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Depletion of Three BMP Antagonists from Spemann's Organizer Leads to a Catastrophic Loss of Dorsal Structures

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Summary

Transplanted Spemann's organizer induces dorsal embryonic cell fates such as the nervous system and somites, but in normal development, elimination of individual organizer signals (such as the bone morphogenetic protein [BMP] antagonists) has surprisingly modest effects on these tissues. Thus, the role of BMP antagonists may be limited to fine tuning the size of the dorsal domain. However, at least five BMP antagonists are specifically expressed in the organizer, and all can mimic aspects of organizer function, suggesting overlapping functions. Here, we deplete the function of three BMP antagonists, chordin, noggin, and follistatin, in *Xenopus tropicalis.* We demonstrate that this results in catastrophic failure of dorsal development and expansion of ventral and posterior fates. We conclude that BMP antagonists are required for formation of the neural plate and dorsal mesoderm. In addition, our results show that neural specification requires the continuous activity of BMP antagonists from blastula through gastrula stages.

Introduction

The blastula embryo activates a cascade of signals, which specify dorsal structures such as the neural plate, the somites, and the notochord (Harland and Gerhart, 1997; De Robertis et al., 2000; Wilson and Edlund, 2001; Stern, 2002; Niehrs, 2004). Spemann's organizer, the dorsal blastopore lip of the early gastrula embryo, appears to be an important source of some of these signals. In a gain-of-function assay, transplants of Spemann's organizer to the ventral side of the embryo can induce a complete secondary axis (Spemann and Mangold, 1924; Gimlich and Cooke, 1983; Smith and Slack, 1983). These transplants respecify host tissues to form neural tissue and somites instead of epidermis and ventral-posterior mesoderm. Meanwhile, the organizer graft only contributes to a fraction of the secondary axis, primarily the notochord. Therefore, signals are secreted by Spemann's organizer to induce surrounding host tissue to form dorsal structures. Among these signals are the BMP antagonists, which are thought to protect the ectoderm from epidermalizing signals and protect the mesoderm from ventralizing signals, allowing the development of a patterned neural plate (Harland and Gerhart, 1997).

There are several BMP antagonists expressed in the organizer, and when added as protein to explants, they mimic the effects of the organizer (Lamb et al., 1993; Smith et al., 1993; Sasai et al., 1994). However, experiments to date that reduce BMP antagonist activity have not shown a significant requirement for these antagonists in specification of the neural plate and dorsal mesoderm (Stern, 2004). Indeed, single mutations of the BMP antagonists in the mouse or zebrafish, and even a double mutant of noggin and chordin, have surprisingly modest effects on the segregation of neural plate and somites (Matzuk et al., 1995; Schulte-Merker et al., 1997; McMahon et al., 1998; Bachiller et al., 2000, 2003). In part, this could be due to redundancy in early signaling, but another possibility is that these antagonists are only involved in fine tuning the amount of tissue devoted to neural plate and dorsal mesoderm.

Even the requirement for an organizer for dorsal development has been called into question. A mutation that eliminates the node of the mouse, along with chordin and noggin expression, still makes a neural plate that is well patterned in the anterior-posterior axis (Klingensmith et al., 1999). Furthermore, extirpation of the organizer from *Xenopus* or zebrafish gastrulae still permits considerable neural induction and patterning (Sater and Jacobson, 1990; Shih and Fraser, 1996; Schneider and Mercola, 1999).

To define dorsal structures, reducing BMP signals may be critical; however, multiple mechanisms have been demonstrated to reduce BMP signaling in addition to the extracellular antagonists. In *Xenopus* and zebrafish, the β-catenin stabilization that follows cytoplasmic rearrangement in the first cell cycle leads to a reduction in BMP mRNA expression (Baker et al., 1999; Leung et al., 2003), which could provide an independent mechanism for patterning. Similarly, in the chick, early fibroblast growth factor (FGF) expression reduces BMP mRNA expression in the prospective neural plate (Wilson et al., 2000). Therefore, transcriptional mechanisms may achieve the same effective result as BMP antagonists, namely reduction in BMP signal transduction in order to specify dorsal structures. Other experiments in the chick suggest that BMP antagonists may mostly function in the fine tuning of neural development. Application of noggin or chordin to the chick epiblast can alter the position of the neural folds but cannot induce ectopic neural tissues. In contrast, FGF8 signaling is able to initiate the events of neural induction (Streit et al., 1998, 2000; Wilson et al., 2000).

Therefore, genetic studies in the mouse and zebrafish as well as overexpression studies in the chick have not identified a substantive role for BMP antagonists in the specification of dorsal structures during normal development. Furthermore, in *Xenopus*, zebrafish, and the chick, alternative mechanisms to reduce BMP signals have been identified that have called into question

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what role the extracellular BMP antagonists play. Loss of function in *Xenopus* has so far shown that reducing chordin function can mildly decrease the size of the neural plate in whole embryos. Furthermore, in sensitized assays where the organizer is transplanted at the late blastula and early gastrula stage, chordin function is required for proper induction of neural tissues in the host (Oelgeschlager et al., 2003). Thus, the experiments to date do not address the extent to which BMP antagonists are required in normal development. In contrast, the early signals mediated by VegT, Smad2, and β-catenin have been shown by loss of function to be essential for the early steps of patterning the embryo (Wylie et al., 1996; Zhang et al., 1998; Whitman, 2001; Xanthos et al., 2002).

The failure to elucidate whether BMP antagonists are critical for embryonic pattern may result from the overlapping function of the multiple antagonists expressed in normal development. To address the importance of this mechanism, we have used morpholino oligonucleotides to knock down the function of multiple antagonists. We find that with elimination of three BMP antagonists, there is a catastrophic failure of dorsal development.

For these experiments, we exploit *Xenopus tropicalis* in which multiple genes can be targeted effectively with morpholino oligonucleotides (MOs). *X. tropicalis* has a simple diploid genome and a short generation time that allows for significant inbreeding. Therefore, variations in target sequence from either tetraploidization or polymorphism can be eliminated, optimizing the effectiveness of antisense MOs. (Amaya et al., 1998; Hirsch et al., 2002; Khokha et al., 2002).

Results

Expression of BMP Antagonists

To define the patterns of expression of BMP antagonists in the organizer of *X. tropicalis*, we stained bisected embryos by in situ hybridization. In cleared embryos, *xnr3* expression can first be detected in the midblastula (stage [st] 8) well before the appearance of the dorsal lip (Figure 1). Concurrently, expression of noggin and chordin can be weakly detected in the midblastula (st 8) and more strongly in the late blastula. The patterns are similar to those in *X. laevis*, with domains of expression in the marginal zone (Smith and Harland, 1992; Smith et al., 1995; Wessely et al., 2001). In *X. tropicalis*, *xnr3* is triplicated (Haramoto et al., 2004) and has a more uniform expression in the marginal zone than the superficial expression found in *X. laevis* (Smith et al., 1995). *Chordin* mRNA is expressed broadly in the mesoderm and anterior endoderm, and *noggin* expression is not present in the deep endomesoderm. The anterior endomesoderm initiates *cerberus* expression at st 9, whereas expression of *follistatin* is restricted to the dorsal mesoderm and first detected at the onset of gastrulation (st 10). In cleared blastula embryos, we see no evidence for exclusive expression of any transcripts in the dorsal ectoderm (Kuroda et al., 2004). The location and timing of expression of these transcripts is consistent with a function in Spemann's organizer.

Single and Double Knockdown of BMP Antagonists

To assess the requirement for BMP antagonists in normal development, we used MOs to knock down gene function in whole embryos (Figure 2). We first compared the phenotypes of *chordin*, *noggin*, or *follistatin* individual knockdowns and then in combination. We examined the expression of *sox2*, which marks the neural plate, in the neurula (st 14–15) embryo. In *noggin* morphants, *sox2* is unaffected (Figures 2A–2C) and only minimally affected in *follistatin* morphants (Figures 2D–2F). However, as in *X. laevis,* the *sox2* domain is slightly narrowed in the *chordin* morphants, especially anteriorly (Figures 2G–2I) (Oelgeschlager et al., 2003).

Consistent with the idea that the antagonists have cooperative functions, all three double morphants show further reductions in the neural plate (Figures 2J–2R). In each case, any phenotype in the neural plate can be rescued by injection of heterologous *noggin* mRNA, suggesting that the MOs are acting specifically. In each of these experiments (single, double, and triple morphants), we injected a constant total mass of MOs (60 ng) regardless of the number of genes targeted. Therefore, we can conclude that the antagonists must synergize to some extent because the neural plates are reduced in double morphants. However, a significant fraction of the neural plate persists, consistent with previous experiments in the mouse and zebrafish (Stern, 2004). Thus, either other signaling pathways are responsible for specifying the majority of the neural plate or additional required BMP antagonist activity persists.

Triple Knockdown and Neural Plate

To address these two possibilities, we injected MOs targeted against *follistatin*, *chordin*, and *noggin* in combination (FCN morphants). For comparison, we also injected embryos with a β-catenin MO, which prevents formation of Spemann's organizer (Heasman et al., 2000; Khokha et al., 2002). Loss of β-catenin signaling eliminates the neural plate and dorsal mesoderm (Heasman et al., 1994); therefore, we can compare FCN morphants with β-catenin morphants with regard to dorsal fate specification. Both FCN morphants and β-catenin morphants lack a morphological neural plate at neurula stages. Expression of *sox2* is restricted to a rim of tissue around the blastopore in both triple morphants and β-catenin morphants (Figures 3A–3D). Sox3, another marker of the neural plate at neurula stages, is also restricted to a rim of tissue surrounding the blastopore lip in triple and β-catenin morphants (Figures 3E–3H). Normally, *sox3* is expressed broadly in the blastula and then becomes restricted to the neural plate. Therefore, the extinction of *sox3* in FCN morphants indicates that development is not significantly delayed. These results strongly suggest that BMP antagonism is required for specification of the neural plate.

Triple Knockdown and Dorsal Mesoderm

Dorsal mesodermal tissues were also eliminated in FCN morphants and β-catenin MO-injected embryos. We tested *myf5* and *myoD*, which are expressed in the so-

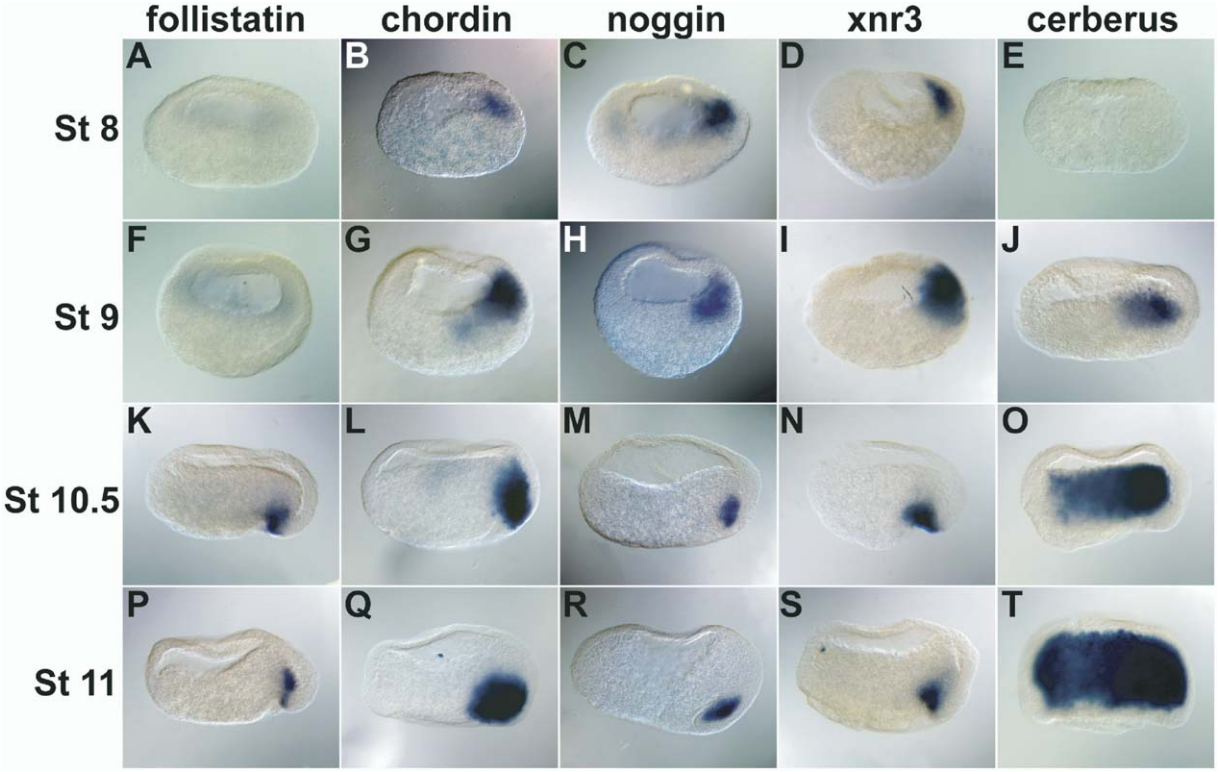

Figure 1. BMP Antagonists Are Expressed in Spemann's Organizer

Blastula and gastrula embryos were bisected, stained, and then cleared to visualize expression of *follistatin* (A, F, K, and P), *chordin* (B, G, L, and Q), *noggin* (C, H, M, and R), *xnr3* (D, I, N, and S), and *cerberus* (E, J, O, and T) at midblastula (A–E), late blastula (F–J), early gastrula (K–O), and midgastrula (P–T). Dorsal is to the right with the animal pole toward the top of the figure. St = stage.

mites, *sonic hedgehog (shh)* expressed in the floorplate and notochord, and *xnot*, which progressively narrows from an initial broad domain to the notochord. All of these transcripts are reduced or absent in both FCN and β-catenin morphants at neurula stages (Figures 3I–3X). Therefore, BMP antagonists are also required for the development of the dorsal mesoderm, the somites, and notochord.

Rescue of the FCN Morphant Phenotype

In order to show that these results are specific to a reduction in BMP antagonism, we performed two rescue experiments. First the FCN morphants were secondarily injected with pufferfish *noggin* mRNA, whose sequence is different from *Xenopus*. Triple morphants injected with *noggin* mRNA develop rescued neural plates and dorsal mesoderm (Figures 4A–4J). Also, if the phenotype of the triple morphant is due to MO toxicity, then additional MOs should exacerbate the effect. Instead, in a second rescue experiment, we subsequently injected additional MOs that are targeted against BMP4 and BMP7 (to attenuate BMP signaling) into half of the FCN morphant embryo and this results in a partial rescue of the neural plate (Figures 4K–4M). These experiments show that the phenotype of the FCN morphant is specific to a loss of BMP antagonism. Therefore, we conclude that BMP antagonists are required for the specification of dorsal structures and not just for fine tuning the size of the neural plate and dorsal mesoderm.

Triple Knockdown and Ventral Tissues

To address whether the loss of BMP antagonists causes a respecification of tissues, we examined ventral and posterior tissues in embryos depleted of three BMP antagonists. BMP signaling activates *BAMBI* and *msx1* in a ventral and posterior domain of the neurula (Suzuki et al., 1997; Onichtchouk et al., 1999). In β-catenin-injected embryos, *BAMBI* and *msx1* are greatly expanded and expressed circumferentially around the embryo, indicating that the embryo is ventralized (Figures 5C, 5D, 5G, 5H, 5S, 5T, 5W, and 5X). In FCN morphants, *BAMBI* and *msx1* are also greatly expanded (Figures 5A, 5B, 5E, 5F, 5Q, 5R, 5U, and 5V) though a dorsal strip of tissue expresses neither *BAMBI* nor *msx1*. *Sizzled* is a marker of the extreme ventral and posterior mesoderm (Collavin and Kirschner, 2003) and is greatly expanded in β-catenin-injected embryos and also in the triple morphants (Figures 5A, 5I–5L, 5Y, and 5Z). As with *BAMBI* and *msx1*, however, *sizzled* expression is absent dorsally in the triple morphants (Figures 5I, 5J, 5U, and 5V). At this stage, prior to the completion of neurulation, the epidermis is located ventrolaterally in a broader region than BAMBI or msx1 and abuts the neural plate. The panepidermal marker cytokeratin is expressed throughout the FCN morphants (Figures 5M–

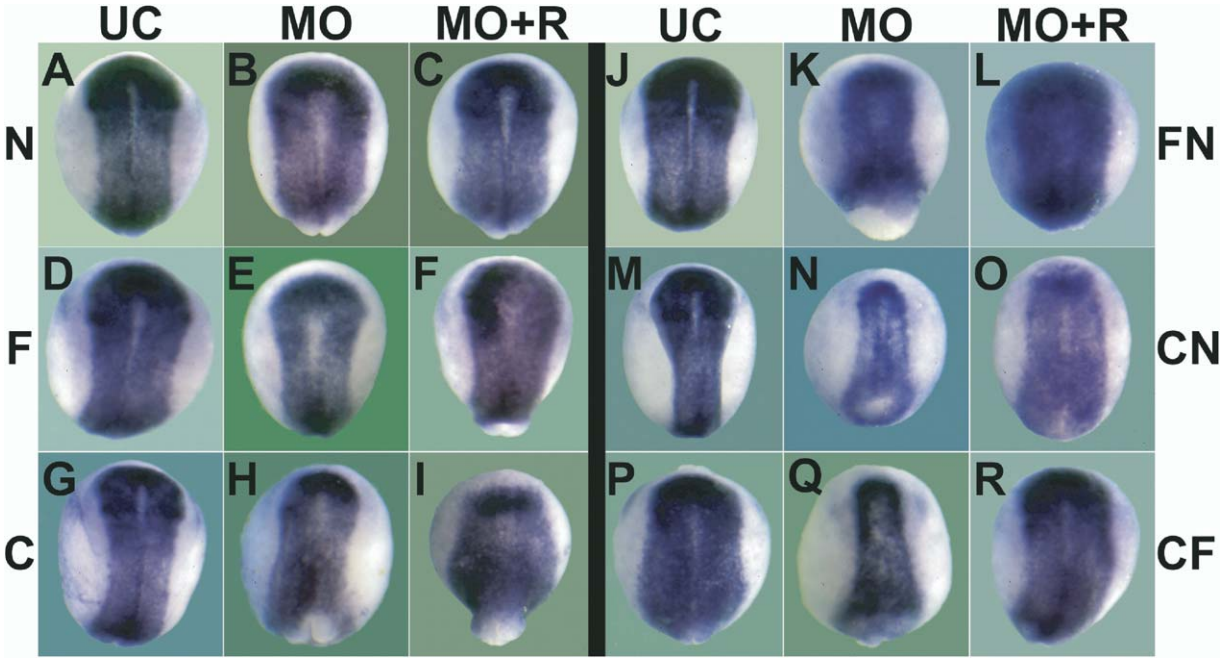

Figure 2. Single Morphants and Double Morphants Still Form a Substantial Neural Plate

All panels show *sox2* expression to visualize the neural plate (dorsal views with anterior to the bottom) in neurula embryos (st 14–15). Uninjected embryos are shown (A, D, and G) with sibling noggin morphants (B), follistatin morphants (E), and chordin morphants (H). Double morphants include follistatin/noggin (K), chordin/noggin (N), and chordin/follistatin (Q) and are shown with sibling uninjected embryos (J, M, and P). Morphants were rescued with pufferfish noggin (C, F, I, L, O, and R). Abbreviations are as follows: F = follistatin, C = chordin, N = noggin, UC = uninjected sibling control embryos, MO = morphant, and MO + R = morphant rescued with noggin mRNA.

5P and 5C′–5F′). These results demonstrate that there is indeed a large expansion of the ventrolateral and posterior territories. However, an incompletely ventralized strip of tissue, which expresses an epidermal marker, persists in the FCN morphants when compared to the β-catenin morphants. This difference between the β-catenin-depleted embryos and the FCN embryos suggests some residual organizer or β-catenin signaling.

Organizer versus Nieuwkoop Center

To address the differences between FCN morphants and β-catenin morphants, we examined expression of genes at the early gastrula stage. In the current understanding of early *Xenopus* patterning, the Nieuwkoop center acts early in development to induce the organizer in the marginal zone (Gimlich and Gerhart, 1984). VegT, Smad2, and β-catenin signals cooperate to establish the organizer (Wylie et al., 1996; Zhang et al., 1998; Heasman et al., 2000; Whitman, 2001; Xanthos et al., 2002). BMP antagonists expressed in the organizer then may affect subsequent patterning. In contrast, reduction of β-catenin ablates the Nieuwkoop center, and Spemann's organizer never forms. We therefore tested whether FCN morphants would differ from β-catenin morphants in organizer establishment. In the early gastrula (st 10.5) a number of markers are expressed in the mesoderm in a regionally specific manner. *Xbra* is expressed radially in the mesoderm of both FCN morphants and the β-catenin morphants (Figures 6A–6D). *Goosecoid* (*gsc*), an early marker of the organizer, is expressed normally in the FCN morphants but is absent in the β-catenin MO-injected embryos (Figures 6E–6H). Two additional targets of β-catenin signaling, *siamois* and *xnr3* (Brannon et al., 1997; McKendry et al., 1997), are expressed in the organizer of triple morphants but are absent in the β-catenin morphants (Figures 6I–6P). These results illustrate that early signaling establishes the organizer normally in the early gastrula despite the reduction in BMP antagonism. However, *vent2*, a BMP target (Rastegar et al., 1999) that is normally excluded from the organizer, is radially expressed in both the triple morphants and β-catenin-injected embryos (Figures 6Q–6T). This illustrates a very early consequence of loss of antagonists, which we interpret to mean that BMP signals are present in the dorsal marginal zone but normally suppressed by antagonists. In addition, *myf5*, which marks the dorsolateral mesoderm in the midgastrula embryo, is greatly reduced in triple morphants (Figures 6U–6X). This observation shows the importance of suppressing BMP signals for future muscle development and rules out the possibility that myf5 and muscle development only require the graded activity of nodal signaling. We therefore conclude from these experiments that even at this early stage, loss of BMP antagonism affects patterning of the mesoderm, and in FCN morphants, dorsal mesoderm is already developing a ventro-lateral character.

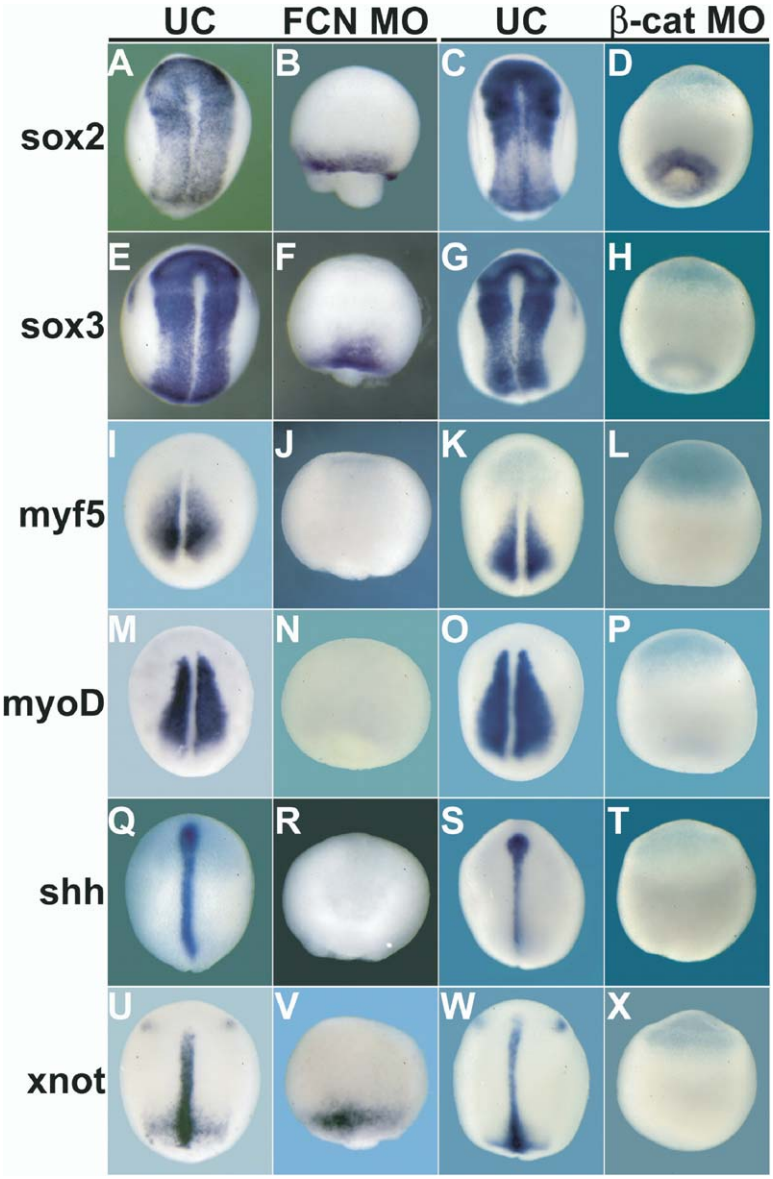

Figure 3. FCN Morphants Have a Catastrophic Loss in Dorsal Development

All panels are dorsal views with anterior to the top of st 14–15 neurula embryos. Expression of multiple dorsal markers (*sox2* [A–D], *sox3* [E–H], *myf5* [I–L], *myoD* [M–P], *shh* [Q–T], and *xnot* [U–X]) are shown in uninjected sibling embryos (A, C, E, G, I, K, M, O, Q, S, U, and W) and follistatin, chordin, and noggin (FCN) triple morphants (B, F, J, N, R, and V) as well as β-catenin morphants (D, H, L, P, T, and X) for comparison.

Discussion

In this study, we demonstrate an essential, cooperative role for BMP antagonists in Spemann's organizer for normal dorsal development. Embryos that cannot effectively reduce the BMP signal on their dorsal side develop excessive amounts of ventral tissue at the expense of dorsal structures. However, early signaling by the Nieuwkoop center appears unaffected, and the initial specification of the organizer occurs normally.

The level of redundancy in BMP antagonists is surprising; at least five antagonists are expressed in the organizer and are assisted by other more generally expressed genes, such as *twisted gastrulation* and *tsukushi* (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001; Blitz et al., 2003; Ohta et al., 2004). In addition, FGFs and β-catenin have been shown to antagonize BMP signaling indirectly through transcriptional regulation (Wilson et al., 2000). Because so many of these activities can cooperate to generate appropriate pattern, loss of any one antagonist has minimal effects on dorsal development as shown in experiments with zebrafish, mouse, and *Xenopus*. In the mouse, elimination of two antagonists has more severe but still relatively minor effects on neural plate formation, which is similar to our results in the frog. However, our results would suggest that elimination of an additional BMP antagonist in the mouse might also lead to a catastrophic loss of dorsal structures.

There are well-documented examples of overlaps of gene function in development, such as that between *myf5* and *myoD* in muscle cell specification (Rudnicki et al., 1993) or the nodal family members *cyclops* and *squint* in zebrafish mesoderm formation (Feldman et al.,

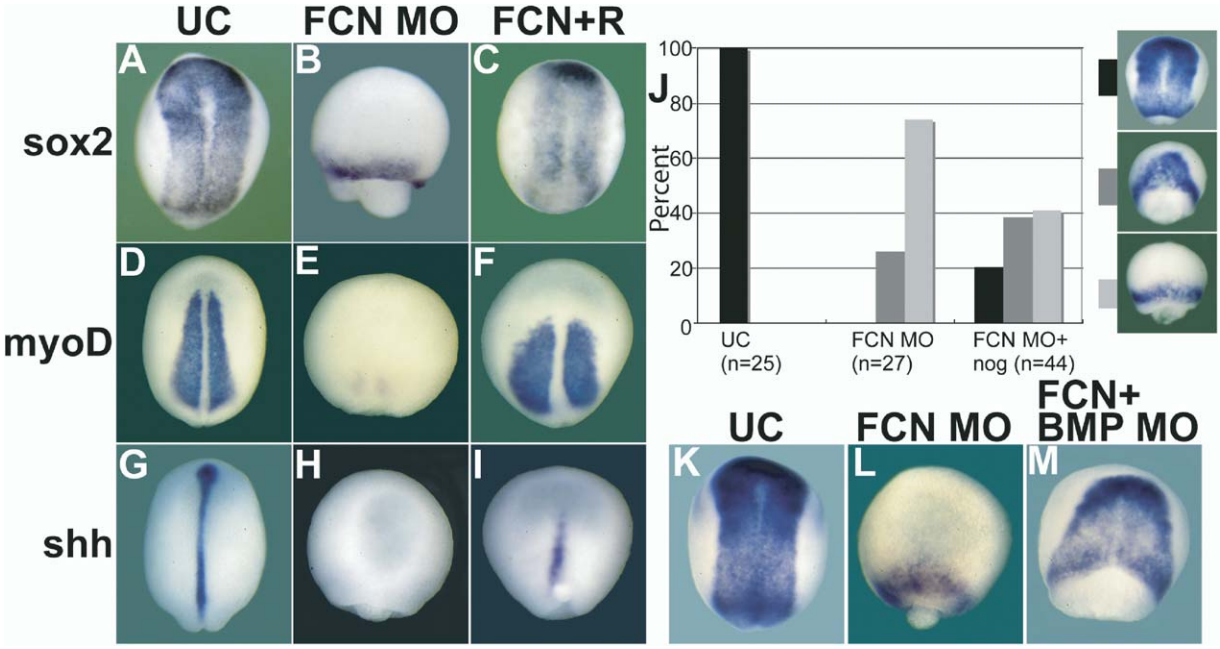

Figure 4. The Phenotype of the FCN Triple Morphant Is Specific to a Loss in BMP Antagonism

All panels are dorsal views of neurula embryos (st 14) with anterior to the top. Triple morphants were subsequently injected with pufferfish noggin mRNA (C, F, and I) and show substantial rescue of dorsal structures (*sox2* [A–C], *myoD* [D–F], and *shh* [G–I]) when compared to triple morphants (B, E, and H) and uninjected sibling embryos (A, D, and G). The distribution of phenotypes seen in these rescue experiments is depicted in a bar graph (J). The vertical axis is the percent of embryos that show a particular phenotype. The horizontal axis shows the experimental groups and the phenotypes seen. The black bar depicts the percentage of embryos with a substantial neural plate, the dark gray bar depicts the percentage of embryos with a minor neural plate, and the light gray bar depicts the percentage of embryos with no neural plate that show only a ring of *sox2* expression. We also tested the phenotype of the triple morphant for specificity by subsequently injecting BMP4,7 MOs (BMP MO), which should reduce BMP signaling. A partial rescue of the neural plate is seen (M) compared to triple morphants (L) and uninjected sibling control embryos (K).

1998). These examples relied on the generation of double mutant animals, which can be quite laborious. Here, we show that the diploid frog, *X. tropicalis*, whose genome is well characterized, may be an ideal animal in which to test for redundancy in two or more genes and overlap in gene function by using morpholino oligonucleotides.

Contributions of Different BMP Antagonists

Knockdown of two antagonists has effects, but the neural plate still forms. However, once three antagonists are inhibited, there is a catastrophic failure of organizer function. Therefore, because dorsal structures are present in the double knockdowns but completely eliminated in the triple, any one BMP antagonist does have a substantial effect on dorsal development provided it is in an appropriate context, i.e., loss of other BMP antagonists. Therefore, this suggests that these BMP antagonists do have overlapping and cooperative functions that are critical for dorsal development.

Based on the expression of ventral-posterior genes, β-catenin morphants appear cylindrically symmetrical without any evidence of dorsal structures. On the other hand, in the FCN morphants, ventral-posterior transcripts are cleared on one side of the embryo even though this region does express an epidermal marker. The mechanism of this clearing may be that other BMP antagonists, such as Xnr3 and Cerberus, may be expressed in sufficient amounts to prevent extreme ventral-posterior fates but insufficient for dorsal cell fates such as the neural plate. Therefore, even with severe loss of BMP antagonism, remaining BMP antagonists may still prevent the most ventral fates from forming. Alternatively, these incompletely ventralized fates may be the consequence of early β-catenin signaling or FGF function.

BMP Antagonists and Neural Specification

We were surprised to see a residual ring of *sox2* and *sox3* expression in the β-catenin-depleted embryos, because these transcripts are often used to score definitive neural tissue. In β-catenin-depleted embryos, neural tissues do not develop (Heasman et al., 1994), implying that this early *sox*-expressing region is not definitive neural tissue. Thus, the early phase of Sox2 and Sox3 marks the competence or potential to become neural. Embryos in which BMP antagonists are depleted show a similar residual ring of *sox2*- and *sox3*-expressing tissue.

Our results also have implications for the timing of neural induction and/or maintenance. Previous work has suggested that neural specification may occur prior to gastrulation or the establishment of Spemann's organizer (Streit et al., 2000; Kuroda et al., 2004). However, in our experiments, gastrulation is a critical time for the continuation of neural specification. Follistatin

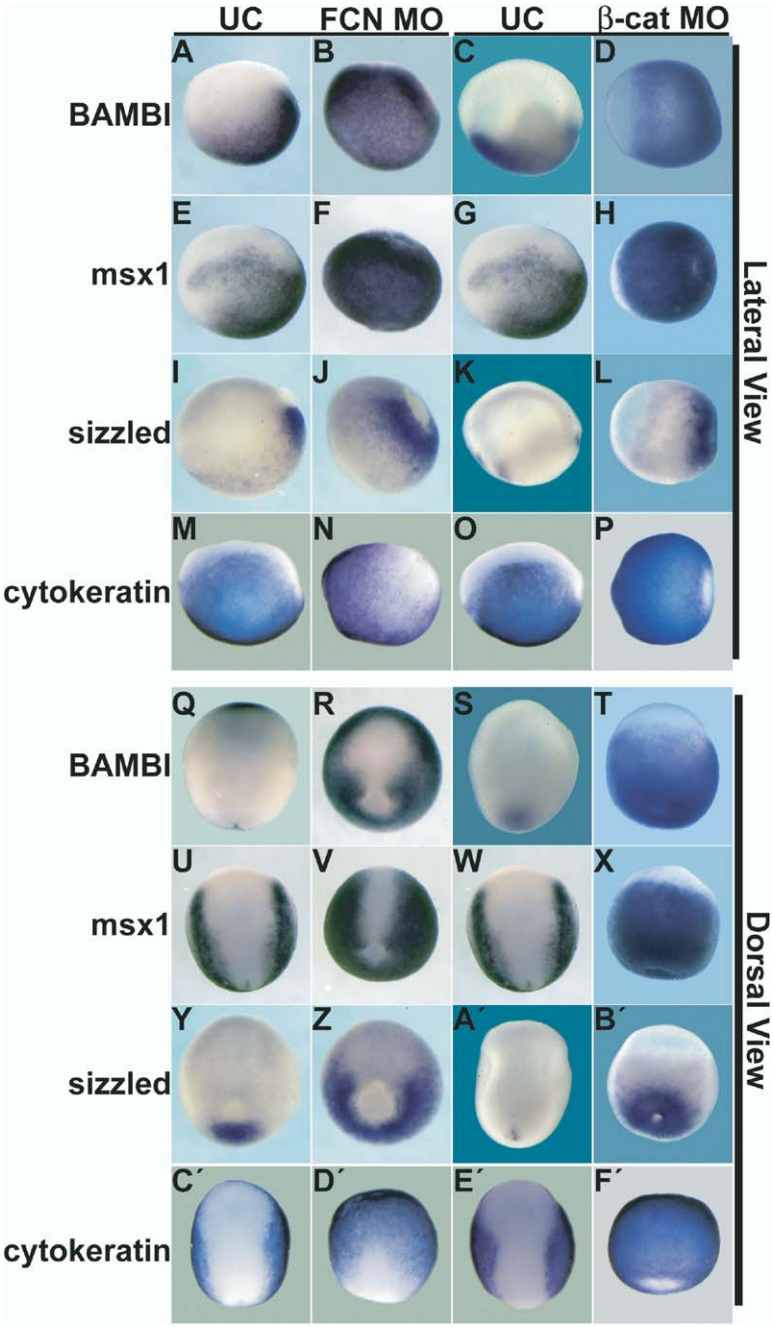

Figure 5. Ventral Tissues Are Expanded in FCN Morphants

(A–P) are lateral views with anterior to the left of neurula (st 13–14) embryos, whereas (Q–F′) are dorsal views with anterior to the top. Expression of multiple ventral markers (*BAMBI* [A–D and Q–T], *msx1* [E–H and U–X], *sizzled* [I–L and Y–B′], and cytokeratin [M–P and C′–F′]) are shown in uninjected sibling embryos (A, C, E, G, I, K, M, O, Q, S, U, W, Y, A′, C′, and E′) and FCN triple morphants (B, F, J, N, R, V, Z, and D′) as well as in β-catenin morphants (D, H, L, P, T, X, B′, and F′) for comparison.

is not expressed until st 10, the onset of gastrulation (Figures 1F and 1K). Yet, comparing the chordin/noggin double morphant (Figure 2N) to the triple morphant (Figure 3B) clearly shows that follistatin is required for specification of the neural plate. Therefore, continuing BMP antagonism during gastrulation is important for normal neural specification.

Early Effects of BMP Antagonists

Finally, the effects of the BMP antagonists are evident at the onset of gastrulation, as revealed by the expansion of *vent2* expression and the reduction in *myf5* expression in FCN morphants. Transcripts of Xnr3, noggin, and chordin are present at the midblastula stage, though they are expressed more strongly at the onset of gastrulation. Our results imply that this early phase of expression of BMP antagonists is important in the early patterning of the gastrula. The results reinforce the idea that organizer signals are required for patterning the marginal zone and argue against the idea that early graded nodal signaling is sufficient for the initial patterning of the prospective muscle region (Agius et al., 2000).

Results in other model systems have shown relatively minor consequences with loss of BMP antagonism on early development, and this had led to the idea that

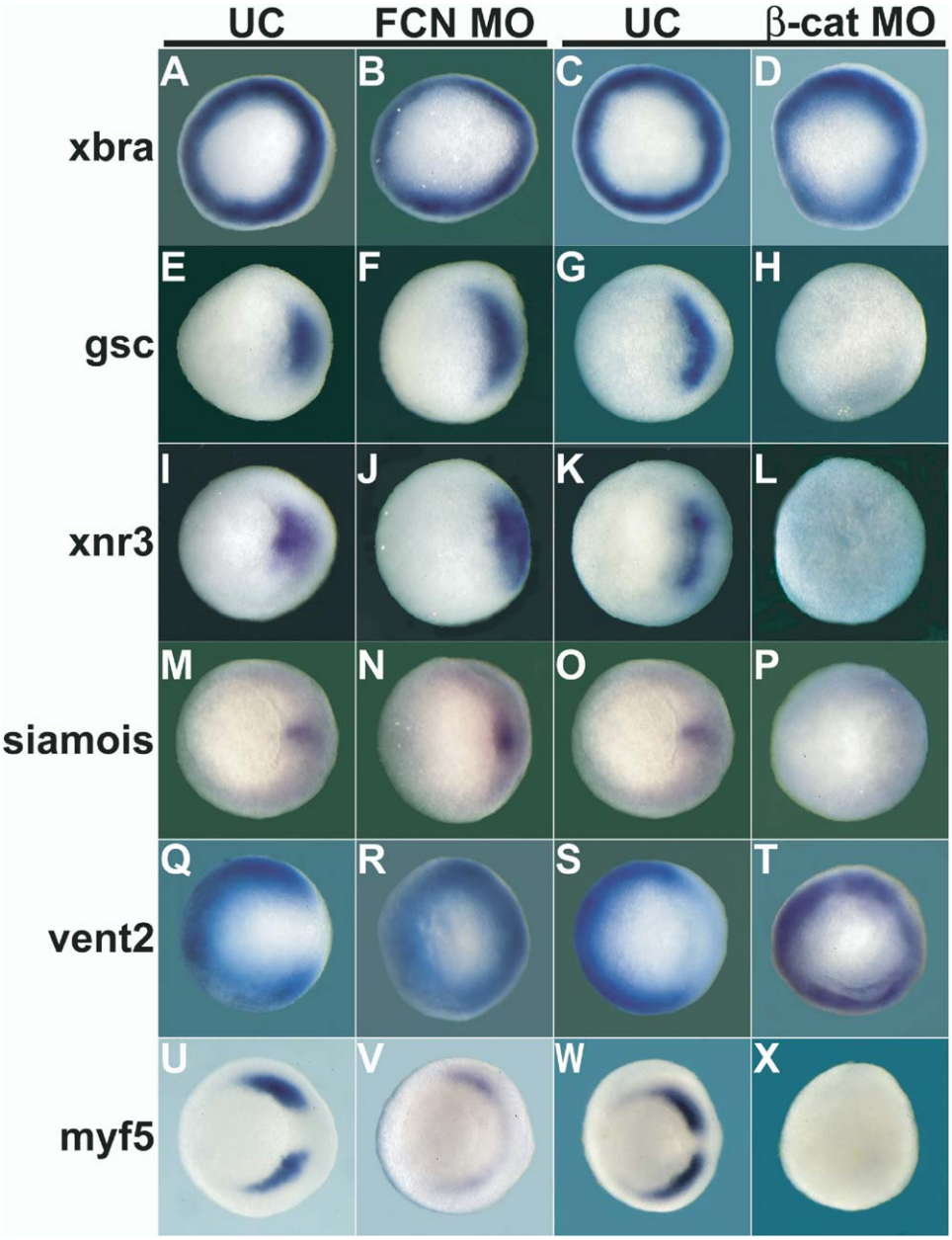

Figure 6. Spemann's Organizer Is Specified in FCN Morphants

All panels are vegetal views of early gastrula embryos (st 10.5 [A–T]) or midgastrula (st 11 [U–X]) with dorsal to the right in embryos where dorsal can be distinguished. Expression of multiple mesodermal markers (brachyury [xbra] [A–D], goosecoid [gsc] [E–H], xnr3 [I–L], siamois [M–P], vent2 [Q–T], and myf5 [U–X]) are shown in uninjected sibling embryos (A, C, E, G, I, K, M, O, Q, S, U, and W) and FCN triple morphants (B, F, J, N, R, and V) as well as β-catenin morphants (D, H, L, P, T, and X) for comparison.

BMP antagonism is not required for neural induction or development of the somites. In this study, we show that the overlapping function of BMP antagonists is essential in Spemann's organizer for normal dorsal development. Early signaling is unaffected, and the initial specification of the organizer occurs normally. However, embryos that cannot effectively reduce the BMP signal on their dorsal side develop excessive amounts of ventral tissue at the expense of dorsal structures. Therefore BMP antagonism is an essential signal from Spemann's organizer that defines dorsal structures in the developing vertebrate embryo.

Experimental Procedures

Frog Husbandry

X. tropicalis were obtained from NASCO and represent F5 inbred Nigerians from the University of Virginia stocks. Animals were housed and fed in a temperature-controlled environment. (see http://tropicalis.berkeley.edu/home for details). We identified ma-

ture females by the presence of a cloaca and mature males by the presence of dark nuptial pads.

Microinjection

Both male and female *X. tropicalis* were primed with 10 units (U) of human chorionic gonadotropin (hCG) 12–24 hr prior to ovulation. Mating was induced with 100 U of hCG in males and 200 U of hCG in females. Approximately 3–5 hr after the injection, females would begin to lay eggs.

Males were euthanized by immersion in benzocaine. Testes were harvested, put in ice cold, fresh L15 supplemented with calf serum to approximately 10%, and crushed. Eggs were gently squeezed out of females. The sperm suspension was applied to the eggs and after two minutes, the eggs were flooded with 1/9 × MR (Modified Ringer's) + 3% Ficoll.

25 min later, the jelly coats of the eggs were removed by immersion in 3% cysteine (pH ~8). Once the jelly coats were removed, the eggs were rinsed in 1/9 × MR and then placed in 1/9 × MR + 3% Ficoll and were ready for injection. *X. tropicalis* embryos have a sticky vitelline envelope so dishes were coated with a thin layer of 1% agarose to avoid shearing the embryos.

X. tropicalis embryos were injected at either the one-cell stage with 3–4 nl of a MO solution, or both cells were injected at the two-cell stage with 1–2 nl of MO solution. Larger volumes were toxic. Also, significant cooling of the embryos (<18°C) to lengthen the cell cycle was toxic so embryos were kept at room temperature.

After injection, the embryos were left in 1/9 × MR + 3% Ficoll for 1–3 hr and then transferred to agarose-coated dishes with 1/20 × MR supplemented with 10 μg/ml of gentamicin. For additional details see http://tropicalis.berkeley.edu/home. Embryos were raised at 22–28°C. Because many of the morphological criteria used in staging embryos were absent in morphants, uninjected sibling embryos were used for staging.

MOs and mRNA Injection

MOs were obtained from Genetools, LLC. All MOs used in this study were targeted against the 5′UTR and/or the start site of transcription. The sequences of the MOs were as follows: chordin, 5′-CAAAGCATTTTTGTGGTAGCCCCGA-3′; noggin, 5′-CACAAGGCAC TGGGAATGATCCATG-3′; and follistatin, 5′-AGAAGCAGCAGAGT CTCAAGTGGAG-3′. MOs were suspended in diethylpyrocarbonate treated 1/9 × MR at a concentration of 4 mM. MOs were injected with miniruby (Molecular Probes) as a lineage tracer. Prior to injection, MOs were warmed to 55°C for 5 min and then kept at 37°C until injection, which helped prevent clogging of microinjection needles. Pufferfish *noggin* was linearized with KpnI and transcribed with Sp6 RNA polymerase (Bauer et al., 1998). GFP mRNA was coinjected with *noggin* mRNA as a tracer.

In order to make effective comparisons of the activity and potential toxicity of MOs, we used a standard 60 ng dose of MO per embryo for all experiments. In experiments where multiple MOs were injected, this 60 ng dose was divided equally among the MOs injected (i.e., if noggin and chordin MOs were injected, then 30 ng of noggin and 30 ng of chordin MO were used). In the rescue experiment with BMP MOs, 20 ng of BMP4 and 20 ng of BMP7 were injected into the two dorsal blastomeres at the four-cell stage of FCN morphant embryos (total MO dose = 100 ng/embryo).

Whole Mount In Situ Hybridization

Whole mount in situ hybridization was done according to the standard protocol with minor modifications (Harland, 1991; Khokha et al., 2002). Embryos were exposed to BM Purple for development of the stain for 4–24 hr at either 22°C or 4°C except st 8 embryos hybridized with noggin or chordin antisense probe. These embryos were exposed to BM Purple for 36–48 hr at 4°C in order to detect these transcripts. Probes from the MRC Geneservice are described further at http://tropicalis.berkeley.edu/home.

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