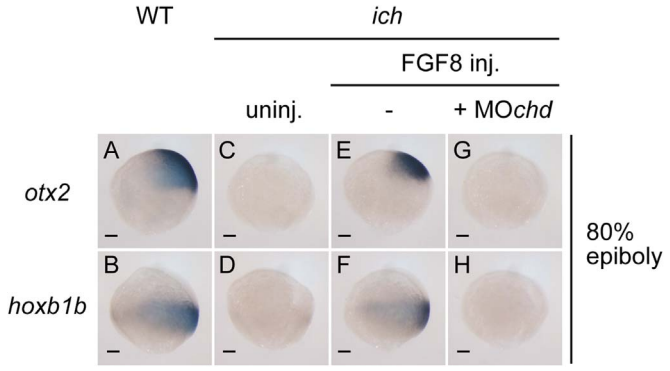

Fig. 1. *Chd* acts downstream of the FGF signaling pathway to induce the anterior and posterior neuroectoderm. Each image represents the expression pattern of *otx2* or *hoxb1b* in (A and B) wild-type embryos, (C and D) uninjected *ichabod* embryos, (E and F) FGF8 mRNA-injected *ichabod* embryos, and (G and H) FGF8 mRNA and MO*dkk1b*-injected *ichabod* embryos. All embryos are shown at 80% epiboly stage in lateral views. Scale bars represent 100 μm.

hybridization was performed following a protocol described by Maegawa et al. (2006). Images of embryos stained by *in situ* hybridization were captured by a SZX16 stereo microscope (Olympus, Tokyo, Japan) coupled to a CCD digital camera MicroPublisher 5.0 (QImaging, Surrey, Canada).

3. Results

3.1. *Overexpression of* chd *was insufficient to induce the formation of a dorsal axis with a complete head structure in* ichabod *embryos*

We previously reported that *chd* is required to induce dorsal axis formation through activation of the FGF signaling pathway (Maegawa et al., 2006). Additionally, we found that FGF induction of neuroectoderm patterned along DV and AP axes, was suppressed by inhibition of *chd* activity with MO*chd* (Fig. 1). These results suggest that *chd* plays a pivotal role in the FGF induction of the dorsal axis. Therefore, we hypothesized that overexpression of *chd* would be sufficient to induce the formation of a dorsal axis with a complete head structure in *ichabod* embryos. To examine this hypothesis, we injected *chd* mRNA into a single cell of *ichabod* embryos at a 4- or 8-cell stage (Fig. 2A–D). Uninjected *ichabod* embryos (control embryos) showed a severe ventralized phenotype (22/22; Fig. 2A). *Ichabod* embryos injected with FGF8 mRNA formed complete head structures (14/20; Fig. 2B). In contrast, *ichabod* embryos injected with *chd* mRNA showed abnormal forebrains and no eyes (14/20; Fig. 2C) and were classified with a class 3 phenotype. Injection of β-catenin mRNA completely rescued the *ichabod* embryo phenotype (18/22; Fig. 2D). Increasing the mRNA concentration of *chd* (10-fold increase) resulted in *ichabod* embryos with a dorsalized phenotype and incomplete head structures (data not shown). These results clearly show that overexpression of *chd* is insufficient for induction of a dorsal axis with a complete head structure, and suggest that other downstream factors of the FGF signaling pathway could be involved in complete head structure formation.

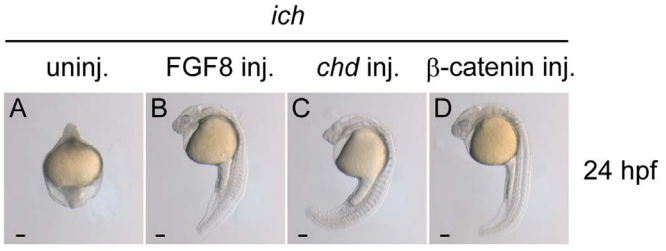

Fig. 2. *Chd* expression is not sufficient to induce the formation of complete head structures in *ichabod* embryos. (A–D) Phenotypic observation of *ichabod* embryos at 24 hpf. Embryos were classified using the phenotypic classification criteria reported by Kelly et al. (2000) and Maegawa et al. (2006): Class 1 embryos show a severe ventralized phenotype; embryos develop neither a head nor a trunk. Class 1a embryos develop a partial trunk and spinal cord but no head structures; class 2 embryos develop a spinal cord and hindbrain but lack head structures anterior to the midbrain; class 3 embryos develop a defective forebrain and no eyes; class 4 embryos develop complete head structures but no notochord; class 5 embryos have no apparent deficiencies and survive until they are adults. (A) Uninjected *ichabod* embryos showed a ventralized phenotype, and were classified as a class 1 phenotype (22/22). (B) Upon injection with FGF8 mRNA, *ichabod* embryos showed a class 4 phenotype (14/20). (C) The majority of the *ichabod* embryos that were injected with *chd* mRNA were classified as a class 3 phenotype (14/20). (D) *Ichabod* embryos injected with β-catenin mRNA showed a class 5 phenotype (18/22). Scale bar represents 100 μm.

3.2. Dkk1b *is a downstream factor of the FGF signaling pathway*

We focused on the zygotic Wnt signaling inhibitor, *dkk1b*, as a possible factor involved in the formation of complete head structures, as a consequence of activation of the FGF signaling pathway. The *dkk1b* gene codes for a protein that is expressed in the embryonic shield and that is involved in the formation of head structures in zebrafish (Caneparo et al., 2007; Seiliez et al., 2006; Shinya et al., 2000). However, it has not yet been reported whether *dkk1b* could be a downstream factor in the FGF signaling pathway, and if it has a role in the FGF-mediated head induction. Consequently, we examined if the expression of *dkk1b* was induced by injection of FGF8 mRNA in *ichabod* embryos (Fig. 3A–D). In wild-type embryos, *dkk1b* is expressed in cells of the embryonic shield (Fig. 3A) (Hashimoto et al., 2000). As expected, *ichabod* embryos did not express *dkk1b* (Fig. 3B). Injection of FGF8 mRNA (Fig. 3C), as well as injection of β-catenin mRNA (Fig. 3D), restored the expression of *dkk1b* at the shield stage in *ichabod* embryos. These results demonstrate that activation of the FGF signaling pathway induces *dkk1b* expression at the shield stage.

3.3. Dkk1b *acts downstream of FGF signaling to induce the formation of complete head structures and proper neuroectoderm patterning along the AP axis*

To determine the role of *dkk1b* in formation of the head, *ichabod* embryos were injected with FGF8 mRNA, and *dkk1b* was knocked-down using MO*dkk1b* (Fig. 4A–C). Injection of FGF8 mRNA rescued *ichabod* embryos, which were able to form complete head structures (35/52; Fig. 4B). When *dkk1b* activity was inhibited with MO*dkk1b*, most of the FGF8 mRNA-injected embryos lacked anterior forebrains and eyes (38/43; Fig. 4C), consistent with results obtained in wild-type embryos (Caneparo et al., 2007). These results demonstrate that Dkk1b has a role in the formation of complete head structures, acting as a downstream factor in response to FGF signaling.

Next, we examined whether *dkk1b* inhibition, after activation of the FGF signaling pathway, affected neuroectoderm patterning along the AP axis in *ichabod* embryos (Fig. 4D–K). We performed whole mount *in situ* hybridization experiments using RNA probes for the anterior neural marker, *otx2*, and the posterior neural marker, *hoxb1b*. In wild-type embryos at mid-gastrula stage, *otx2* expression was observed at

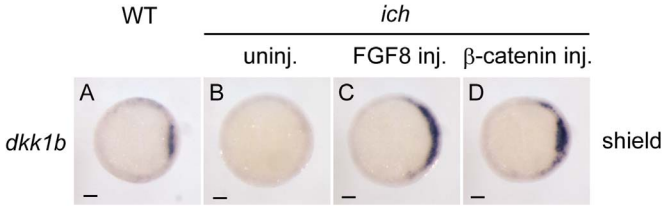

Fig. 3. *Dkk1b* is a downstream component of the FGF signaling pathway. Expression of *dkk1b* in (A) a wild-type embryo, (B) an uninjected *ichabod* embryo, (C) an FGF8 mRNA-injected *ichabod* embryo, and (D) a β-catenin mRNA-injected *ichabod* embryo. Embryos are at the shield stage, shown in animal pole views. Scale bar represents 100 μm.

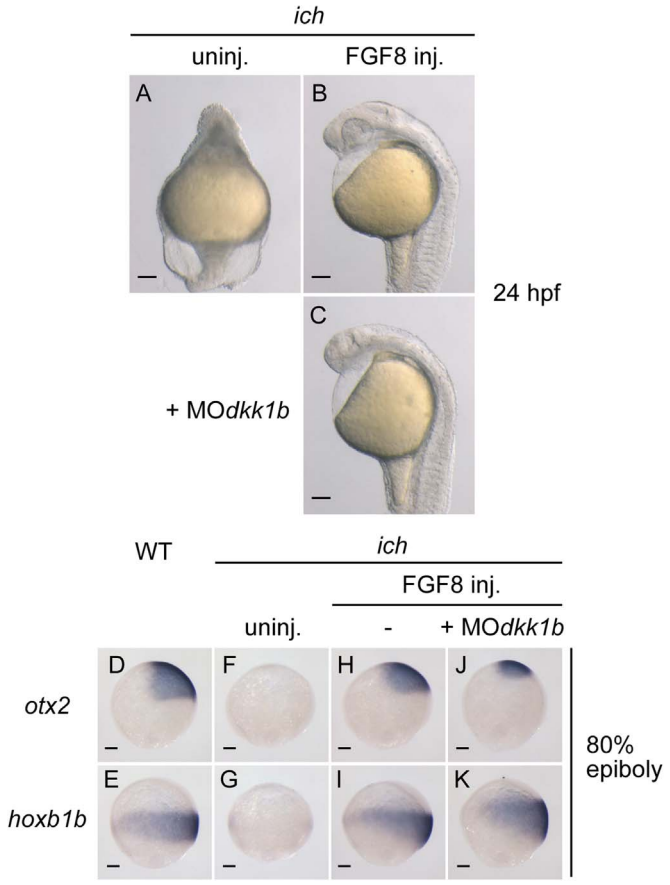

Fig. 4. *Dkk1b* acts downstream of the FGF signaling pathway to induce the formation of complete head structures and neuroectoderm patterning. Phenotypic representation of (A) uninjected (50/52), (B) FGF8 mRNA-injected (35/52), and (C) FGF8 mRNA and MO*dkk1b*-injected *ichabod* embryos at 24 hpf (38/43). Each image represents the expression pattern of *otx2* or *hoxb1b* in (D and E) wild-type embryos, (F and G) uninjected *ichabod* embryos, (H and I) FGF8 mRNA-injected *ichabod* embryos, and (J and K) FGF8 mRNA and MO*dkk1b*-injected *ichabod* embryos. (D–K) All embryos are shown at 80% epiboly stage in lateral views. Scale bars represent 100 μm.

the anterodorsal region (Fig. 4D); in contrast, *hoxb1b* expression was observed at the posterodorsal region (Fig. 4E). *Ichabod* embryos lacked *otx2* and *hoxb1b* expression (Fig. 4F and G). Injection of FGF8 mRNA partially restored *otx2* expression in *ichabod* embryos (Fig. 4H, compared with F), consistent with previous studies (Maegawa et al., 2006). Similarly, *hoxb1b* expression was also restored by injection of FGF8 mRNA (Fig. 4I, compared with G); and the FGF8-induced expression domain of *hoxb1b* was similar to wild-type embryos (Fig. 4I compared with E). The expression domain of *hoxb1b* appeared larger (Fig. 4K compared with I) when *dkk1b* activity was inhibited. In contrast, *otx2* expression was confined to a smaller region near the animal pole (Fig. 4J compared with H). These results demonstrate that *dkk1b* expression, via activation by FGF, is necessary for full anteriorization of the neuroectoderm in *ichabod* embryos, suggesting that the FGF signaling pathway induces neuroectoderm patterning along the AP axis through *dkk1b*.

3.4. *Synergistic activities of Chd and Dkk1b induce the formation of a dorsal axis with a complete head structure*

Both *chd* and *dkk1b* are required for the FGF-induced formation of a dorsal axis with a complete head structure. Accordingly, we examined whether co-injection of *chd* and *dkk1b* mRNA in *ichabod* embryos could induce the formation of a dorsal axis with a complete head structure (Fig. 5A–J). Uninjected *ichabod* embryos showed a severely ventralized phenotype (63/63; Fig. 5A). *Ichabod* embryos that were injected only with *dkk1b* mRNA still showed a ventralized phenotype, specifically, a class 1a phenotype (48/64; Fig. 5B and C). As already shown, injection of *chd* mRNA partially rescued *ichabod* embryos (55/64; Figs. 2D, 5D and E). Therefore, neither *chd* nor *dkk1b*, by themselves, can induce the formation of complete head structures in *ichabod* embryos. However, co-injection of both mRNAs induced the formation of a dorsal axis with a complete head structure (47/58; Fig. 5F and G). Injection of β-catenin mRNA was also able to rescue *ichabod* embryos (positive control) (48/62; Fig. 5H and I). The phenotypic distribution observed for each condition is shown in Fig. 5J. These results demonstrate that synergistic activities of *chd* and *dkk1b* are sufficient to induce the formation of complete head structures in *ichabod* embryos. Taken together, we conclude that *chd* and *dkk1b* are essential downstream factors of the FGF signaling pathway that work together to induce the formation of a dorsal axis with a complete head structure. Although β-catenin mRNA can fully rescue *ichabod* embryos, including formation of a notochord (class 5 embryos, Fig. 5J), *ichabod* embryos injected with *chd* plus *dkk1b* mRNAs fail to form notochord.

3.5. Dkk1b *is a downstream factor of* chd *induced by the FGF signaling pathway*

Next, we investigated the epistatic relationship between *chd* and *dkk1b*. We examined whether *chd* could induce *dkk1b* expression (and vice versa) in *ichabod* embryos (Fig. 6A–F). Injection of *chd* mRNA induced *dkk1b* expression, which was an unexpected finding (Fig. 6C). Conversely, injection of *dkk1b* mRNA failed to induce *chd* expression in *ichabod* embryos (Fig. 6F). These results demonstrate that *dkk1b* expression is a downstream response to *chd* expression.

Then, we examined whether *chd* inhibition had an effect on *dkk1b* expression (and vice versa) in *ichabod* embryos injected with FGF8 mRNA (Fig. 7A–H). *Dkk1b* expression was induced by an injection of FGF8 mRNA (Figs. 3C and 7C); however, inhibition of *chd* activity suppressed *dkk1b* expression at the shield stage (Fig. 7D). Conversely, inhibition of *dkk1b* activity did not suppress *chd* expression (Fig. 7H). These results demonstrate that FGF signaling requires *chd* activity for induction of *dkk1b* in *ichabod* embryos at the shield stage.

3.6. *Activation of the FGF signaling pathway maintains* dkk1b *expression levels until early somitogenesis, while* chd *fails to maintain* dkk1b *expression*

We showed that *dkk1b* is a downstream factor of *chd*. These findings suggest that an injection of *chd* mRNA should rescue *ichabod* embryos and induce the formation of complete head structures. However, our results clearly show that *chd* injection is not sufficient for formation of complete head structures in these embryos (Figs. 2 and 5). Therefore, we hypothesized that *chd* is insufficient to maintain *dkk1b* expression levels after the shield stage.

To clarify this hypothesis, we examined whether *chd*-induced *dkk1b* expression was maintained after the early-gastrulation period (Fig. 8A–O). Whole mount *in situ* hybridization analyses were performed at three different stages: 65% epiboly, 90% epiboly, and 5-somite stage. *Dkk1b* expression in wild-type embryos was found at both sides of the embryonic shield at the 65% epiboly stage (Fig. 8A), at the edge of the prechordal plate, including the polster at the 90% epiboly stage (Fig. 8B), and at the mid-hindbrain boundary at the 5-somite stage (Fig. 8C) (Hashimoto et al., 2000; Shinya et al., 2000). *Ichabod* embryos had undetectable *dkk1b* expression levels at 65% and 90% epiboly stages (Fig. 7D and E). Furthermore, at a stage equivalent to the 5-somite stage, *dkk1b* expression was extensively found around the vegetal pole (Fig. 8F). As expected, injection of FGF8 mRNA induced *dkk1b* expression at 65% epiboly, 90% epiboly, and 5-somite stages in *ichabod* embryos (Fig. 8G–I). Injection of *chd* mRNA induced low expression of *dkk1b* in the embryonic shield at 65% epiboly stage

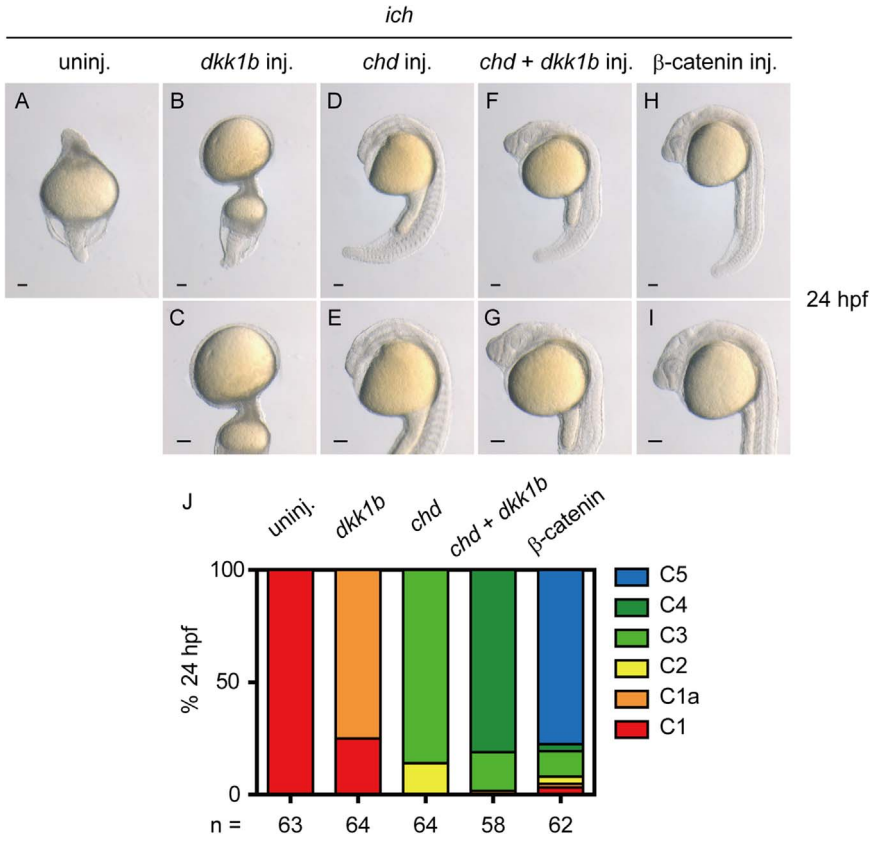

Fig. 5. Synergistic activities of *chd* and *dkk1b* mRNA induce the formation of a dorsal axis with a complete head structure in *ichabod* embryos. Phenotypic representation of (A) uninjected (63/63), (B and C) *dkk1b* mRNA-injected (48/64), (D and E) *chd* mRNA-injected (55/64), (F and G) *chd* and *dkk1b* mRNA-injected (47/58), and (H and I) β-catenin mRNA-injected *ichabod* embryos (48/62). (C, E, G, and I) Magnified images of the head region of B, D, F, and H. All embryos are shown at 24 hpf. Scale bars represent 100 μm. (J) Phenotypic classes obtained for each treatment.

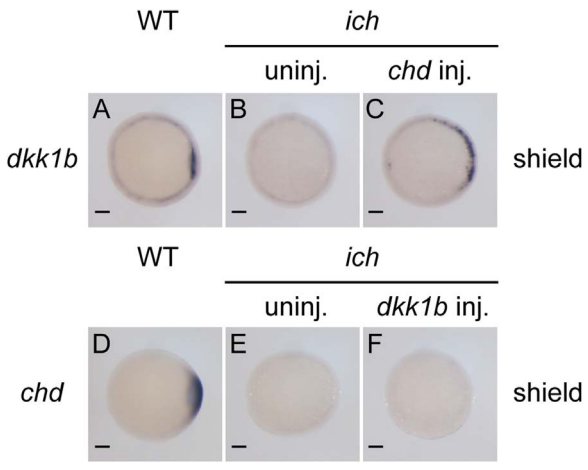

Fig. 6. *Dkk1b* is a downstream factor of *chd*. Expression of *dkk1b* in (A) wild-type embryos, (B) uninjected *ichabod* embryos, and (C) *chd* mRNA-injected *ichabod* embryos. Expression of *chd* in (D) wild-type embryos, (E) uninjected *ichabod* embryos, and (F) *dkk1b* mRNA-injected *ichabod* embryos. All embryos are shown at the shield stage in animal pole views. Scale bars represent 100 μm.

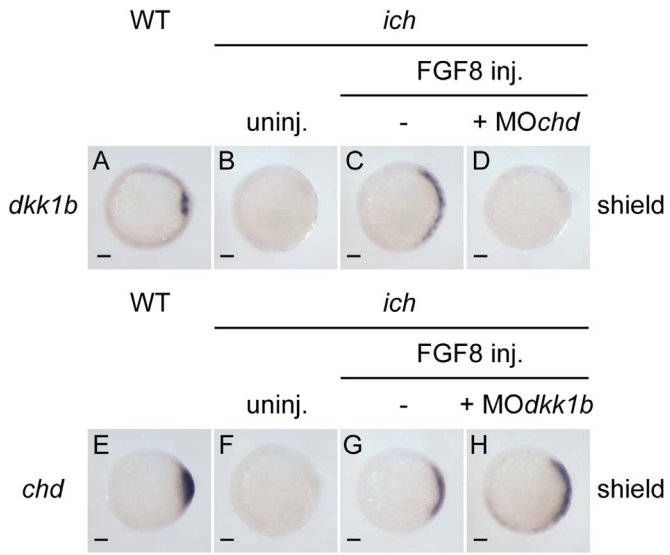

Fig. 7. FGF-induced *dkk1b* expression is dependent on *chd*. Expression of *dkk1b* in (A) wild-type embryos, (B) uninjected *ichabod* embryos, (C) FGF8 mRNA-injected *ichabod* embryos, and (D) FGF8 mRNA and MO*chd*-injected *ichabod* embryos. Expression of *chd* in (E) wild-type embryos, (F) uninjected *ichabod* embryos, (G) FGF8 mRNA-injected *ichabod* embryos, and (H) FGF8 mRNA and MO*dkk1b*-injected *ichabod* embryos. All embryos are shown at the shield stage in animal pole views. Scale bars represent 100 μm.

(Fig. 8J). However, *dkk1b* was no longer detected at 90% epiboly stage (Fig. 8K). Moreover, *chd* injected-*ichabod* embryos had abnormal *dkk1b* expression in the hindbrain region at the 5-somite stage (Fig. 8L). Injection of β-catenin mRNA induced *dkk1b* expression in *ichabod* embryos, serving as a positive control (Fig. 8M–O). These results demonstrate that *chd* fails to maintain *dkk1b* expression in cells of the shield and, subsequently, in the cells of the prechordal plate after mid-gastrula stage. In contrast, activation of the FGF signaling pathway is able to maintain the *dkk1b* expression from the onset of gastrulation to early somitogenesis.

We further examined the relationship between *chd* and *dkk1b* expression and FGF signaling by testing the effect of the dominant negative FGF receptor, XFD, on the expression of *dkk1b* in β-catenin mRNA-injected *ichabod* embryos (Fig. 8P–W). Whole mount *in situ*

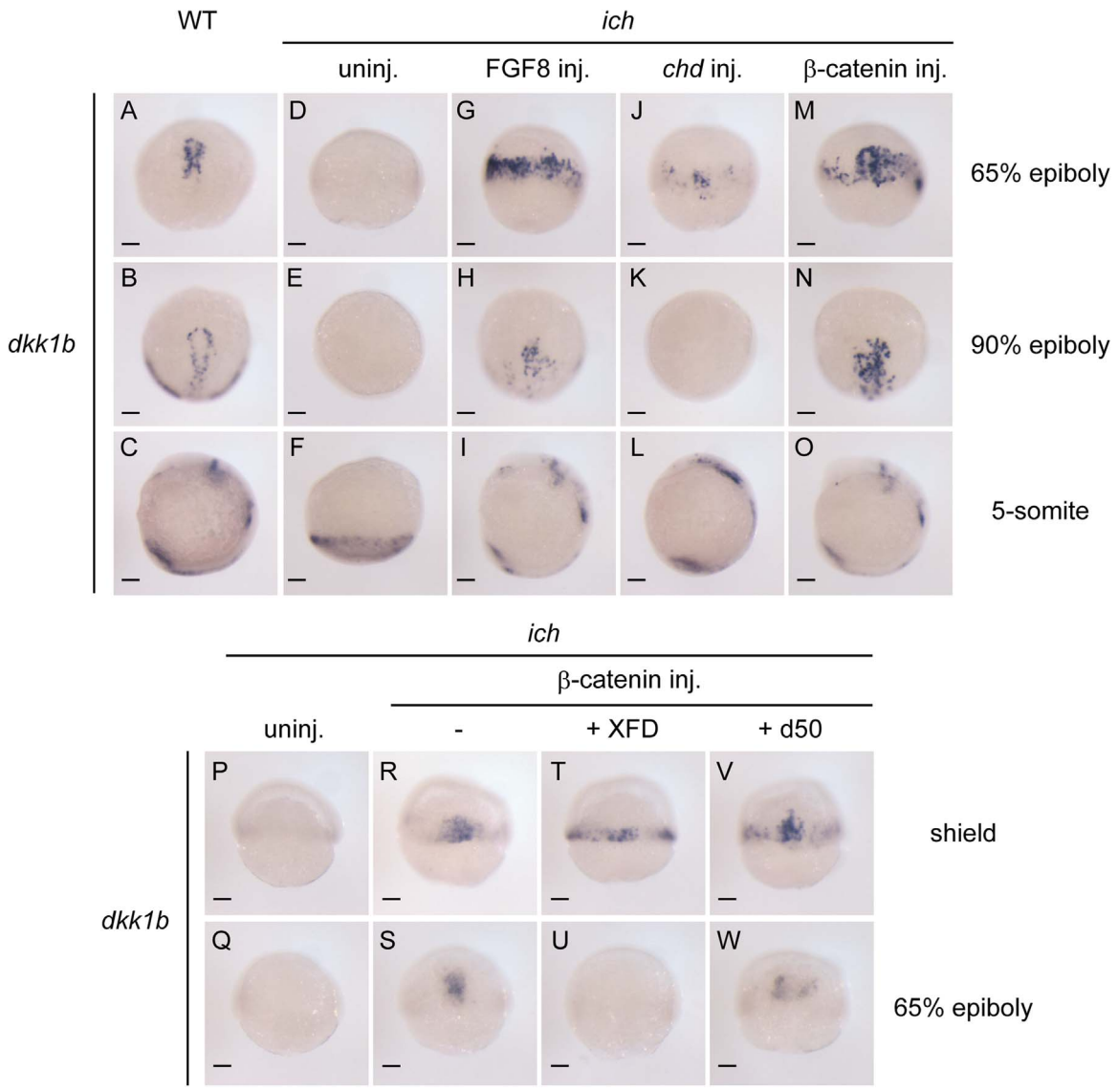

Fig. 8. Activation of the FGF signaling pathway maintains *dkk1b* expression levels from mid-gastrulation to early somitogenesis, whereas *chd* fails to maintain *dkk1b* expression levels after mid-gastrula stage. Expression of *dkk1b* in (A–C) wild-type embryos, (D–F, P and Q) uninjected *ichabod* embryos, (G–I) FGF8 mRNA-injected *ichabod* embryos, (J–L) *chd* mRNA-injected *ichabod* embryos, (M–O, R and S) β-catenin mRNA-injected *ichabod* embryos, (T and U) β-catenin and XFD mRNA-injected *ichabod* embryos, and (V and W) β-catenin and d50 mRNA-injected *ichabod* embryos. Embryos are shown at the shield stage (P, R, T and V, dorsal views), 65% epiboly (A, D, G, J, M, Q, S, U and W, dorsal views), 90% epiboly (B, E, H, K and N, animal views), and 5-somite stage (C, F, I, L and O, lateral views). Scale bars represent 100 μm.

hybridization analyses were performed at the shield stage and 65% epiboly stage. Injection of XFD mRNA did not affect β-catenin-mediated induction of *dkk1b* expression at the shield stage (Fig. 8T). In contrast to the lack of effect of XFD on *dkk1b* at the shield stage, this inhibitor of FGF signaling did obliterate *dkk1b* expression at 65% epiboly stage (Fig. 8U). The non-functional FGF receptor, d50, did not affect induction and maintenance of *dkk1b* expression at the shield and 65% epiboly stages (Fig. 8V and W). These results demonstrate that FGF signaling is required for maintenance, if not initial expression, of *dkk1b* expression induced by β-catenin.

4. Discussion

4.1. Activation of the FGF signaling pathway, through chd and dkk1b, induces neuroectoderm patterning along the DV and AP axes

It has been previously shown that localized activation of the FGF signaling pathway by the injection of FGF8 mRNA into a single cell at the 4- or 8-cell stage is sufficient to induce the formation of the neuroectoderm in *ichabod* embryos (Maegawa et al., 2006; Tsang et al., 2004). The present study reveals that the induction of the anterior and posterior neuroectoderm by FGF8 is suppressed when *chd* activity is inhibited (Fig. 1). Inhibition of FGF signaling strongly suppresses the expression of *chd,* a downstream factor of this signaling pathway (Londin et al., 2005; Maegawa et al., 2006). Furthermore, overstimulation of the FGF signaling pathway enhances *chd* expression (Koshida et al., 2002; Londin et al., 2005). Considering that FGF signaling has different roles in the induction of anterior and posterior neuroectoderm, depending on the developmental stage (Londin et al., 2005; Rentzsch et al., 2004), there is a possibility that the FGF signaling pathway regulates the timing of *chd* expression to determine neuroectodermal patterning along the AP axis.

We here demonstrated that FGF signaling is also involved in neuroectoderm patterning along the AP axis by induction of *dkk1b* (Fig. 4D–K). A number of previous studies have shown that activation of the FGF signaling pathway enhances posteriorizing activity of zygotic Wnt/β-catenin signaling in *Xenopus* and zebrafish embryos (Koshida et al., 1998; McGrew et al., 1997; Rhinn et al., 2005). The potential of

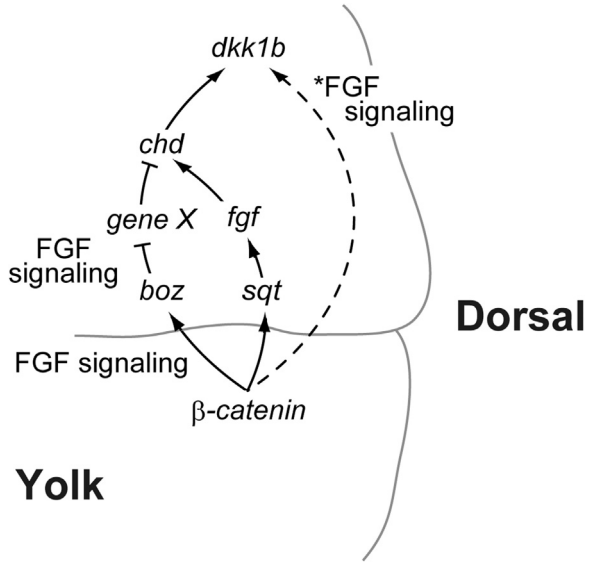

Fig. 9. A model of FGF-mediated induction and maintenance of the head organizer genes, *chd* and *dkk1b*. β-catenin induces a dorsal axis with a complete head structure at least through the three molecular pathways. All the pathways are dependent to some extent on FGF signaling. *Sqt* can activate shield stage expression of *chd* by inducing *fgf8*. *Boz* requires FGF signaling to relieve inhibition of *chd* expression. *Dkk1b* expression at the shield stage normally requires *chd* expression, which is dependent on FGF signaling. However, in embryos in which FGF signaling is inhibited, shield stage expression of *dkk1b* can occur (dotted line). Maintenance of *dkk1b* expression requires FGF signaling. The asterisk indicates that maintenance, but not initial induction of *dkk1b* by β-catenin, requires FGF signaling in this arm of the pathway. The step indicated by the arrow between *chd* and *dkk1b* represents induction of *dkk1b* at the shield stage, but there is little if any effect of *chd* on maintenance of *dkk1b*. Thus, two head organizer genes, *chd* and *dkk1b*, are induced and maintained through the FGF signaling in the dorsal organizer, resulting in the formation of a dorsal axis with a complete head structure in zebrafish.

FGF signaling to anteriorize embryos has not been extensively studied so far. Our results revealed that FGF signaling can contribute to anteriorization of neural tissues through the regulation of the Wnt antagonist, Dkk1b.

4.2. Activation of the FGF signaling pathway induces inhibitors for the BMP and Wnt signaling pathways

We showed that localized activation of the FGF signaling pathway induces the expression of *chd* and *dkk1b*, which encode essential factors for the formation of head structures in *ichabod* embryos. *Dkk1b* expression induced by the FGF signaling pathway at the shield stage is completely dependent on *chd* expression (Fig. 7) and expression of *dkk1b* is a downstream response to expression of *chd* (Fig. 6). (However, in the absence of FGF signaling, *dkk1b* expression at the shield stage can occur independently of *chd* expression, see below.) In *Xenopus*, the induction of a secondary axis with a complete head structure depends on the simultaneous inhibition of both the BMP and Wnt signaling pathways; inhibition of the BMP signaling pathway alone is insufficient (Glinka et al., 1997). Our results show that in zebrafish, expression of a gene encoding a BMP inhibitor (*chd*) results in expression of a gene encoding a Wnt inhibitor (*dkk1b*). Moreover, treatment of *ichabod* embryos with the BMP pathway inhibitor, LDN193189 (1 μM or 2 μM), resulted in *dkk1b* expression at the shield stage (unpublished observations), indicating that expression of *dkk1b* is a downstream response to the inhibition of the BMP signaling pathway. This epistatic relationship could be one of the molecular mechanisms underlying spatio-temporal coordination of DV and AP patterning in zebrafish.

4.3. Distinguishing the controls of dkk1b induction and dkk1b maintenance

FGF signaling induces expression of *chd* in *ichabod* embryos (Maegawa et al., 2006), and as described above, *chd* expression can induce some degree of *dkk1b* expression at the shield stage in these embryos (Figs. 6C and 8J). Expression of *chd*, although required for shield stage expression of *dkk1b* in FGF8 mRNA injected *ichabod* embryos (Fig. 7C and D), is not sufficient for formation of a fully anteriorized head in these embryos (Figs. 2C, 5D and E). These findings indicate that full anteriorization resulting from FGF signaling must involve more than induction of *chd*. Expression of *dkk1b* at 90% epiboly stage is observed when such embryos are injected with FGF8 mRNA but not with *chd* RNA (Fig. 8H and K). Inhibition of FGF signaling by expression of XFD further distinguishes control of *dkk1b* expression at the shield stage from its maintenance at later embryonic stages. FGF pathway inhibition does not appear to affect *dkk1b* expression at the shield stage in β-catenin injected *ichabod* embryos, whereas it obliterates *dkk1b* expression at 65% epiboly stage (Fig. 8P–W). Maintenance of *dkk1b* in these later stage embryos is thus dependent on FGF signaling.

Our observation that XFD expression had little or no effect on shield stage *dkk1b* expression in *ichabod* embryos was somewhat surprising since prior work showed that *chd* expression at the shield stage was completely inhibited in these embryos (Maegawa et al., 2006) and that *chd* expression is essential for *dkkb1* expression in FGF8 mRNA-injected embryos (Fig. 7C and D). To explain this finding, we propose that there is a latent *chd*-independent route to shield stage expression of *dkk1b* downstream of β-catenin that is revealed when FGF signaling is inhibited. Although *dkk1b* is observed at the shield stage under these conditions, it does not persist at 65% epiboly stage (Fig. 8P–W), indicating that *dkk1b* mRNA, even if can be induced at an earlier stage through an FGF-independent pathway, still requires FGF signaling for its maintenance as epiboly progresses. Since *chd* expression alone does not maintain *dkk1b* expression as epiboly proceeds (Fig. 8J and K), FGF-dependent maintenance of *dkk1b* does not involve *chordin*.

4.4. Molecular pathway in FGF-mediated head induction

We present a model of the molecular pathway in FGF-mediated head induction caused by the maternal Wnt/β-catenin signaling (Fig. 9). The model of this pathway has three arms, one dependent mainly on *sqt*, a second dependent on *boz*, and a third independent of these genes. At least one step of each arm is dependent on FGF signaling. As *sqt* induces the expression of FGF3 and FGF8 to form a dorsal axis with a complete head structure in *ichabod* embryos (Maegawa et al., 2006), it appears that FGF signaling is an indispensable downstream component of *sqt*-dependent dorsalizing functions of the maternal Wnt/β-catenin signaling pathway (Maegawa et al., 2006). Furthermore, *boz* induces weak expression of FGF3 and FGF8. Activation of the FGF signaling pathway is also required to maintain the expression of *boz,* induced by β-catenin mRNA-injection (Maegawa et al., 2006). These two arms of the pathway both ultimately promote the expression of *chd,* through FGF signaling, to repress Bmp activity at the dorsal side of the embryo (Maegawa et al., 2006). The present study demonstrates two novel functions of FGF signaling in head induction; one is to induce *dkk1b* expression at the shield stage through *chd* activity (Figs. 3 and 7) and the other is to maintain *dkk1b* expression (Fig. 8). It has already been reported that the *sqt* and *boz* genes play important roles in maintaining *dkk1b* expression in zebrafish (Hashimoto et al., 2000). Thus, the FGF signaling pathway is likely responsible for the maintenance of *dkk1b* expression levels through the action of *sqt* and *boz*. Moreover, *in silico* analysis, using Genomatix Matinspector (http://www.genomatix.de/solutions/genomatix-software-suite.html) reveals that the *dkk1b* putative promoter contains consensus binding sites of Ets1 and AP1, which are downstream targets of the FGF signaling pathway (unpublished results). FGF signaling very well might maintain the

expression of *dkk1b* through the activation of transcription factors such as Ets1 and AP1.

The third arm of control of *dkk1b* expression is illustrated in Fig. 9 by a dotted line. In the absence of FGF signaling, β-catenin is able to induce *dkk1b* expression at the shield stage. However, maintenance of expression at 65% epiboly stage does require FGF signaling. It is quite possible that FGF-independent shield stage expression of *dkk1b* involves direct interactions of β-catenin/Lef-1 with the *dkk1b* promoter. In *silico* analysis revealed that there are eleven putative Lef-1 binding sites in a region upstream of the gene (using Genomatix Mainspector). In at least one other case, *dkk1* of the mouse, a *Dkk1* promoter has been shown to contain Lef1 sites that are important for transcriptional regulation of the gene (Lieven et al., 2014). Such a mode of regulation may also operate in zebrafish *dkk1b*. The robust nature of shield stage expression of *dkk1b* in response to *chd* expression, and its dependence on *chd* expression, probably indicate that unless FGF signaling is inhibited, the direct activation of *dkk1b,* represented by the dotted line of Fig. 9, probably has a minor role. FGF signaling, however, even in these embryos, is required for maintenance of *dkk1b* during epiboly. *Chd* appears to play a minor role, if any, in the maintenance of *dkk1b,* whereas persistence of *dkk1b* transcript in β-catenin RNA-injected embryos still requires FGF signaling. Thus, FGF signaling plays an important role in both the induction and maintenance of head organizer genes.

We have shown that localized activation of FGF signaling by injection of FGF8 mRNA at 4–8 cell stage cannot completely rescue *ichabod* mutants, as the majority of the rescued embryos failed to form notochord. Interestingly, activation of FGF signaling induced notochord with complete head structures when FGF8-expressing cells were transplanted into *ichabod* embryos at the 1k-cell stage (unpublished observation). This result suggests that a localized region of FGF signaling may be sufficient for inducing a complete dorsal organizer capable of inducing a notochord. In the studies reported here and in Maegawa et al. (2006), FGF8 mRNA was injected into one cell at the 4–8 cell stage, which would create a more widespread, less discrete region of FGF signaling in the embryo. It will be of interest to use a cell transplantation approach with *ichabod* embryos to further explore whether FGF signaling is sufficient to carry out all aspects of dorsal organizer activity.

We conclude that the activation of the FGF signaling pathway induces the formation of a dorsal axis with a complete head structure in *ichabod* embryos by inducing *chd* expression and by maintaining *dkk1b* expression levels. Further studies are needed to understand the molecular mechanisms modulating the expression of *dkk1b* via the activation of the FGF signaling pathway.

Acknowledgements

We thank Dr. Hibi for providing zebrafish *dkk1b* gene plasmids. We appreciate our lab members' efforts in zebrafish care. S.M. was supported by Grant-in-Aid for Young Scientists (A) No. 22687016, Japan Society for the Promotion of Science. E.W. was supported by NIH, United States grant HD39272).

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