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RESEARCH ARTICLE

Xenopus hairy2b Specifies Anterior Prechordal Mesoderm Identity Within Spemann's Organizer

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Spemann's organizer is a region of the gastrula stage embryo that contains future anterior endodermal and dorsal mesodermal tissues. During gastrulation, the dorsal mesoderm is divided into the prechordal mesoderm and the chordamesoderm. However, little is known regarding how this division is established. We analyzed the role of the anterior prechordal mesoderm-specific gene *Xhairy2b* in the regionalization of the organizer. We found that mesoderm-inducing transforming growth factor-β signaling induced *Xhairy2b* expression. On the other hand, the ectopic expression of *Xhairy2b* induced the expression of organizer-specific genes and resulted in the formation of a secondary dorsal axis lacking head and notochord structures. We also showed that Xhairy2b down-regulated the expression of ventral mesodermal, anterior endodermal, and chordamesodermal genes. In Xhairy2b-depleted embryos, defects in the specification of anterior prechordal mesoderm identity were observed as the border between the prechordal mesoderm and the chordamesoderm was anteriorly shifted. These results suggest that Xhairy2b establishes the identity of the anterior prechordal mesoderm within Spemann's organizer by inhibiting the formation of neighboring tissues. *Developmental Dynamics 234:102–113, 2005.* © 2005 Wiley-Liss, Inc.

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INTRODUCTION

Spemann's organizer was first discovered in amphibians, and its functional homologues have been identified in a wide variety of vertebrate species (reviewed by Beddington and Robertson, 1998; Niehrs, 2004). The organizer is required for patterning the nascent mesoderm and converting the dorsal ectoderm into neural tissues (Harland and Gerhart, 1997). Different inductive properties are present in different subdomains of the amphibian organizer and developed the notion of distinct head and trunk organizers. Similar to amphibians, the mouse and the chick also have head and trunk organizer equivalents, and the head organizer (AVE/anterior hypoblast) is physically separated from the trunk organizer (node; reviewed by Beddington and Robertson, 1998; Niehrs, 2004). In *Xenopus*, however, the head and trunk organizers (anterior endoderm and dorsal mesoderm) are partially intermingled, and these regions are not readily discernible in the early gastrula stage (Bouwmeester et al., 1996). As gastrulation progresses, these regions are divided into distinct endodermal and mesodermal tissues. How is this division between the head and trunk organizers established? One transcription factor involved in the organizer division is Hex, which functions by directly suppressing the expression of the dorsal mesodermal gene *goosecoid* in the anterior endoderm (Brickman et al., 2000). Another example is the anti-dorsalizing morphogenetic protein (ADMP), which is a secreted molecule expressed in the dorsal mesoderm, that functions in this region to antagonize head formation and maintain dorsal mesoderm identity (Dosch and Niehrs, 2000).

During gastrulation, the dorsal mesoderm is further divided into the pre-

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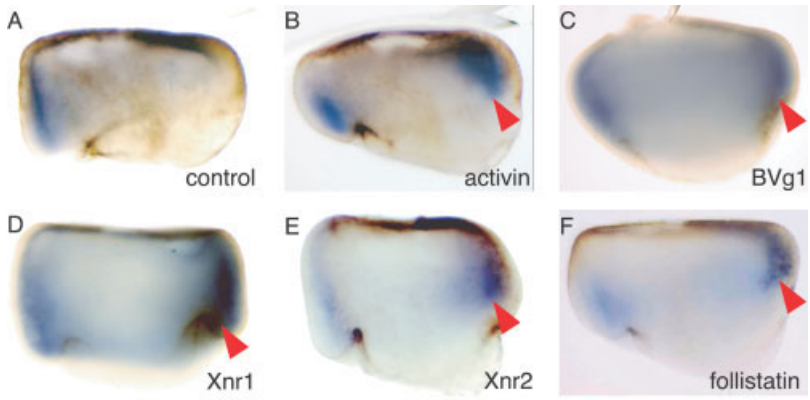

Fig. 1. Organizer-inducing factors activate *Xhairy2b* expression. A–F: Spatial expression patterns of *Xhairy2b* by whole-mount in situ hybridization at stage 10.5. Cleared embryos are shown in lateral view and dorsal is to the left. **A:** Uninjected control embryo. **B–F:** Ectopic induction of *Xhairy2b* expression in embryos ventrally microinjected with mRNAs encoding organizer-inducing factors. mRNAs of *activin* (B), *BVg1* (C), *Xnr1* (D), *Xnr2* (E), and *follistatin* (F) were injected at the eight-cell stage. Arrowheads show the ectopic expression of *Xhairy2b*.

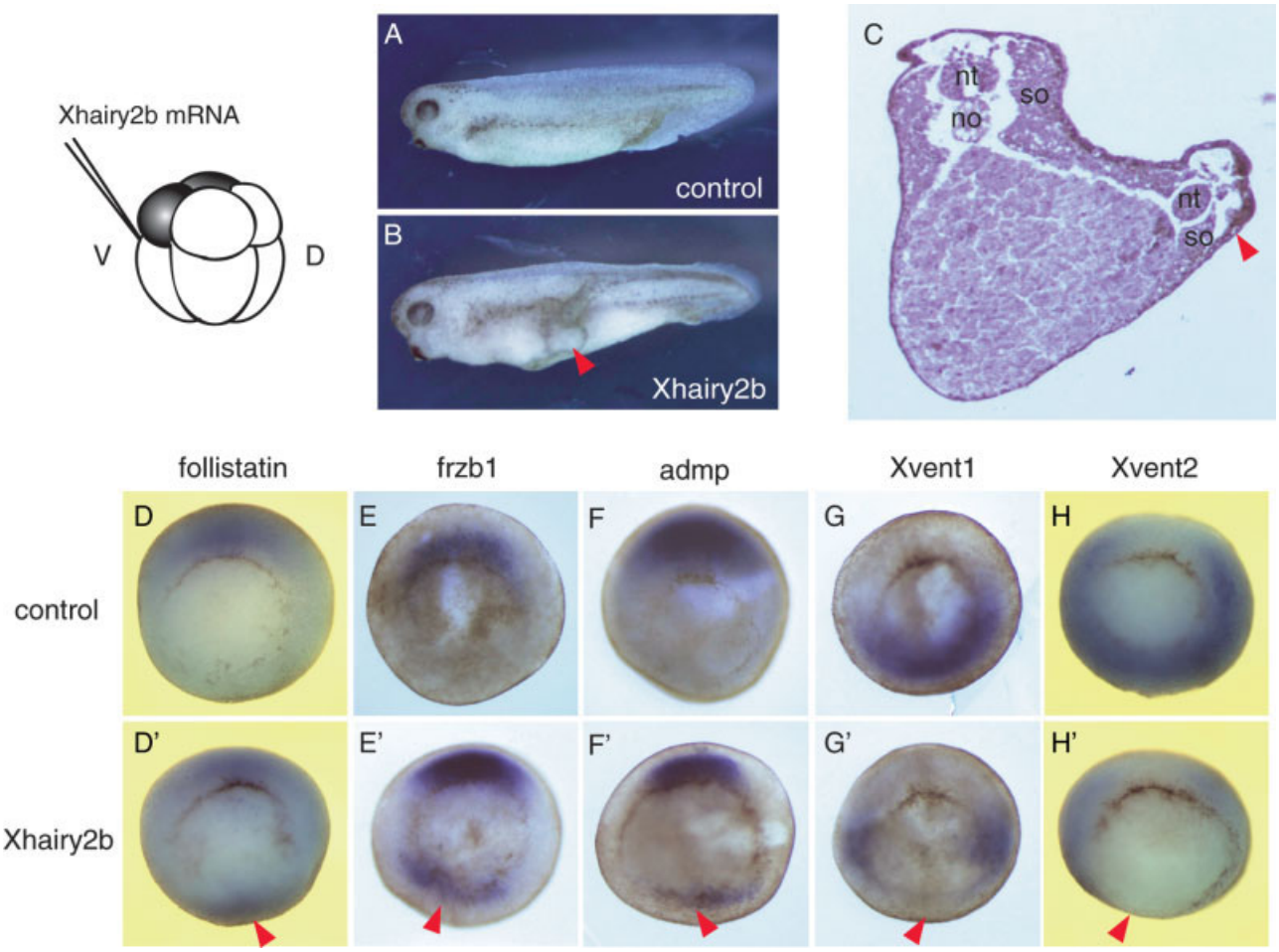

Fig. 2. Ventral expression of Xhairy2b induces a secondary axis and inhibits ventralizing factors. **A,B:** Uninjected control embryo and *Xhairy2b* mRNA ventrally injected embryo, respectively, at stage 35. *Xhairy2b* mRNA ventral injection results in the induction of secondary axes lacking head structures (arrowhead). **C:** Transverse section of the embryo shown in B. Secondary axis (arrowhead) has neural tube and somitic mesoderm but lacks notochord. no, notochord, nt, neural tube, so, somite. **D–H,D′–H′:** Uninjected control embryos (D–H) and *Xhairy2b* mRNA (800 pg) ventrally injected embryos (D′–H′) are shown in vegetal view, dorsal side up at stage 10.5. D′–F′: Expression of organizer genes, *follistatin* (D′), *frzb1* (E′), and *admp* (F′) is ectopically induced by Xhairy2b (arrowheads). G–H′: The expression of *Xvent1* and *Xvent2* in the ventral region (G,H) is reduced by Xhairy2b expression (G′,H′, arrowheads).

chordal mesoderm and the chordamesoderm, which are necessary for the specification of the rostral diencephalon and the floor plate, respectively (Dale et al., 1997; Dodd et al., 1998). In chick, axial mesodermal cells that initially migrate out of Hensen's node are a mixed population of prechordal mesodermal and chordamesodermal cells (Foley et al., 1997). Transforming growth factor-β (TGF-β) signaling from the anterior endoderm plays a critical role in the specification of the prechordal mesoderm by maintaining the expression of prechordal mesoder-

TABLE 1. Ectopic Induction of Xhairy2b by Organizer-inducing Factors*

Ventrally injected mRNA	pg	Xhairy2b n	Xhairy2b Normal expression (%)	Xhairy2b Ectopic expression (%)
activin	2	40	28	72
BVg1	400	24	8	92
Xnr1	100	35	0	100
Xnr2	10	34	12	88
follistatin	5600	61	49	51
chordin	800	68	99	1
noggin	800	77	99	1
tBR	800	80	100	0
β-catenin	200	52	87	13
twin	40	34	88	12
Xnr3	8000	50	94	6

*The results of three to eight experiments are summarized.

mal genes, such as *goosecoid*, and repressing chordamesoderm characteristics (Vesque et al., 2000). Similarly, in early *Xenopus* gastrula, prechordal mesodermal and chordamesodermal cells are also intermingled in the organizer region. It is known that Goosecoid, a transcriptional repressor expressed in the prechordal mesoderm, functions in the specification of these subdivisions. In early to mid-gastrula, *Xbra* and *goosecoid* expression domains overlap in the dorsal mesoderm. However, in late gastrula, *Xbra* expression is directly suppressed by Goosecoid and restricted to the chordamesoderm and the ventrolateral mesoderm (Cho et al., 1991; Smith et al., 1991; Artinger et al., 1997; Latinkic et al., 1997; Zoltewicz and Gerhart, 1997).

The prechordal mesoderm plays important roles in the induction and patterning of anterior neural tissue (reviewed by Kiecker and Niehrs, 2001). Several genes expressed in the prechordal mesoderm have been isolated, and many of their functions have been painstakingly studied. Many prechordal mesodermal genes encode secreted molecules, including Chordin (Sasai et al., 1994), Xdkk1 (Glinka et al., 1998), Frzb1 (Leyns et al., 1997; Wang et al., 1997), ADMP (Moos et al., 1995), and Crescent (Shibata et al., 2000). The prechordal mesoderm also specifically expresses many transcription factors, including Goosecoid (Cho et al., 1991), Otx2 (Blitz and Cho, 1995), and Xlim1 (Taira et al., 1992). From a comparison of the expression domains of these genes, distinct subdomains can be found within the prechordal mesoderm. For instance, *Xhairy2b* is expressed in the anterior portion of the prechordal mesoderm, whereas *chordin* is expressed in the posterior region, and the expression domains of these two genes never overlap (this issue). This finding leads us to postulate that the embryonic region that is generally called "prechordal mesoderm" can be functionally subdivided into at least two portions.

In this study, we address how *Xhairy2b* functions in the establishment of the subdivisions in Spemann's organizer. We show that *Xhairy2b* expression is induced by TGF-βs, such as Activin, BVg1, and Nodal family members. Follistatin signaling also induces *Xhairy2b* expression, but neither bone morphogenetic protein (BMP) inhibitors nor β-catenin induces *Xhairy2b* expression in early gastrula. We also show that Xhairy2b could ectopically induce dorsal mesoderm-specific genes, such as *follistatin*, *frzb1*, and *admp*, and secondary dorsal axis lacking head and notochord structures. Furthermore, Xhairy2b represses the expression of genes specific to neighboring tissues, such as the ventral mesoderm and the anterior endoderm, thereby inhibiting the differentiation of the dorsal mesoderm into another fate at the early gastrula stage. Loss-of-function experiments demonstrate that the defects in the formation of the anterior prechordal mesoderm are observed at the late gastrula stage. These results suggest that, during *Xenopus* gastrulation, Xhairy2b functions in the regionalization of the mesoderm by inhibiting the formation of various neighboring tissues, and this function is essential for the specification of the anterior prechordal mesoderm.

RESULTS

Organizer-Inducing TGF-β Signals Activate *Xhairy2b* Expression

Xhairy2b is predominantly expressed in the deep layer of the dorsal lip (Spemann's organizer) at the early gastrula stage (Tsuji et al., 2003; see also Fig. 1A). As numerous growth factors are known to induce the expression of genes involved in organizer function (Harland and Gerhart, 1997; Fagotto et al., 1997; Asashima et al., 1999), we sought to examine how the expression of *Xhairy2b* is regulated by these molecules. To this end, we ectopically expressed these growth factors in early gastrula and examined *Xhairy2b* induction by whole-mount in situ hybridization. Each mRNA was injected at a concentration sufficient to induce the secondary dorsal axis.

We found that general mesoderm-inducing TGF-βs, such as Activin (Sokol et al., 1990; Thomsen et al., 1990), BVg1 (Thomsen and Melton, 1993; Dale et al., 1993), and *Xenopus* nodal-related factors (Xnrs) 1 and 2 (Jones et al., 1995), could induce

TABLE 2. Repression of Anterior Endodermal and Chordamesodermal Genes by Xhairy2b*

	β-catenin injection			β-catenin and Xhairy2b injection		
induction of mRNA	n	non ectopically induction (%)	ectopically induction (%)	n	non or reduced ectopically induction (%)	ectopically induction (%)
Xdkk1	20	10	90	50	60	40
Xhex	20	20	80	50	88	12
cerberus	20	20	80	31	10	90
chordin	19	11	89	39	59	41
Xnot	23	9	91	59	61	39
noggin	10	10	90	10	10	90

*The results of two to six experiments are summarized.

Xhairy2b expression at the early gastrula stage (Fig. 1B–E; Table 1; Activin 72%, n = 40; BVg1 92%, n = 24; Xnr1 100%, n = 35; Xnr2 88%, n = 34). As it has been reported that Activin-like signaling induces the expression of Notch and its target genes (Abe et al., 2004) and Notch signaling induces the expression of *Xhairy2b* (Lopéz et al., 2005), the induction of *Xhairy2b* expression by general mesoderm-inducing TGF-βs might be through Notch signaling.

Next, neither the ectopic expression of β-*catenin* (Heasman et al., 1994; Funayama et al., 1995) nor its direct downstream target genes *twin* (Laurent et al., 1997) and *Xnr3* (Smith et al., 1995; Ecochard et al., 1995) induced *Xhairy2b* expression at the early gastrula stage (Table 1; β-catenin 13%, n = 52; Twin 12%, n = 34; Xnr3 6%, n = 50). However, the secondary axis induced by β-catenin had the expression of *Xhairy2b* at the late neurula stage (data not shown). Taken together, the results indicate that β-catenin signaling might not participate in the first induction of *Xhairy2b* expression.

We also examined the ability of BMP antagonists to stimulate *Xhairy2b* expression by their ectopic expression in the ventral marginal zone. Interestingly, we found that Follistatin, a dual Activin/BMP antagonist (Nakamura et al., 1990; Hemmati-Brivanlou et al., 1994; Iemura et al., 1998), induced *Xhairy2b* expression (Fig. 1F; Table 1; 51%, n = 61), whereas the BMP antagonists Chordin (Sasai et al., 1994) and Noggin (Smith and Harland, 1992) did not (Table 1; Chordin 1%, n = 68; Noggin 1%, n = 77). Consistent with the latter results, tBR2, a dominant-negative BMP type 1 receptor (Ishikawa et al., 1995), also failed to induce *Xhairy2b* expression (Table 1; 0%, n = 80). Follistatin is known to differ from other known BMP antagonists in at least two respects: one is that only Follistatin antagonizes ADMP (Dosch and Niehrs, 2000), and the other is that Follistatin forms a tertiary complex with BMP and its receptor (Iemura et al., 1998). These differences may have caused the induction of *Xhairy2b* expression at the early gastrula stage.

These results suggest that the expression area of *Xhairy2b* at the early gastrula stage is defined by the signaling interaction of Follistatin with general mesoderm-inducing TGF-βs, and not by the maternal β-catenin signaling.

Xhairy2b Induces Ectopic Expression of Organizer Factors and Secondary Trunk

As *Xhairy2b* is expressed within Spemann's organizer (Tsuji et al., 2003) and induced by secreted signaling molecules that also induce Spemann's organizer, we suspected that Xhairy2b may function in axis formation. Therefore, we ectopically expressed *Xhairy2b* ventrally to determine whether Xhairy2b has axis-inducing activity. We found that Xhairy2b induced the secondary axes lacking head and notochord structures (51%, n = 191; Fig. 2A–C; Table 2). One major function of Spemann's organizer in early development is to influence surrounding tissues through inductive interactions (Harland and Gerhart, 1997). As Xhairy2b is a transcription factor and, therefore, cannot directly influence surrounding cells, we sought to determine which organizer-specific secreted molecule was induced by Xhairy2b. We examined the expression of the BMP antagonists *chordin*, *noggin*, and *follistatin*, which are normally expressed in the organizer (Smith and Harland, 1992; Sasai et al., 1994; Iemura et al., 1998). Among them, only *follistatin* expression was induced by the ectopic expression of *Xhairy2b* (Fig. 2D,D′; 50%, n = 70). This result, taken together with Follistatin's induction of *Xhairy2b* expression, suggests that *Xhairy2b* expression can be maintained by a positive feedback loop between Xhairy2b and Follistatin. We also examined the expression of the Wnt antagonists *frzb1* (Leyns et al., 1997; Wang et al., 1997) and *Xdkk1* (Glinka et al., 1998) and the multifunctional (BMP, Wnt, and Nodal) antagonist *cerberus* (Hsu et al., 1998; Piccolo et al., 1999). Among them, only *frzb1* expression was induced by Xhairy2b (Fig. 2E,E′; 59%, n = 58). Furthermore, the expression of the *anti-dorsalizing morphogenetic protein* (*admp*; Moos et al., 1995), which is a member of the BMP family expressed in the organizer, was also induced (Fig. 2F,F′; 43%, n = 40). These data suggest that Xhairy2b induces incomplete secondary axes by means of its establishment of a secondary Spemann's organizer that expresses *follistatin*, *frzb1*, and *admp*, but not several other organizer-specific secreted factors.

Xhairy2b Suppresses Expression of Ventral Mesodermal Markers

It is known that Spemann's organizer not only provides active signal for the specification of the dorsal state, but also signals to inhibit mesoderm ventralization (reviewed by Niehrs, 2004). Therefore, we examined whether Xhairy2b could repress the expression of the ventral mesoderm-specific genes *Xvent1* (Gawantka et al., 1995) and *Xvent2* (Onichtchouk et al., 1996). As expected, these genes were strongly down-regulated by the ectopic expression of *Xhairy2b* (Fig. 2G–H′; *Xvent1* 83%, n = 30; *Xvent2* 79%, n = 29), although it is not clear if this repression was directly or indirectly mediated by Xhairy2b. As Xhairy2b induces *follistatin* expression, and Follistatin can antagonize BMP signaling (Fig. 2D,D′; Iemura et al., 1998), the repression of *Xvent1* and *2* might occur by means of Follistatin.

Xhairy2b Suppresses Expression of Anterior Endodermal Markers and Head Structural Formation

As *Xhairy2b* is expressed dorsally and has axis-inducing activity, we expected that its overexpression in the dorsal marginal zone would either enhance dorsal fate or show no significant effect. However, we found that the dorsal injection of *Xhairy2b* mRNA caused head defects (82%, n = 215). In *Xhairy2b*-injected embryos, the expression of anterior neural marker genes, such as *nkx2.4* (hypothalamus; Small et al., 2000), *otx2* (eye, forebrain, and midbrain; Blitz and Cho, 1995), and *en2* (midbrain–hindbrain boundary; Hemmati-Brivanlou et al., 1991), was absent or significantly reduced (*nkx2.4* 55%, n = 56; *otx2* 38%, n = 58; *en2* 36%, n = 59; Fig. 3A–C′). On the other hand, the expression of *krox20* (Bradlay et al., 1993) in hindbrain was detected in all injected embryos (n = 54, Fig. 3D,D′). These data indicate that an excess of *Xhairy2b* can suppress the head structures. This head defect might be caused by a deficit in the anterior endoderm, which is known to be necessary for the head formation (reviewed by Beddington and Robertson, 1998). To confirm this possibility, the expression of anterior endodermal markers, *Xhex* (Newman et al., 1997), *Xdkk1*, and *cerberus*, was analyzed in *Xhairy2b* dorsally injected embryos. Among them, the endogenous expression of *Xhex* and *Xdkk1* was suppressed by Xhairy2b but that of *cerberus* was not (Fig. 3F–H′). As shown in Figure 3F–H′, because the expression of *Xhex* and *Xdkk1* was partially intact in the organizer, the capability of Xhairy2b for head suppression might be underestimated. To analyze precisely the head deformation caused by Xhairy2b, we coinjected β-*catenin* and *Xhairy2b* mRNA on the ventral side of the embryo and analyzed the induction of the secondary axis and the ectopic expression of the anterior endodermal genes described above. As the ventral expression of β-*catenin* is known to generate the entire organizer (Guger and Gumbiner, 1995), this coinjection experiment can induce the distribution of Xhairy2b throughout the organizer tissue. In these embryos, the coexpression of *Xhairy2b* almost completely suppressed the induction of *Xhex* and *Xdkk1* expression but not that of *cerberus* expression, whose expression is induced by β-*catenin* alone (Fig. 3L–N′; Table 3). As expected, the formation of head structures in the secondary axis induced by β-*catenin* overexpression was strongly inhibited in the *Xhairy2b* and β-*catenin* coexpressing embryos, although a secondary trunk was induced (78%, n = 226; Fig. 3E,E′; Table 2). Consistent with the results obtained by the *Xhairy2b* dorsal injection, the expression of *krox20* was not affected (100%, n = 17), whereas the expression of *en2* and *otx2* was significantly affected by Xhairy2b coinjection (*en2* 50%, n = 16; and *otx2* 94%, n = 16; data not shown). These results confirm that Xhairy2b has the ability to suppress the formation of forebrain to midbrain. Furthermore, we investigated whether the head defects caused by *Xhairy2b* mRNA coinjection could be rescued by *Xhex*, *Xdkk1*, or *cerberus* mRNAs. As head development could be rescued only by *Xhex* and *Xdkk1* (data not shown), it was speculated that the depletion of Xhex and Xdkk1 caused the observed head defects. From these results, Xhairy2b is able to suppress the expression of anterior endodermal markers, and this suppression is responsible for the defect of the anterior head structures (forebrain to midbrain).

Expression Patterns of *Xhairy2b*, *Xhex*, *Xdkk1*, and *chordin* Suggest That *Xhairy2b* Is Expressed in Dorsal Mesoderm Distinct From *Xhex*- and *Xdkk1*-Expressing Region

As shown above, Xhairy2b seems to function in trunk induction and head repression at the early gastrula stage. Generally in *Xenopus*, the dorsal mesodermal region has trunk-inducing activity and the anterior endodermal region has head-inducing activity (Bouwmeester et al., 1996; Beddington and Robertson, 1998). To more precisely compare the expression domains of *Xhex*, *Xdkk1*, *chordin*, and *Xhairy2b* at the early gastrula stage, in situ hybridization of neighboring sections was performed. *Xhairy2b* was expressed in the dorsal mesoderm, which expressed *chordin*, and *Xdkk1* and *Xhex* were expressed in the anterior endoderm, clearly distinct from *Xhairy2b* (Fig. 4A–F). These observations also support the idea that Xhairy2b may play a role in sustaining the trunk organizing identity by repressing the expression of genes specific for the anterior endoderm in the dorsal mesoderm.

Xhairy2b Suppresses Expression of Chordamesodermal Markers

Surprisingly, Xhairy2b also suppressed the expression of *chordin* and *Xnot* (von Dassow et al., 1993), but not that of *noggin*, which are known as dorsal mesodermal genes (Fig. 3I–K′). These results were confirmed by β-*catenin* and *Xhairy2b* coinjection experiments (Fig. 3O–Q′; Table 3). Histological analysis indicated that Xhairy2b did not inhibit the formation of notochord structure (data not shown), although *Xhairy2b*-injected embryos often showed defects in convergent extension movement, resulting in spina bifida (51%, n = 215; Fig. 3A–D′). This defect of the axial mesoderm might be caused by the depletion of *chordin* and *Xnot* (Fig. 3I–K′; Table 3). In fact, the expression domain of *chordin* in the dorsal mesoderm

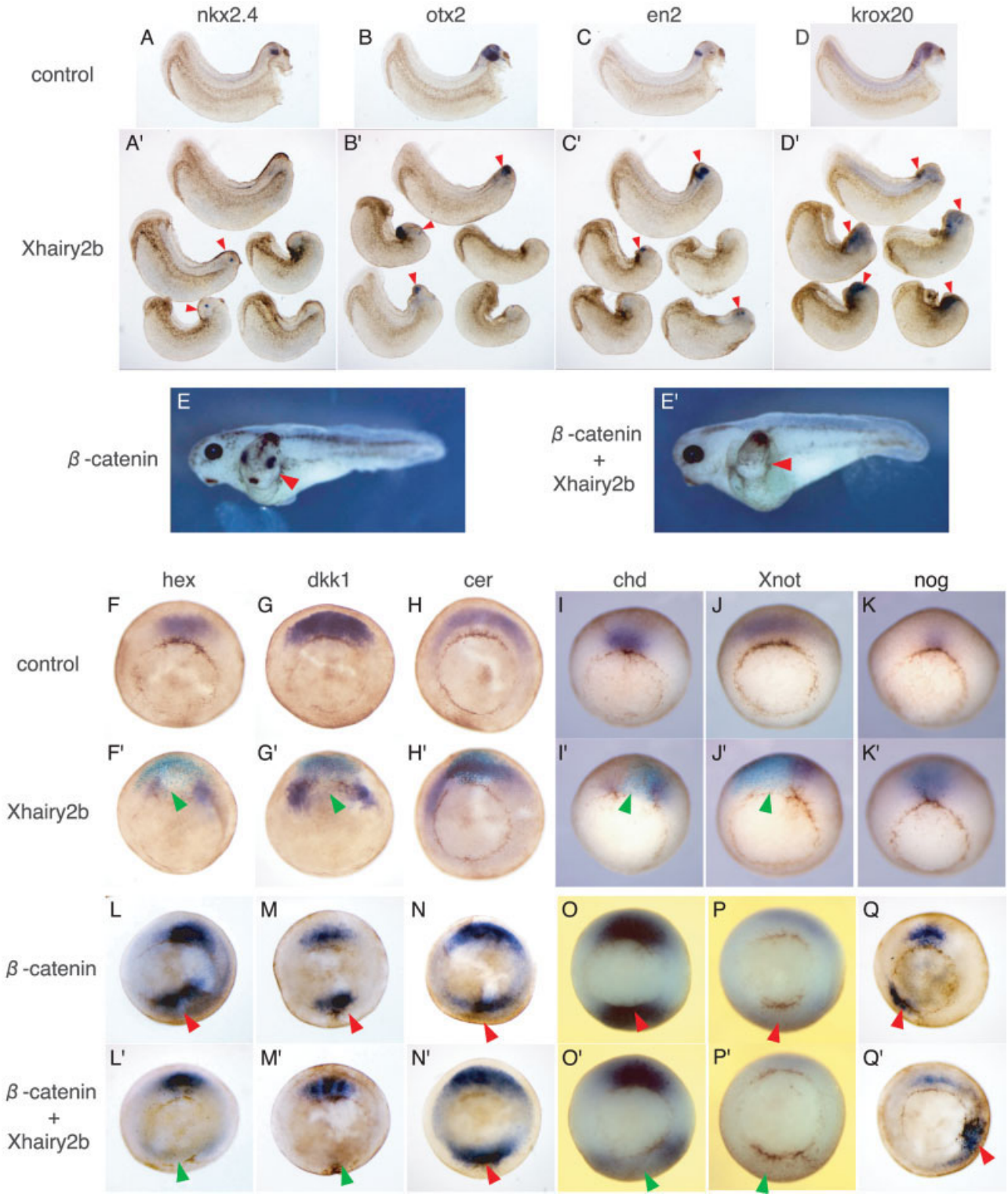

Fig. 3. Xhairy2b expression inhibits head formation and represses expression of some trunk and head organizer genes. **A–D′:** Uninjected control embryos (A–D) and 800 pg of *Xhairy2b* mRNA dorsally injected embryos (A′–D′) at stage 30. The overexpression of *Xhairy2b* results in a deficit of the expression of *nkx2.4* (A,A′), *otx2* (B,B′), and *en2* (C,C′) but not that of *krox20* (D,D′). Arrowheads indicate the remainder of the expression. E,E′: β-*catenin* ventrally injected embryo (E) and β-*catenin* and *Xhairy2b* coinjected embryo (E′). β-*catenin* expression induces a secondary axis containing a complete head (arrowhead in E), whereas the coinjection of *Xhairy2b* and β-*catenin* induces a secondary axis lacking head structures (arrowhead in E′). **F–Q′:** Uninjected control embryo (F–K), 800 pg of *Xhairy2b* mRNA dorsally injected embryos (F′–K′), 400 pg of β-*catenin* mRNA ventrally injected embryos (L–Q), and 400 pg of β-*catenin* and 800 pg of *Xhairy2b* mRNA ventrally coinjected embryos (L′–Q′) are shown in vegetal view, dorsal side up at stage 10.5. The expression of *Xhex* (F,F′), *Xdkk1* (G,G′), *chordin* (I, I′), and *Xnot* (J,J′) is repressed by Xhairy2b (green arrowheads), whereas that of *cerberus* (H,H′) and *noggin* (K,K′) is not affected by Xhairy2b. Light blue staining is coinjected β-gal. These genes are ventrally induced by β-*catenin* expression (red arrowheads in L–Q). The ectopic expression of *Xhex* (L′), *Xdkk1* (M′), *chordin* (O′), and *Xnot* (P′) is repressed by *Xhairy2b* coexpression with β-*catenin* (green arrowheads). Meanwhile, the expression of *cerberus* (N′) and *noggin* (Q′) is not affected (red arrowheads).

TABLE 3. Suppression of Head Structures by Xhairy2b

Ventrally injected mRNA	n	Normal (%)	Secondary trunk (%)	Secondary axis with one eye or/and cementgland (%)	Complete secondary axis (%)
β-catenin	261	11	6	10	73
β-catenin + Xhairy2b	226	5	78	9	8

TABLE 4. Induction of Secondary Axis by Xhairy2b

Ventrally injected mRNA and mo	n	Normal (%)	Secondary trunk (%)	Spina bifida (%)	Dead (%)
control	194	100	0	0	0
Xhairy2b mRNA	191	19	51	22	8
Xhairy2b mRNA + 5mis mo	61	20	49	16	15
Xhairy2b mRNA + Xh2b mo	184	95	2	1	3

seemed to overlap with the area of *Xhairy2b* expression at the early gastrula stage (Fig. 4E,F). However, with the progress of gastrulation movements and the subdivision of the dorsal mesoderm into the chordamesoderm and the prechordal mesoderm, the expression of *chordin* and *Xhairy2b* became clearly separated from each other. *Xhairy2b* expression was restricted to the anterior prechordal mesoderm and *chordin* was predominantly expressed in more posterior tissues, such as the posterior prechordal mesoderm and the chordamesoderm (Fig. 5D,E). Considering this expression pattern, it is possible that, at the late gastrula stage, Xhairy2b represses the expression of genes specific for neighboring tissues, including the posterior prechordal mesoderm, thereby participating in defining the identity of the anterior prechordal mesoderm.

Xhairy2b Is Essential for Specification of Anterior Prechordal Mesoderm

As shown above, it seems that Xhairy2b may function in the establishment of endomesodermal patterning by repressing the expression of genes specific for neighboring tissues, such as the ventral mesoderm, the anterior endoderm and the posterior prechordal mesoderm (and chordamesoderm). To examine whether this repression is essential for the establishment of these endomesodermal subdivisions, we inhibited the translation of endogenous *Xhairy2b* by dorsal coinjection of antisense morpholino oligonucleotide (mo) directed against *Xhairy2b*. We tested the efficacy of morpholino in blocking the in vivo translation of injected Xhairy2b–Myc Tag (MT) constructs containing the binding site for the morpholino. Indeed, the translation of Xhairy2b–MT was repressed by coinjecting Xhairy2b mo and not a five-mismatch control morpholino (Fig. 5A,B). On the other hand, the translation of -mo Xhairy2b mRNA, which does not contain the morpholino binding site, was not suppressed by the Xhairy2b mo (Fig. 5A,B). In addition, we confirmed that Xhairy2b mo did not influence the translation of Xhairy2a, a pseudo-allele of *Xhairy2b* (data not shown). Further-

Fig. 5. Xhairy2b morpholino oligonucleotide (mo) specificity and change of axial mesoderm in Xhairy2b-depleted embryos. **A:** Alignment of the injected *Xhairy2b* mRNA constructs and morpholino oligos (1: five-mismatch control mo; 2: Xhairy2b mo). Xhairy2b–Myc Tag (MT) constructs (3) contain the 5′-untranslated region binding site for the Xhairy2b morpholino, whereas the -mo Xhairy2b–MT constructs (4) do not. **B:** Western blot analysis of Xhairy2b–MT. The efficacy of the morpholinos is tested by blocking the in vivo translation of Xhairy2b–MT mRNA and morpholino-injected embryos. Uninjected control (lane 1) and embryos injected with Xhairy2b–MT mRNA (lane 2), Xhairy2b–MT mRNA+five-mismatch control mo (lane 3), Xhairy2b–MT mRNA+Xhairy2b mo (lane 4), -mo Xhairy2b–MT mRNA (lane 5), or -mo Xhairy2b–MT mRNA+Xhairy2b mo (lane 6). The translation of Xhairy2b–MT is repressed by coinjecting Xhairy2b mo but not the five-mismatch control mo. On the other hand, the translation of -mo Xhairy2b mo, which does not contain the morpholino binding site, is not suppressed by Xhairy2b mo. Coomassie staining of the cell pellets served as a loading control. **C,C′:** In situ hybridization of *chordin* and *pax2* in control mo-injected embryo (C) and *Xhairy2b* morpholino antisense oligo-injected embryo (C′) at stage 13. The expression of *chordin* is expanded in the mo-injected embryo. Dorsal view and anterior to the top. **D–E′:** Neighboring section in the mid-sagittal plane of control mo-injected embryo (D,E) and Xhairy2b mo-injected embryo (D′,E′). At the early neurula stage, the mesodermal expression of *Xhairy2b* is restricted to the anterior prechordal mesoderm and is distinct from the expression of *chordin*. In the mo-injected embryo, the expression of *chordin* (D′) and *Xhairy2b* (E′) is overlapped in the anterior prechordal mesoderm. Arrowheads indicate the posterior limits of Xhairy2b expression in the axial mesoderm. Anterior is to the left. **F,F′:** *goosecoid* expression in control mo-injected (F) and Xhairy2b mo-injected (F′) embryos. The *goosecoid* expression is significantly decreased in Xhairy2b mo-injected embryo. Light blue staining is in situ hybridization for coinjected YFP mRNA. **G–I′:** Mid-sagittal section of stage 14 control mo-injected embryo (G–I) and Xhairy2b mo-injected embryo (**G′–I′**). Anterior is to the left. Prechordal mesoderm-specific *goosecoid* expression is decreased in the posterior region (G,G′) and chordamesoderm-specific *Xbra* and *Xnot* expression is anteriorly increased (H–I′). Arrowheads indicate the limit of the gene expression domain. The dotted line is the border between the anterior neuroectoderm and the prechordal mesoderm. **J:** Schematic diagram of the axial mesodermal tissue in Xhairy2b mo-injected embryo. Anterior prechordal mesoderm (apm) is decreased, and the region of the posterior prechordal mesoderm (ppm) and the notochord (nc) is anteriorly expanded.

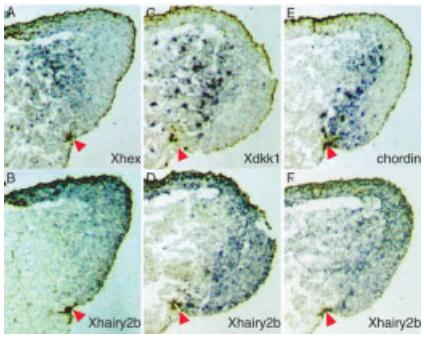

Fig. 4. Comparison of the expression domain of *Xhairy2b* with that of anterior endodermal genes and chordamesodermal genes at the early gastrula stage. A–F: Neighboring sections in the mid-sagittal plane of stage 10.5 embryos. Dorsal is to the right. Arrowheads indicate the blastopore lip. **B,D,F:** *Xhairy2b* is expressed in the ectoderm and the deep layer of the dorsal blastopore lip at the early gastrula stage. **A,C:** The expression of *Xhex* (A) and *Xdkk1* (C) in the anterior endoderm is hardly overlapped with the expression of *Xhairy2b*. **E:** The expression of *chordin* in the deep layer of the organizer is overlapped with that of *Xhairy2b*.

more, the development of secondary axes following *Xhairy2b* mRNA injection was morphologically inhibited by coinjecting Xhairy2b mo and not the five mismatch control morpholino (Table 4). Taken together, it is indicated that this mo is useful for inhibiting the translation of endogenous *Xhairy2b*.

Then, we sought to examine the in vivo effects of mo-mediated depletion of endogenous Xhairy2b on marker gene expression. When Xhairy2b was depleted, *chordin* expression was significantly increased in the Xhairy2b-depleted embryos at the late gastrula stage (stage 13, Fig. 5C,C′), although the expression patterns of the ventral mesodermal genes (*Xvent1* and *Xvent2*) and the anterior endodermal genes (*Xhex* and *Xdkk1*) were not significantly affected at the early gastrula stage (data not shown). Neighboring midsagittal sections of late gastrula embryos revealed that, in control mo-injected embryos, *chordin* expression was never overlapped with *Xhairy2b* expression in the axial mesoderm (Fig. 5D,E), but in the Xhairy2b-depleted embryos, *chordin* expression was overlapped with *Xhairy2b* expression (40%, n = 15, Fig. 5D′,E′). In these embryos, the amount of detected *Xhairy2b* mRNA appeared to have increased, although the expression region of *Xhairy2b* remained un-

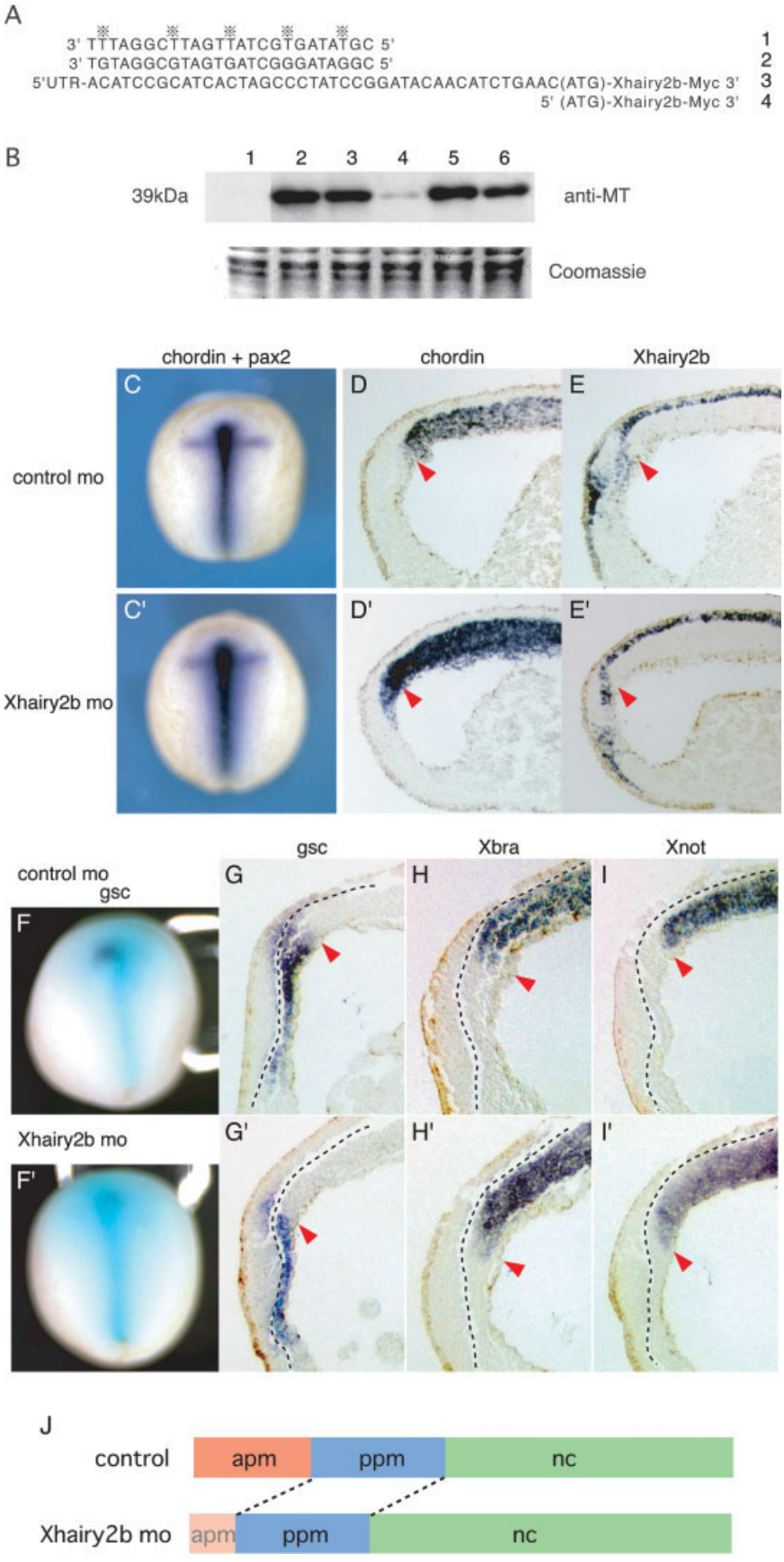

Fig. 5.

changed. To determine whether the character of the prechordal mesoderm and the chordamesoderm was changed, the expression of genes specific for the prechordal mesoderm and the chordamesoderm was analyzed in Xhairy2b mo-injected embryos. The prechordal mesoderm-specific expression of *goosecoid* was significantly decreased and remained only in the most rostral region (Fig. 5F–G′). Alternatively, the expression domain of *Xbra*, which was restricted in the chordamesoderm by means of the direct transcriptional repression by Goosecoid, was expanded anteriorly (Fig. 5H,H′). In addition, the region of chordamesoderm-specific *Xnot* expression was also expanded (Fig. 5I′). Although it is uncertain whether the anterior prechordal mesoderm is absent from the Xhairy2b-depleted embryos, it seems that the region of the anterior prechordal mesoderm became defective, and as a result, the posterior prechordal mesoderm and the chordamesoderm were anteriorly shifted in the Xhairy2b mo-injected embryos (Fig. 5J). These data suggest that Xhairy2b is necessary for the specification of anterior prechordal mesoderm identity.

DISCUSSION

Xhairy2b Induces a Subset of BMP and Wnt Antagonists but Fails to Induce Head and Notochord Structures

Our results show that Xhairy2b can induce such organizer-specific molecules as Follistatin, Frzb1, and ADMP, but not Chordin, Noggin, Xddk1, or Cerberus. As it is known that the ventral expression of *follistatin* induces secondary axes containing the notochord structure (Kablar, 1999) and that BMP antagonism in collaboration with a Wnt antagonist can induce a cyclopic head (Glinka et al., 1997), the secondary axis induced by Xhairy2b should contain head and notochord structures. However, we observed otherwise (Fig. 2A–C). In addition, the overexpression of *Xhairy2b* resulted in the repression of genes required for notochord and head formation (Fig. 3F–Q′). This finding may be explained by the behavior of *admp*, another gene induced by Xhairy2b (Fig. 2F,F′). ADMP is known to repress both trunk and head inducers; however, Follistatin attenuates ADMP signaling to levels sufficient for the repression of head but not of trunk (Dosch and Niehrs, 2000). Therefore, the interaction of ADMP with Follistatin may repress the formation of head and notochord structures. As HES family members usually work as transcriptional repressors, it is also possible that Xhairy2b directly down-regulates the genes essential for head and notochord formation.

Xhairy2b Represses Expression of Anterior Endodermal Genes at Early Gastrula Stage and Chordamesodermal Genes at Late Gastrula Stage

We have shown that the ectopic injection of Xhairy2b suppressed the expression of anterior endodermal genes and chordamesodermal genes. To evaluate the function of endogenous Xhairy2b, the expression pattern of each gene should be taken into consideration. Of interest, we found that *Xhairy2b* and *Xdkk1* coexisted in the prechordal mesoderm at the early neurula stage (Glinka et al., 1998; Tsuji et al., 2003). This finding is unexpected because Xhairy2b represses the expression of *Xdkk1* at the early gastrula stage (Fig. 3G,G′). These observations indicate that there must be unknown mechanisms underlying the repression by Xhairy2b of *Xdkk1* expression in the anterior endoderm and not in the prechordal mesoderm. One possible explanation is that Xhairy2b represses *Xdkk1* expression through *Xhex* repression in the dorsal mesoderm. Although the expression domain of *Xdkk1* is mostly overlapped with that of *Xhex* in early gastrula, *Xdkk1* expression additionally appears in mesodermal tissues during gastrulation (Glinka et al., 1998). As it is assumed that the *Xdkk1* expression at late gastrula is independent of Xhex, both Xdkk1 and Xhairy2b are able to coexist at the late gastrula stage.

Our results also showed that Xhairy2b repressed the expression of genes specific for the chordamesoderm and the posterior prechordal mesoderm (*chordin* and *Xnot*) at early gastrula (Fig. 3I–K′). However, *Xhairy2b* was expressed in the same domain as *chordin* at the early gastrula stage (Fig. 4E,F). How can these seemingly contradictory events be explained? It is known that the onset of *chordin* expression (stage 9) is much earlier than that of *Xhairy2b* expression (stage 10). Thus, it follows that cells have already accumulated *chordin* mRNA at the onset of *Xhairy2b* expression. The perdurance of *chordin* mRNA from these earlier stages may explain this observation. By the time sufficient levels of Xhairy2b protein are available for repressing *chordin* expression, high levels of *chordin* mRNA may already be present, thereby accounting for the coexistence of *chordin* mRNA with its repressor, Xhairy2b.

How Does *Xhairy2b* Function in Organizer Subdivision During Gastrulation?

Although the subdivisions of the *Xenopus* organizer are roughly distinguishable from each other, cells are partially intermingled at their boundaries during gastrulation. As both of these tissues seem to receive similar inductive signals, mechanisms must exist to prohibit these different cell types from differentiating into neighboring cell types. In this study, we showed that Xhairy2b participated in the establishment of the endomesodermal subdivision in gastrula *Xenopus* embryo by repressing the expression of genes specific for neighboring tissues. At the early gastrula stage, the ectopic expression of *Xhairy2b* repressed the expression of genes specific for the ventral mesoderm (*Xvent1* and *2*) and the anterior endoderm (*Xdkk1* and *Xhex*; Figs. 2G–H′, 3F–H′), indicating that Xhairy2b established dorsal mesoderm identity by inhibiting the formation of neighboring tissues. However, these subdivisions were not affected in the Xhairy2b-depleted embryos. It is known that dorsal mesoderm-specific gene products, such as Goosecoid and BMP antagonists (Chordin, Noggin, and Follistatin), repress the expression of *Xvent1* and *2* directly or indirectly. *Xhex* and *Xdkk1* are also known to be down-regulated by the dorsal mesodermal molecule ADMP (Smith and Harland, 1992; Sasai et al., 1994; Gawantka et al., 1995; Onicht-

chouk et al., 1996; Iemura et al., 1998; Dosch and Niehrs, 2000). These findings indicate that various region-specific molecules, including Xhairy2b, collaborate in establishing functional subdivisions of the endomesoderm during gastrulation.

The overexpression of *Xhairy2b* suppressed the expression of genes specific for both the posterior prechordal mesoderm and the chordamesoderm. Furthermore, in the Xhairy2b-depleted embryos, the expression of genes suppressed by Xhairy2b was anteriorly increased, indicating that the anterior prechordal mesoderm identity was defective and both the posterior prechordal mesoderm and the chordamesoderm were shifted anteriorly at the late gastrula stage. These results suggest that this suppression by Xhairy2b is essential for the identification of the anterior prechordal mesoderm.

The HES family of basic helix–loop–helix transcription factors is known to function in the establishment of various tissue identities during gastrulation (reviewed by Iso et al., 2003). The zebrafish HES-related gene *her5* is known to function in the establishment of the endodermal/endmost mesendodermal germ layer by inhibiting cell participation to the endmost-fated mesendoderm (Bally-Cuif et al., 2000). Xhairy2a (the pseudo-allele of Xhairy2b) is also known to function in the promotion of floor plate development by repressing notochord fate (Lopéz et al., 2005). In this study, we showed that Xhairy2b functions in the identification of the anterior prechordal mesoderm by inhibiting the formation of neighboring tissues. In these ways, the HES-mediated suppression of gene expression may be adopted as a universal strategy for the establishment of tissue identities during development.

EXPERIMENTAL PROCEDURES

Plasmid Construction

Plasmids containing genes used in this study were as follows. Using forward and reverse primers based on the published sequence, the coding region of each gene was amplified by RT-PCR. Sequences of the primers are shown below.

Xhairy2b: F(5′CCCGAATTCCACCATGCCTGCAGATAGTATGGAGAAG), R(5′CCCGGCGCGCCTCACCATGGTCGCCACACGGACTC); ADMP: F(5′GCCCATCGATCCACCATGGACCTTAGGAAGATGTTGGG), R(5′GCCCCTCGAGTTAGTGGCACCCGCAGCTGC); Frzb1: F(5′CCCGAATTCCACCATGTCTCCAACAAGGAAATTGGAC), R(5′CCCGGCGCGCCCTAACTACGCGCTTGTCTGGAATT); Xdkk1: F(5′CCCGAATTCCACCATGTCTCCAACAAGGAAATTGGAC), R(5′CCCGGCGCGCCCTAACTACGCGCTTGTCTGGAATT); Cerberus: F(5′CCCGAATTCCACCATGTTACTAAATGTACTCAGGATCTG), R(5′CCCGGCGCGCCTTAATGGTGCAGGGTAGTAGATG); Xvent1: F(5′CCCGAATTCCACCATGGTTCAACAGGGATTCTCTATTG), R(5′CCCGGCGCGCCTTACATATACTGAGCCCCAAAGAG); Xvent2: F(5′CCCGAATTCCACCATGACTAAAGCTTTCTCCTCTGTTG), R(5′CCCGGCGCGCCCTAATAGGCCAGAGGTTGCCC); Xnot: F(5′CCCGAATTCCACCATGTTACACAGCCCAGTCTTCCC), R(5′CCCGGCGCGCCCTAATTTATGTTCATTAGGCTCC); and Xhex: F(5′CCCGAATTCCACCATGCAGTACCAGCACCCCAGCTCCTC), R(5′CCCGGCGCGCCTTAATGTGCACAGTTGTAATATCCTTTGTCG).

These polymerase chain reaction products were digested with *Eco*RI/*Asc*I (Xhairy2b, Frzb1, Xdkk1, Cerberus, Xvent1, Xvent2, Xnot, and Xhex) or *Cla*I/*Xho*I (ADMP), and ligated into pCS2AT+ that was constructed by inserting annealed oligonucleotides (5′TCGAGGGCGCGCCGATATCTCTAGACGCCCTATAGTGAGTCGTATTAC3′ and 5′GTAATACGACTCACTATAGGGCGTCTAGAGATATCGGCGCGCCC3′) into *Xho*I-*Sna*BI-digested pCS2+. This strategy creates new *Asc*I and *Eco*RV sites in the polylinker I region. Xhairy2a/b–MT plasmids were constructed, producing C-terminally fused six repeats of a Myc Tag and were subcloned in pCS2 using PCR strategies.

Embryological Manipulations

Embryos were in vitro fertilized, dejellied, and cultured as described (Hawley et al., 1995). Staging was accomplished according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967), and embryos were fixed with MEMFA (Harland, 1991). Capped mRNAs for microinjection were synthesized from linearized plasmids using an mMESSAGE mMACHINE Kit (Ambion). mRNA at the indicated doses was microinjected into two ventral or dorsal blastomeres at the four- or eight-cell stage. The Xhairy2b morpholino antisense oligo was obtained from Gene Tools. The Xhairy2b mo had the sequence 5′-CGGATAGGGCTAGTGATGCGGATGT-3′. The five-mismatch Xhairy2b mo had the sequence 5′-CGTATAGTGCTATTGATTCGGATTT-3′. The morpholino oligos were resuspended in sterile water to a concentration of 0.1 mM each and injected with YFP mRNA to confirm the injected region. A total of 8 nl of mos (6.9 ng) was injected into the dorsal marginal zone at the four- or eight-cell stage. For Western blot experiments, 800 pg of Xhairy2b–MT mRNA was injected with 6.9 ng of each mo. Western blotting was performed with the anti–[c-myc]-peroxidase (Roche). Whole-mount in situ hybridization was performed as described previously (Harland, 1991) with minor modifications. The section in situ hybridization protocol of Butler et al. (2001) was used with minor modifications.

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