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Hes6 is required for *MyoD* induction during gastrulation

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Abstract

The specification of mesoderm into distinct compartments sharing the same lineage restricted fates is a crucial step occurring during gastrulation, and is regulated by morphogenic signals such as the FGF/MAPK and activin pathways. One target of these pathways is the transcription factor *XmyoD*, which in early gastrulation is expressed in the lateral and ventral mesoderm. Expression of the hairy/enhancer of split transcription factor *hes6*, is also restricted to lateral and ventral mesoderm in gastrula stage *Xenopus* embryos, leading us to investigate whether it has a role in *XmyoD* regulation. *In vivo*, Xhes6 is required for FGF-mediated induction of *XmyoD* expression but not for induction of early mesoderm. The WRPW domain of Xhes6, which binds Groucho family transcriptional co-regulators, is essential for the *XmyoD*-inducing activity of *Xhes6*. Two Groucho proteins, Xgrg2 and Xgrg4, are expressed in lateral and ventral mesoderm, and inhibit expression of *XmyoD*. Xhes6 binds both Xgrg2 and Xgrg4 and relieves their inhibition of *XmyoD* expression. We also find that lowering *Xhes6* expression levels blocks normal myogenic differentiation at tail bud stage. We conclude that Xhes6 is essential for *XmyoD* induction and acts by relieving Groucho-mediated repression of gene expression.

Keywords: *hes6*; *Xenopus*; Patterning; Gastrulation; Groucho

Introduction

The specification of mesoderm into spatially defined compartments that will develop into different tissues is a crucial process occurring in early gastrulation. An early marker of mesodermal specification is the transcription factor *XmyoD*, which in early to mid gastrula embryos identifies the lateral mesoderm that will give rise to the somites and ventral marginal zone mesoderm which later forms the lateral plate mesoderm, mesenchyme and blood (Frank and Harland, 1991; Hopwood and Gurdon, 1990; Hopwood et al., 1991; Scales et al., 1990). The expression of *XmyoD* is regulated by mesoderm-inducing/patterning signals such as members of FGF, TGFβ/activin and Wnt protein families. A key challenge in understanding mesodermal specification is to determine how such signals are integrated to generate distinct mesodermal domains such as the future myotome.

Three mesodermal-inducing factors, FGF, activin and Xwnt8 have been shown to regulate expression of *XmyoD* (Cornell and Kimelman, 1994; Fisher et al., 2002; Hoppler et al., 1996). As gastrulation proceeds, FGF/MAP kinase (MAPK) signaling is further required for dorsal/ventral patterning of mesodermal fate within the domain of *XmyoD* expression, inhibiting lateral mesoderm from adopting blood island fate (Isaacs et al., 2007; Kumano et al., 2001; Kumano and Smith, 2000). *FGF3*, *FGF 8* and *FGF4* (also known as *eFGF*) are all expressed in the mesoderm of early gastrula embryo (Fisher et al., 2002; Isaacs et al., 1995; Lombardo et al., 1998). There is strong evidence for eFGF in particular being essential for regulating expression of *XmyoD*, as blocking translation of eFGF protein or inhibiting FGF signaling with the pharmacological inhibitor SU5402 results in a dramatic downregulation of *XmyoD* transcription (Fisher et al., 2002; Isaacs et al., 2007). Unique among mesodermal-inducing factors, eFGF is responsible for the

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"community effect", where myogenic differentiation requires clusters of early muscle precursors. Without eFGF signaling from adjacent precursor cells, terminal muscle differentiation is blocked (Standley et al., 2001). Furthermore, phosphorylated ERK, a target of FGF signaling, is detectable in the region of *XmyoD* expression (Christen and Slack, 1999). More recently, it has been shown that FGF8 is required for normal induction of *XmyoD* at gastrulation in *Xenopus* and also controls *myoD* expression in somite stage *Zebrafish* embryos (Fletcher et al., 2006; Groves et al., 2005; Hamade et al., 2006). These observations provide convincing evidence for regulation of *XmyoD* expression by FGF. Activin can induce both the pan-mesodermal marker *Xbra* and *XmyoD* but it requires an intact FGF signaling pathway to do so (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). Likewise, *Xwnt8* is necessary for *XmyoD* expression but in embryos in which FGF signaling has been blocked, *XmyoD* is not induced, despite *Xwnt8* levels being normal (Hoppler et al., 1996; Isaacs et al., 1994).

The mesodermal compartments in the early gastrula embryo have clearly defined boundaries, for example between the lateral and ventral mesoderm and the notochord. However, little is known about how diffusible morphogens are able to demarcate mesodermal compartments. One possible explanation is that the effects of morphogens are modulated by regulatory factors whose expression is confined to specific mesodermal compartments. Such factors may act to adjust the level of mesoderm-inducing activity above or below the threshold levels required to specify the appropriate subtype of mesoderm (Green and Smith, 1990; Umbhauer et al., 1995).

Candidate regulators of *XmyoD* expression include *Hairy/Enhancer of split-related* (*hes*) genes, which encode a family of basic helix–loop–helix (bHLH) type transcriptional regulators. Hes proteins share highly conserved domains, including a basic region of the bHLH domain, containing a characteristic proline residue and a C-terminal WRPW motif that binds to the transcriptional repressor Groucho/TLE family of proteins (Dawson et al., 1995; Grbavec and Stifani, 1996; Paroush et al., 1994). Several members of the family, such as *hes1*, *hes5* and *hes7* are direct targets of the Notch signaling pathway, whilst others such as *hes6* are not regulated by Notch, but are induced by FGF signaling (Kawamura et al., 2005; Koyano-Nakagawa et al., 2000). Hes6 also differs from other hes proteins in having a shorter loop within its DNA binding domain. This feature is highly conserved between mammalian Hes6 and its *Xenopus* homologue, Xhes6, that confers DNA binding properties distinct from other Hes proteins (Bae et al., 2000; Cossins et al., 2002; Koyano-Nakagawa et al., 2000). Overexpression of Xhes6 in *Xenopus* embryos expands the myotome but blocks myogenic terminal differentiation at the tail bud stage (Cossins et al., 2002).

We have previously observed that both *Xhes6* and *XmyoD* share the same pattern of gene expression in early gastrula stage *Xenopus* embryos (Cossins et al., 2002). This observation, combined with the strong body of evidence that *XmyoD* is induced by FGF signaling in gastrulation, led us to speculate that, as well as being induced by the morphogen FGF (Kawamura et al., 2005), Xhes6 may have a novel role in regulating FGF-induced gene expression within the mesodermal compartment. To test this hypothesis we first showed that expression of both *Xhes6* and *XmyoD* requires FGF signaling. We then investigated the effect of Xhes6 function on mesoderm specification, both *in vitro*, in the well-defined ectodermal explant (animal cap) assay system and *in vivo*, using *Xhes6* morpholino oligonucleotides (MO) to knock down expression of Xhes6 protein. We find that Xhes6 acts by relieving Groucho-mediated inhibition of *XmyoD* expression, permitting induction of *XmyoD* by FGF/MAP kinase signaling. This function is mediated by the ability of Xhes6 to bind Groucho family transcriptional repressor proteins Xgrg2 and Xgrg4.

Results

Xhes6 expression in the lateral and ventral mesoderm is regulated by FGF and nodal signaling

As reported previously, *Xhes6* is co-expressed with *XmyoD* in the lateral and ventral mesoderm of early gastrula stage embryos (Figs. 1A, B; Cossins et al., 2002). This led us to investigate if *Xhes6* expression was regulated by the same signaling pathways that regulate *XmyoD* transcription. Overexpression of mRNAs encoding either the TGFβ/Nodal family member, Xnr2, or eFGF resulted in an increased expression not only of *XmyoD*, as reported previously, but also of *Xhes6* (Figs. 1C–F) (Fisher et al., 2002; Isaacs et al., 2007; Kofron et al., 1999). Furthermore, inhibition of FGF signaling by overexpression of the dominant negative FGF receptor, XFD, abolished the expression of both *XmyoD* and *Xhes6* (Figs. 1G, H) (Amaya et al., 1991; Isaacs et al., 1994). Thus, *Xhes6* is a target of both nodal and FGF signaling, and requires FGF signaling for its expression, consistent with previous reports that a *hes6*-related gene is an FGF target in *Zebrafish* (Kawamura et al., 2005).

Xhes6 increases expression of XmyoD in vivo

The observation that *Xhes6* transcription is both colocalised with *XmyoD* expression and is regulated by the same mesodermal patterning signals led us to investigate whether Xhes6 plays a role in regulating *XmyoD* expression. Untagged and N-terminally myc-tagged Xhes6 proteins were found to have equivalent effects on *XmyoD* expression (data not shown). Myc-tagged forms of Xhes6 were used in the experiments reported here. We began by injecting embryos with mRNA encoding wild-type and mutant forms of Xhes6 and assaying the expression of *XmyoD* together with the mid mesodermal marker *Xbra*, the ventro-lateral mesodermal marker *Xwnt8* and the dorsal lip mesoderm marker *Chordin* at early gastrula stage. We found that overexpression of Xhes6 results in modest induction of *XmyoD* (Fig. 2D) and smaller increases in *Xbra* and *Xwnt8* expression (Figs. 2H, J), but had no effect on the *chordin* expression (Fig. 2L). The zone of ectopic *XmyoD* expression remains confined to mesoderm, suggesting that Xhes6 requires additional factors whose expression is restricted to the

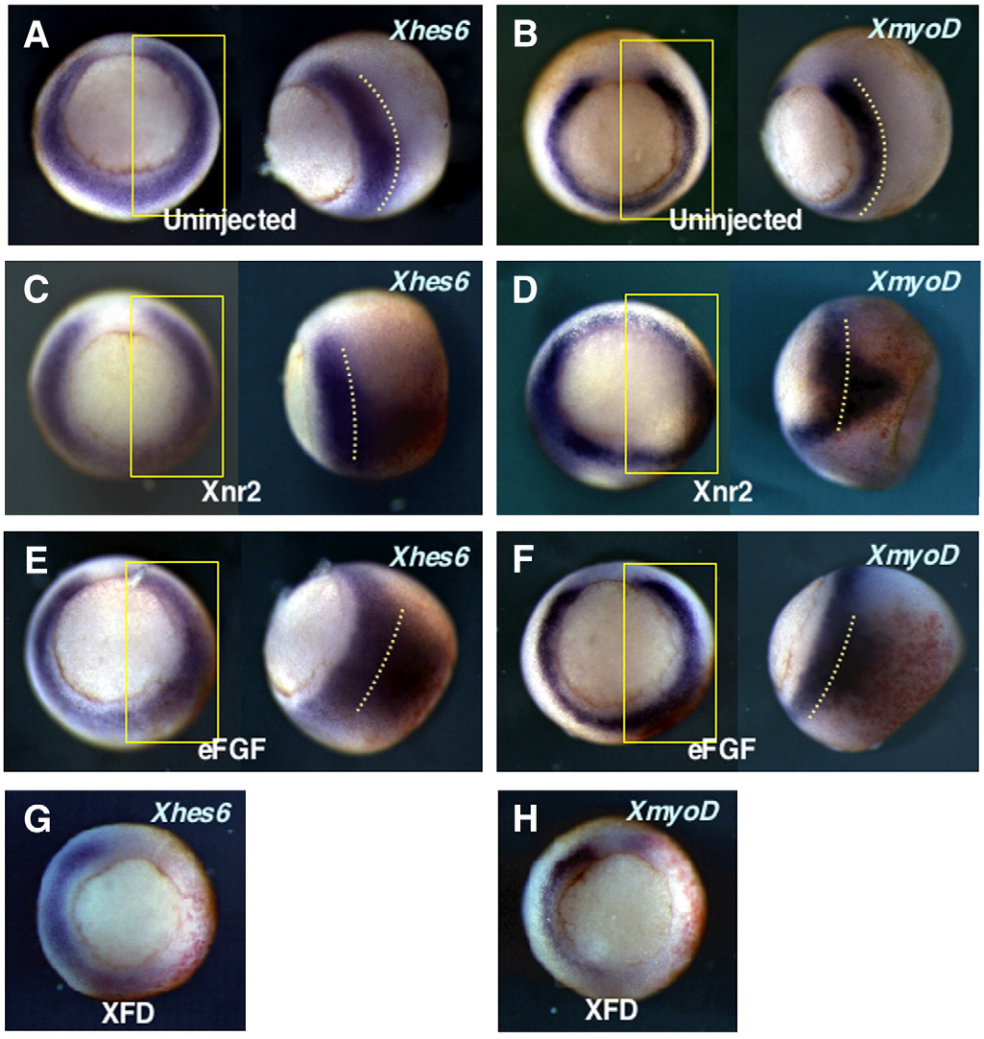


Fig. 1. *Xhes6* is induced by mesoderm inducing signals and its expression is dependent on FGF-signaling. 5 pg of Xnr2 (C, D), 1 pg of eFGF (E, F) or 125 pg of XFD (G, H) mRNA was injected into a blastomere at 2-cell stage, along with β-galactosidase mRNA (red staining) and analyzed at gastrula stage for expression of *Xhes6* (A, C, E and G) and *XmyoD* (B, D, F and H) by whole-mount *in situ* hybridisation. The side view of the area within the yellow box is shown on the right of each panel.

mesoderm to upregulate *XmyoD* expression rather than being able to induce mesoderm directly.

Next we investigated which domains of Xhes6 were required for its activity using the mutants shown in Fig. 2A (Cossins et al., 2002; Koyano-Nakagawa et al., 2000). Expression of each of the myc-tagged constructs was readily detectable when the corresponding mRNAs were injected into whole embryos, though ΔWRPW had a higher level of expression than either Xhes6 or DBM, consistent with previous reports (Fig. 2B) (Kang et al., 2005). The DNA binding mutant (DBM) of Xhes6 in which the basic amino acids have been mutated to acidic residues was found to have activity equivalent to wild-type Xhes6, indicating that direct DNA-binding is not absolutely required for mesoderm induction (Fig. 2E; Table 1). In contrast, a mutant in which the C-terminal WRPW motif had been deleted (ΔWRPW) had substantially reduced activity (Fig. 2F; Table 1), indicating that Xhes6 requires the Groucho-binding WRPW domain for significant regulation of *XmyoD* expression.

Xhes6 induces mesodermal gene expression in vitro

In parallel with the above *in vivo* studies, we investigated the ability of Xhes6 to induce mesodermal gene expression *in vitro*, using the well-characterized assay of mRNA microinjection into *Xenopus* embryos followed by dissection of animal pole explants (animal caps) at the blastula stage. When cultured in the absence of mesoderm-inducing factors, animal caps adopt an epidermal fate (Slack et al., 1987). When injected with Xhes6 mRNA, we found that both *Xbra* and *Xwnt8* were induced (Supplementary Figs. 1A, B), but there was no increase in expression of *chordin* (Supplementary Figs. 1C). Expression of *Mix1* and *Mix2*, markers of the mesoderm/endoderm boundary was only minimally increased whilst the endoderm markers such as *Mixer* and *Sox17alpha* were not induced (Supplementary Figs. 1D, E and data not shown).

Strikingly, the pattern of genes induced by Xhes6 in animal caps parallels that seen when caps are treated with FGF, which induces *Xwnt8*, but not changes in gene expression following

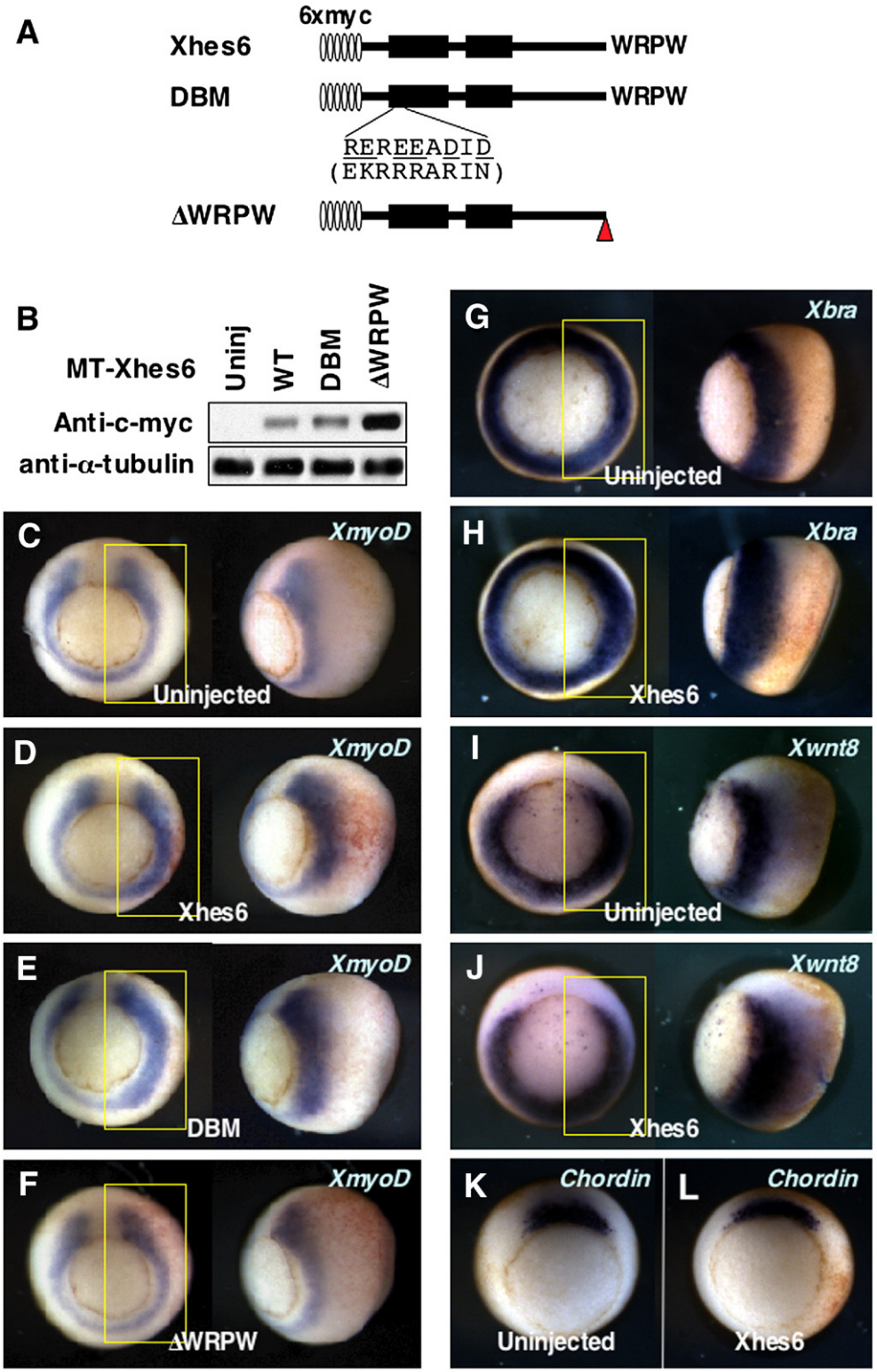


Fig. 2. Over-expression of Xhes6 increases *XmyoD* expression *in vivo*. (A) Constructs used. Xhes6 constructs were myc tagged at the N-terminus. In the DBM mutant, the basic amino acids in the DNA domain were mutated to acidic residues (underlined) whilst in the ΔWRPW mutant, the C-terminal WRPW protein–protein interaction motif was replaced by a stop codon (red triangle). (B) Western blotting with an anti-myc antibody to detect Xhes6 protein (upper panel) and an anti-α-tubulin antibody loading control (lower panel). (C–L) Effects of overexpression of wild-type and mutant Xhes6 500 pg of Xhes6, DBM or ΔWRPW mRNA was injected into marginal zone of a blastomere at 2-cell stage, along with β-galactosidase mRNA (red staining) and analyzed at gastrula stage for expression of *XmyoD* (C–F), *Xbra* (G, H), *Xwnt8* (I, J) and *Chordin* (K, L) by whole-mount *in situ* hybridisation. The side view of the area within the yellow box is shown on the right of each panel.

Table 1
Effect of overexpression of wild-type or mutant Xhes6 on *XmyoD* expression at gastrula stage

	Increased XmyoD expression (%)				Average (%)	Total (*n*)
	Exp. 1	2	3	4		
Xhes6	15/18 (83)	11/15 (73)	14/16 (88)	8/10 (80)	81	59
DBM	6/12 (50)	13/16 (81)	9/16 (56)	8/9 (89)	69	53
ΔWRPW	1/8 (12.5)	2/16 (13)	2/15 (13)	0/9 (0)	9.8	48
Injected RNA (pg)	125	125	250	125		
Injected stages	2-cell	4-cell	4-cell	8-cell		

treatment with activin, which induces, *chordin*, *mix1* and *mixer* (Supplementary Fig. 1).

Xhes6 enhances the effects of exogenous FGF

The above observations suggest that Xhes6 may cooperate with FGF signaling to induce mesodermal genes in animal caps. We therefore examined whether Xhes6 enhances the ability of FGF to induce mesoderm and *XmyoD* in animal caps. In these experiments, caps were treated with a low dose of FGF that produced minimal but detectable induction of *Xbra* (Fig. 3A). Both the wild-type and the DBM mutant of Xhes6 substantially enhanced the induction of *Xbra* by FGF (Fig. 3A), whilst the ΔWRPW mutant had a greatly decreased effect. A similar enhancement of FGF effects on *Xwnt8* and *XmyoD* expression was seen (Figs. 3B, C), the activity of the ΔWRPW mutant was again substantially lower, suggesting that most of the activity of *Xhes6* is WRPW domain-dependent. Thus, Xhes6 enhances the ability of FGF to induce genes normally expressed in lateral and ventral mesoderm in animal caps. This effect does not require Xhes6 to bind to DNA but does require an intact WRPW domain.

Xhes6 cooperates with the MAPK pathway to induce mesodermal gene expression

Several signal transduction pathways downstream of FGF have been described (Carballada et al., 2001; Schlessinger, 2000). However, signaling via the Ras–Raf–MAPK pathway is essential for the mesoderm-inducing activity of FGF *in vitro* (LaBonne et al., 1995; Umbhauer et al., 1995). Furthermore, expression of the MAPK target protein phospho-ERK is confined to the mesoderm at gastrula stage, raising the possibility that Xhes6 interacts with MAPK signaling *in vivo* (Christen and Slack, 1999).

We therefore investigated whether Xhes6 could enhance mesoderm induction brought about by the MAPK pathway alone, using a constitutively active mutant of MEK1, i.e. MEK1$^{S\rightarrow E}$ (Cowley et al., 1994; Umbhauer et al., 1995). The dose of MEK1$^{S\rightarrow E}$ mRNA injected into embryos was titrated to produce a low-level induction of both *Xbra* and *XmyoD* (Figs. 3D, E). This low dose of MEK1$^{S\rightarrow E}$ was then co-injected into embryos with *Xhes6* mRNA (Figs. 3D, E). Animal caps co-expressing Xhes6 and MEK1$^{S\rightarrow E}$ exhibited a greater level of *Xbra* and *XmyoD* induction that are then produced by either Xhes6 or MEK1$^{S\rightarrow E}$ alone (Figs. 3D, E). We conclude that Xhes6 enhances the ability of MAPK signaling to induce both *Xbra* and *XmyoD in vitro*.

We went on to investigate the relationship between Xhes6 and MAPK signaling *in vivo*. mRNAs encoding MEK1$^{S\rightarrow E}$, wild-type or mutant Xhes6 were injected singly or in combination into the marginal zone of a cell in 2-cell stage embryos (Fig. 3; Supplementary Table 1). Firstly, we analysed the effect of injecting mRNA encoding the activated MAPK mutant, MEK1$^{S\rightarrow E}$, alone, at the low dose which resulted in increased *XmyoD* expression in about one-third of embryos. This dose of MAPK not only induces *XmyoD* but also *Xhes6* (Figs. 3F, G).

If Xhes6 regulates *XmyoD* expression, we reasoned that a further increase in the level of *Xhes6* expression beyond that induced by MEK1$^{S\rightarrow E}$ alone might further increase *XmyoD* expression. To test this prediction, the effects of co-expression of Xhes6 and MEK1$^{S\rightarrow E}$ were examined. As expected, injection of Xhes6 mRNA expanded the region of *XmyoD* expression in gastrula embryos (Fig. 3H; Supplementary Table 1), whilst the ΔWRPW mutant had little effect on *XmyoD* expression (Fig. 3J; Supplementary Table 1). However, the extent of *XmyoD* expression was considerably expanded by co-injection of Xhes6 and MEK1$^{S\rightarrow E}$ mRNA (Fig. 3I; Supplementary Table 1), beyond the extent seen with either Xhes6 or MEK1$^{S\rightarrow E}$ alone. In contrast co-injection of *ΔWRPW* produced no enhancement of the effect seen with MEK1$^{S\rightarrow E}$ alone (Fig. 3K; Supplementary Table 1).

Xhes6 is also able to enhance the ability of MEK1$^{S\rightarrow E}$ to induce *Xbra* in animal caps (Fig. 3D), so we investigated whether a similar effect occurred *in vivo*. Whilst injection of mRNA encoding Xhes6 or MEK1$^{S\rightarrow E}$ alone had only a small effect on *Xbra* induction *in vivo* (Fig. 2H and data not shown), co-injection of Xhes6 and MEK1$^{S\rightarrow E}$ mRNA resulted in a significant increase in *Xbra* expression (data not shown), consistent with the results of the corresponding animal cap assays. This suggests that the reason why Xhes6 only had a minor effect on *Xbra* induction *in vivo* is that the level of endogenous MAPK activity limits *Xbra* expression (Fig. 2H).

Xhes6 requires FGF/MAPK signaling to induce mesodermal genes

The above results led us to investigate whether Xhes6 overexpression requires FGF signaling to induce *XmyoD* in whole embryos. Embryos were injected with mRNA encoding the dominant negative FGF receptor, XFD, at 2-cell stage to inhibit endogenous FGF signaling and assayed for *Xbra* and *XmyoD* expression at early gastrula stage (Amaya et al., 1991). Xhes6 overexpression was unable to rescue the loss of either *Xbra* or *XmyoD* expression resulting from inhibition of endogenous FGF signaling (Figs. 4A–D and data not shown), indicating that Xhes6 requires endogenous FGF signaling to induce mesodermal gene expression *in vivo*.

We also investigated further whether Xhes6 requires MAPK activity to induce mesodermal gene expression in animal caps.

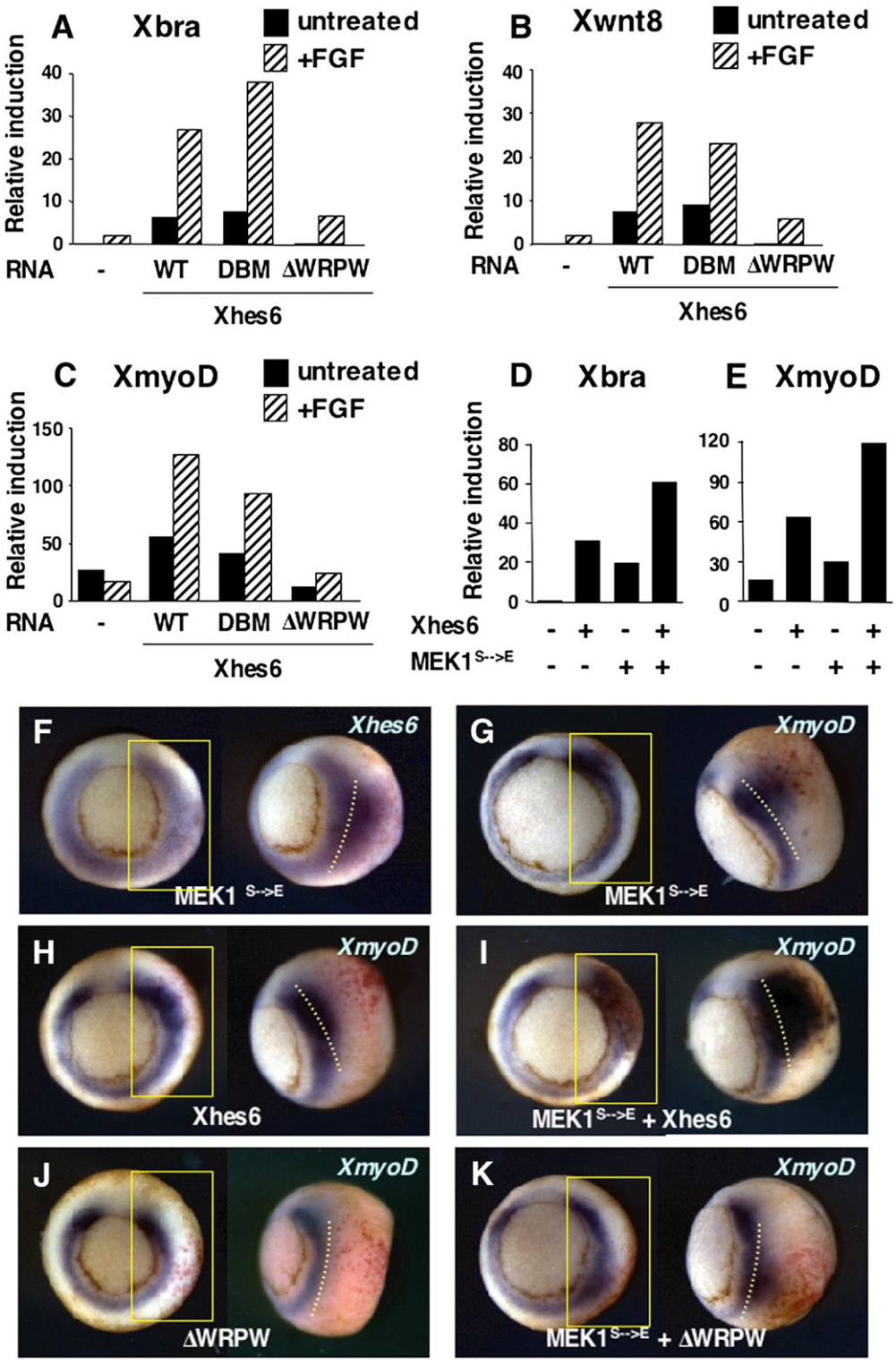
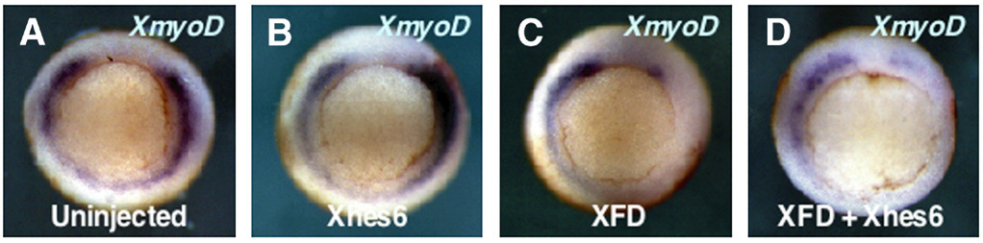


Fig. 3. Xhes6 enhances FGF/MAPK mediated mesoderm induction in animal caps. (A–E) Mesodermal marker gene induction. Animal caps were prepared from embryos injected with mRNA encoding wild-type or mutant Xhes6 as in Fig. 2. Animal caps were cultured in the presence or absence of FGF and collected at stage 11 for real-time RT–PCR analysis. The expression of each mRNA was normalised to level of *ODC* mRNA: (A) *Xbra*, (B) *Xwnt8*, (C) *XmyoD*. Data are from a typical experiment performed in triplicate. (D, E) Xhes6 enhances mesoderm induction by an activated mutant of MEK1. Both blastomeres of 2-cell stage embryos were injected with mRNAs encoding wild-type Xhes6, $MEK^{S\rightarrow E}$ or a combination of both. Animal caps were dissected from uninjected or injected embryos at stage 9. Real-time RT–PCR analysis of *Xbra* (D) and *XmyoD* (E) expression, normalised to *ODC* mRNA, in stage 11 animal caps from embryos injected with mRNA encoding wild-type Xhes6 or $MEK^{S\rightarrow E}$ alone or in combination, is shown. Data are from a typical experiment performed in triplicate. (F–J) 500 pg of Xhes6 or ΔWRPW mRNA, alone or in combination with $MEK1^{S\rightarrow E}$ mRNA was injected into the marginal zone of one blastomere at 2-cell stage, along with β-galactosidase mRNA (red staining). Gastrula stage embryos were analysed for expression of *Xhes6* (E) or *XmyoD* (G–K) by whole-mount *in situ* hybridisation. The side view of the area within the yellow box is shown on the right of each panel.



E

	Increased (%)	Decreased (%)	Normal (%)	total (n=)
Uninjected	0	0	25 (100)	25
Xhes6	32 (80)	2 (5)	6 (15)	40
XFD	0	27 (96)	1 (4)	28
Xhes6+XFD	0	27 (84)	5 (16)	32

Fig. 4. Xhes6 requires FGF signaling to induce *XmyoD*. (A–E) 500 pg of Xhes6 mRNA was injected alone or in combination with 125 pg of XFD mRNA into either the marginal zone of one blastomere at 2-cell stage, along with β-galactosidase mRNA (red staining). Gastrula stage embryos were analysed for *XmyoD* expression (A–D). (E) Summary of *in situ* hybridisation results.

Treatment of animal caps with the pharmacological inhibitor of MAPK activity, U0126, blocks the ability of Xhes6 to induce mesodermal genes, such as *Xbra* and *Xwnt8*, indicating that MAPK signaling is essential for mesoderm induction by *Xhes6* (Supplementary Figs. 1A, B) (Favata et al., 1998). In contrast, the activin receptor ALK5 inhibitor, SB431542, only partially inhibits Xhes6 activity (Supplementary Figs. 1A, B) (Inman et al., 2002). This demonstrates that Xhes6-mediated induction of mesodermal genes is fully dependent on FGF/MAPK signaling, but only partially dependent on signaling downstream of activin.

Xhes6 acts on downstream targets of the FGF/MAPK and activin/Smad2 pathways to induce XmyoD

The results above indicate that Xhes6 induces *XmyoD* by interaction with the FGF/MAPK and/or activin signaling pathways or their downstream targets. To distinguish between these possibilities, we examined the effects of Xhes6 on the levels of the phosphoprotein targets of both pathways (Supplementary Fig. 2). We examined levels of the phosphorylated form of the MAPK target ERK in animal caps treated with FGF, but found no differences in the level of phospho-ERK in the presence of either wild-type or mutant Xhes6 (Supplementary Fig. 2A). This indicates that Xhes6 interacts with targets further downstream in the FGF/MAPK pathway to enhance mesodermal gene expression. Similar results were found when the effect of Xhes6 expression on levels of phospho-Smad2 following activin treatment was examined. In early *Xenopus* embryos, Smad2 is solely responsible for activin signal transduction (Howell et al., 2001), yet there was no significant difference in phospho-Smad2 levels in the presence of Xhes6 (Supplementary Fig. 2B). Thus Xhes6 either interacts with targets of the FGF and activin pathways, downstream of MAPK and Smad2, respectively, or acts in parallel with these pathways to promote mesoderm formation and induction of *XmyoD* expression in animal caps.

Xhes6 is required for induction of XmyoD

Taken together, our results support a role for Xhes6 in regulating *XmyoD* expression by acting downstream of or in parallel with the FGF/MAPK and activin signaling pathway(s). This raises the question as to whether Xhes6 is required for *XmyoD* expression in early gastrula embryos. To address this, we used *Xhes6* antisense morpholino oligonucleotides (MOs) to block translation of Xhes6 protein.

To design the MO, the 5′ sequence of *Xhes6* mRNA was determined by 5′ RACE PCR; the published *Xhes6* clone was found to be truncated by 92 bp at its 5′ end. The full-length cDNA encodes an in-frame initiation codon 72 bp 5′ of the initiation codon indicated in the clone described previously (Fig. 5A; Koyano-Nakagawa et al., 2000). The additional residues contained no conserved motifs and no functional differences were detected between full-length *Xhes6* and the clone described originally (Fig. 5A and data not shown). Of the two potential initiation codons, the first lacked a Kozak sequence whilst the second codon in the same frame is in the optimal context for initiation of transcription. It is possible that ribosomes may bypass the first AUG and initiate translation at the 2nd AUG, to generate two forms of the protein (Kozak, 1987).

We therefore designed two Xhes6 MO, MO-1 and MO-2, directed to the first and second in-frame initiation codons of *Xhes6*, respectively (Fig. 5A). To test for their ability to block translation embryos were injected with Xhes6-HA mRNA either alone or together with MO-1, MO-2 or a control MO. Western blotting of extracts from stage 11 embryos revealed that Xhes6-HA protein was undetectable in the presence of MO-1 (Fig. 5B), whilst standard or 5-bp mismatched control MO had no effect on Xhes6-HA levels (Fig. 5B and data not shown). MO-2 inhibited *Xhes6*-HA translation by approximately 50%, whilst levels of Xhes6-HA were unaltered by a 5-bp mismatched MO-2 control (5-mis MO-2, data not shown).

We then investigated the effects of injection of Xhes6 MOs on *Xbra* and *XmyoD* expression in gastrula embryos. Control

MO, MO-1 or MO-2 was injected into one blastomere of 8-cell stage embryos to target the marginal zone of mesoderm. These embryos were then analysed for *XmyoD* induction by *in situ* hybridisation at gastrula stage (Figs. 5H–L). Embryos injected with a standard control MO (STD-CTL) or 5-mis MO-2 retained normal *XmyoD* expression (Figs. 5I, J; Table 2). However, *XmyoD* expression was significantly reduced in the region of the lateral and ventral marginal zone targeted by MO-1 or

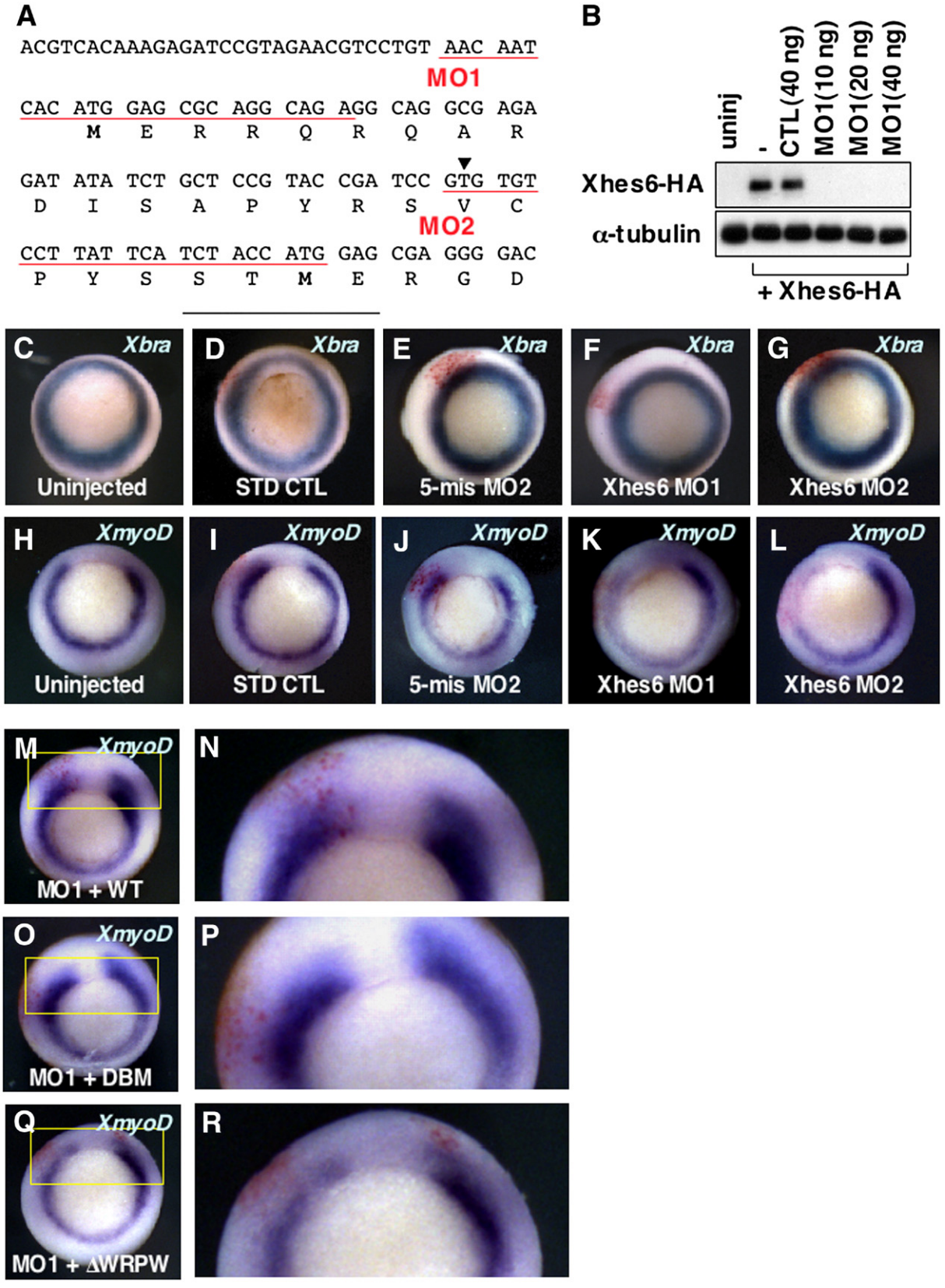


Table 2
Effect of antisense Xhes6 MO on *XmyoD* expression at gastrula stage and rescue of the Xhes6 MO phenotype

MO/RNA	Decreased (%)	Normal (%)	Total (*n*)
Uninjected			74
STD	1 (2.5)	39 (98)	40
5-mis MO-2	2 (7.1)	26 (93)	28
MO-1	55 (82)	12 (18)	67
MO-2	29 (94)	2 (6)	31
Rescue			
MO-1 + WT	9 [a] (43)	12 (57)	21
MO-1 + DBM	9[a] (39)	14 (61)	23
MO-1 + ΔWRPW	14 (78)	4 (22)	18

[a] Embryos showed decreased XmyoD expression at Xhes6 MO1 injected site, but the less extent.

MO-2, in 82% ($n=67$) and in 94% ($n=31$) of stage 11 embryos, respectively (Figs. 5K, L; Table 2). The control MO had no effects on *Xbra* expression (Figs. 5D, E) and in contrast to their effects on *XmyoD* expression, neither MO-1 nor MO-2 altered levels of *Xbra* transcript (Figs. 5F, G). Similarly, neither the control MO, nor MO-1 nor MO-2 caused a significant alteration in the levels of levels of *Xwnt8*, *Chordin* or *XSox17a*, whilst a reduction in expression of the myogenic transcription factor *Xmyf5* was seen in 30–40% of embryos (Supplementary Fig. 3 and data not shown).

To determine whether the effects seen were due to specific depletion of endogenous Xhes6, we attempted to rescue the MO-1 phenotype with 5′ truncated wild-type and mutant forms of Xhes6, which lack sequence complementarity to MO-1 (see Materials and methods; Fig. 5A). Co-injection of MO-1 with 5′ truncated Xhes6 mRNA restored a normal pattern of *XmyoD* expression in the majority of embryos (Figs. 5M, N and Table 2). To determine which aspect of Xhes6 function is required for mesoderm induction, we attempted rescue with mutant forms of Xhes6. Xhes6 DBM co-injected with MO-1 rescued *XmyoD* expression in most embryos (Fig. 5O, Table 2). In contrast, the ΔWRPW mutant demonstrated minimal rescuing activity; only 22% of embryos co-injected with ΔWRPW mRNA and MO-1 showed normal *XmyoD* expression, a similar proportion to that seen when MO-1 was injected alone (Figs. 5Q, R; Table 2). Thus Xhes6 is required for the expression of *XmyoD*, but not for the expression of *Xbra*, in gastrula stage embryos. These observations suggest that Xhes6 regulates the specification of mesoderm but not the initiation of mesoderm induction. Interestingly Xhes6 does not need to bind to DNA for this activity but does require protein–protein interactions mediated by the WRPW domain.

Xhes6 is required for XmyoD induction by eFGF

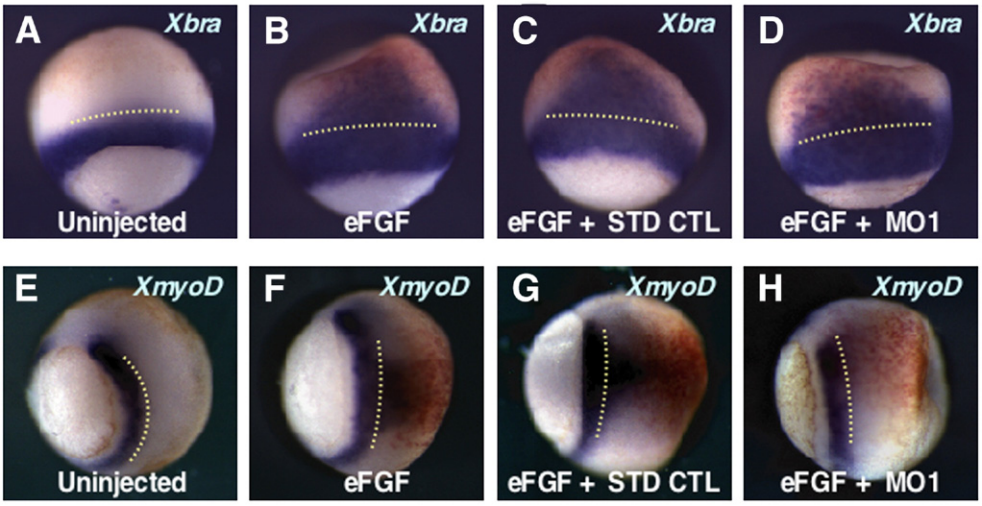
Having established that Xhes6 is required for *XmyoD* induction, we next addressed whether Xhes6 is essential for *XmyoD* induction by FGF. mRNA encoding eFGF was injected into the animal pole of 2-cell stage blastomeres, either alone or in combination with control MO or MO-1 targeting *Xhes6* translation. Expression of *Xbra* and *XmyoD* was then analysed at mid gastrula stage (Fig. 6). eFGF alone or in combination with the control MO expanded the region of both *Xbra* and *XmyoD* expression (Figs. 6B, C and F, G). However, the Xhes6 MO blocked the effect of eFGF on ectopic *XmyoD* expression whilst having no effect on *Xbra* expression (Figs. 6D, H) consistent with its effect on endogenous *Xbra* expression (Figs. 5F, G). Thus eFGF-mediated induction of *XmyoD* requires *Xhes6*.

Xhes6 is co-expressed with Xgrg2 and Xgrg4 in lateral and ventral mesoderm

One striking finding from this study is that the WRPW domain of Xhes6 is very important for Xhes6-mediated mesoderm induction. This WRPW domain has shown to bind to members of the Groucho family of transcriptional repressors (Fisher et al., 1996; Gratton et al., 2003). This led us to investigate whether interactions between Xhes6 and Groucho proteins could explain the ability of Xhes6 to enhance induction of *XmyoD*.

We began by examining the expression pattern of *Xenopus* Groucho homologues, *Xgrg2* and *Xgrg4*, which have the highest degree of sequence conservation with the well-characterized *Drosophila* Groucho protein (Hasson et al., 2005). *Xgrg2* mRNA was detected throughout the gastrula stage embryo except in endoderm, its expression overlapping with the domain of *Xhes6* expression in the lateral and ventral mesoderm (Figs. 7A, B). *Xgrg4* is also detectable at low levels in lateral and ventral mesoderm, as reported previously (Fig. 7C; Molenaar et al., 2000). *Xhes6* is thus co-expressed with both *Xgrg2* and *Xgrg4* in the lateral and ventral mesoderm of gastrula stage embryos.

Fig. 5. Antisense morpholino oligos against Xhes6 block *XmyoD* expression in lateral and ventral mesoderm. (A) The 5′ region of *Xhes6* cDNA. The previously reported 5′ end of the transcript is indicated by the triangle; an additional 81 base pairs of sequence were identified by 5′RACE PCR. Two initiation codons are present (bold). The sequences targeted by Xhes6 antisense morpholino oligonucleotides, MO-1 and MO-2 are underlined in red. (B) Antisense morpholino oligonucleotide (MO) validation. Both cells of 2-cell stage embryos were injected with 1 ng Xhes6-HA mRNA together with either no MO (−), a standard control MO (CTL) or Xhes6 MO-1 in the amounts shown. Embryos were lysed at stage 11 and analyzed for Xhes6-HA protein expression by western blotting with an anti-HA antibody. The same membrane was also blotted with anti-α-tubulin antibody as a loading control. (C–L) Phenotype of Xhes6 MO. Whole-mount *in situ* hybridisations showing *XmyoD* in uninjected or MO-injected embryos. 40 ng of each indicated MO together with 0.25 ng of β-galactosidase mRNA, as a tracer, were injected into marginal zone of one blastomere of embryos at the 8-cell stage. Embryos were analyzed by whole-mount *in situ* hybridisation to detect mRNA encoding *Xbra* (C–G) or *XmyoD* (H–L) at gastrula stage; red colour indicates presence of β-galactosidase detected by salmon-gal, the yellow arrow indicates the targeted region. STD-CTL, standard control MO (D, I); 5-mis MO-2, a 5 base pair mismatched control version of Xhes6 MO-2 (E, J); Xhes6 MO-1 (F, K) and Xhes6 MO-2 (G, L) are directed against *Xhes6* mRNA as shown in panel A. (M–R) Rescue of Xhes6 MO phenotype. 40 ng of MO-1 was injected with mRNA encoding Xhes6 (M, N), DBM (O, P) or MT-ΔWRPW (Q, R) together with β-galactosidase mRNA into the marginal zone of one blastomere of 8-cell stage embryos. Gastrula stage embryos were analysed by whole-mount *in situ* hybridisations for *XmyoD*; red colour indicates presence of β-galactosidase, the yellow box outlines the targeted regions shown enlarged in panels I, K and M.



I

Xbra

	Increased (%)	Decreased (%)	Normal (%)	total (n=)
Uninjected	0	0	22 (100)	22
eFGF	32 (97)	0	1 (3)	33
eFGF+STD	42 (100)	0	0	42
eFGF+MO1	24 (77)	0	7 (23)	31

XmyoD

	Increased (%)	Decreased (%)	Normal (%)	total (n=)
Uninjected	0	0	38 (100)	38
eFGF	31 (66)	0	16 (34)	47
eFGF+STD	43 (62)	1 (1)	26 (37)	70
eFGF+MO1	1 (2)	3 (6)	46 (92)	50

Fig. 6. Xhes6 morpholino inhibits ectopic XmyoD expression by FGF-signaling. 1 pg of eFGF was injected alone or with 40 ng of STD CTL or Xhes6 in the animal pole of a blastomere at 2-cell stage, along with β-galactosidase mRNA (red staining) and analyzed at gastrula stage for *Xbra* (A–D) and *XmyoD* (E–H) expression by whole-mount *in situ* hybridisation. The side view of the area within the yellow box is shown on the right of each panel. (I) Summary of *in situ* hybridisation results.

Xgrg2 and Xgrg4 inhibit XmyoD induction by MAPK signaling

Next, we examined the effects of overexpression of untagged or epitope-tagged Xgrg2 and Xgrg4 on mesoderm induction and specification. Injection of mRNA encoding-tagged or untagged forms of either protein was found to inhibit *XmyoD* expression in about one third of gastrula embryos, consistent with Groucho inhibiting *XmyoD* induction (Figs. 7D, F, and data not shown). We used tagged forms of Groucho in further experiments. One explanation for the modest penetrance of the *Xgrg* phenotype is that the function of Groucho proteins is known to be inhibited following phosphorylation by activated ERK which is a downstream effector of MAPK (Hasson et al., 2005). The MAPK pathway is activated in the lateral and ventral mesoderm, and therefore may attenuate the function of Xgrg proteins in this region (Christen and Slack, 1999). To test this possibility, we constructed mutant forms of Xgrg2 and Xgrg4 in which the serine residues at each of the consensus ERK target sites were converted to either alanine or aspartate, creating constitutively active, non-phosphorylatable mutants (Xgrg2AAA and Xgrg4AA) and pseudophosphorylated mutants (Xgrg2DDD and Xgrg4DD), respectively. We then examined the effects of injection of mRNA encoding the mutants on *XmyoD* expression at gastrula stage. Xgrg2AAA, and Xgrg4AA, both resistant to phosphorylation by ERK, inhibited *XmyoD* expression in the majority of embryos, whilst the pseudo-phosphorylated mutants had minimal activity (Figs. 7E, G; Table 3). These observations suggest that the low penetrance of the wild-type Xgrg2 and Xgrg4 phenotypes may be due to MAPK activity inhibiting Groucho function in the lateral and ventral mesoderm.

Inhibition of *XmyoD* expression by Xgrg proteins is consistent with their acting as negative regulators of *XmyoD* induction by pathways including FGF/MAPK (Fisher et al., 2002). To investigate if this is the case, we examined the effect of the constitutively active Xgrg mutants on induction of *XmyoD* by the constitutively active MEK1$^{S\rightarrow E}$ mutant (Fig. 7H). We found that both Xgrg2 and Xgrg4 mutants decreased, but did not abolish the ability of MEK1$^{S\rightarrow E}$ to induce *XmyoD* (Figs. 7I, J). The fact that inhibition of MEK1$^{S\rightarrow E}$ is only

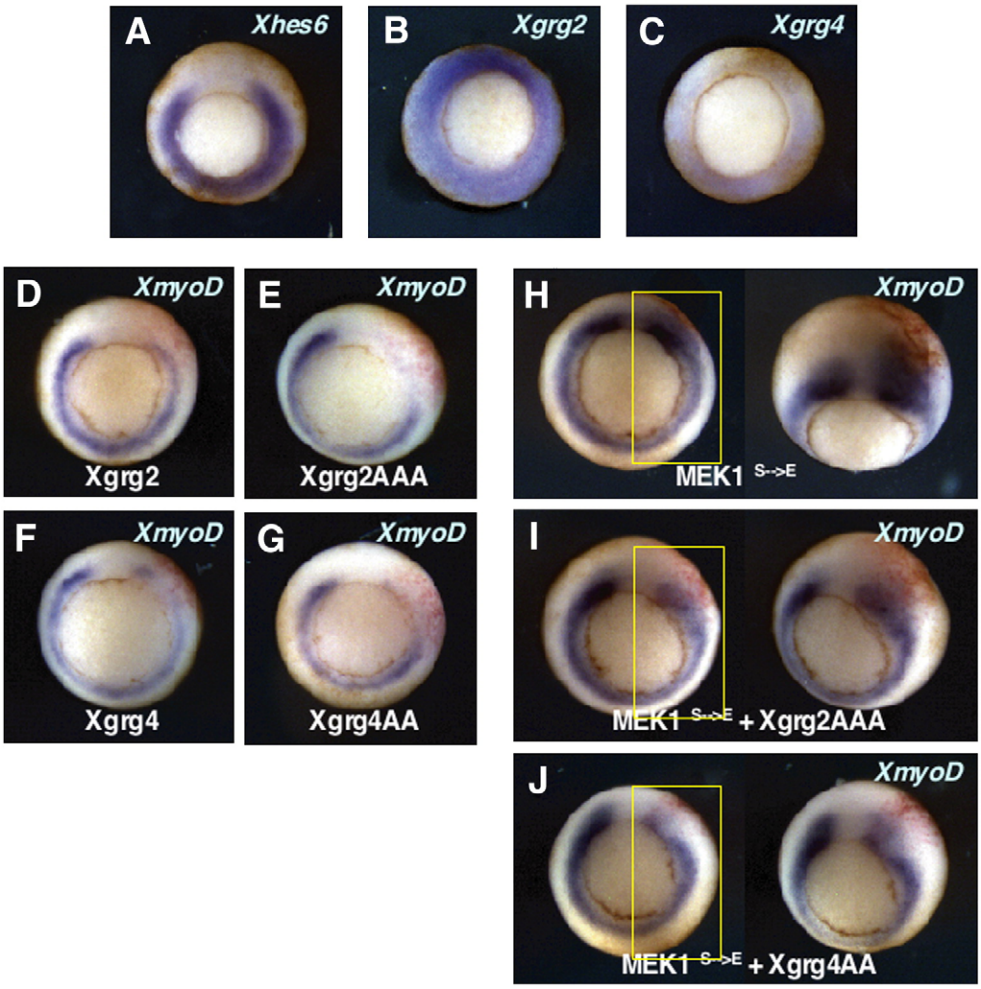


Fig. 7. Xgrg2 and Xgrg4 inhibit *XmyoD* induction by MAPK signaling. Gastrula stage embryos were analysed for *Xhes6* (A), Xgrg*2* (B), *Xgrg4* (C) and *XmyoD* (D–J) expression by whole-mount *in situ* hybridisation. (A–C) Uninjected embryos. (D–G) 1 ng flag-tagged Xgrg2, Xgrg4 or non-phosphorylatable mutant (Xgrg2AAA or Xgrg4AA) was injected with β-galactosidase mRNA (red staining) into marginal zone of a blastomere at 2-cell stage. (H–J) 1 ng of active MEK1 ($MEK1^{S\rightarrow E}$) mRNA was injected alone or with flag-tagged Xgrg2AAA or Xgrg4AA into a blastomere at the 2-cell stage.

partial may be due to $MEK1^{S\rightarrow E}$-inducing Xhes6 which then inhibits the function of the Groucho mutants (Fig. 3F).

Xhes6 relieves inhibition mediated by Xgrg2 and Xgrg4

The results described above offer a mechanism for Xhes6 to regulate mesodermal patterning. Xhes6 might bind Xgrg2 and Xgrg4 proteins to relieve their repression of *XmyoD* within the region of *Xhes6* expression. If this is the case, we postulated that overexpression of wild-type Xhes6, but not the ΔWRPW mutant, should overcome the ability of the activated Xgrg to inhibit *XmyoD* expression. mRNAs encoding constitutively active Xgrg mutants were injected into 2-cell stage embryos together with wild-type Xhes6 or ΔWRPW mutant and *XmyoD* expression was analysed (Figs. 8A–H). While constitutively active Xgrg2 an Xgrg4 both inhibited *XmyoD* expression (Figs. 8B, F; Table 3), *XmyoD* expression was restored with co-injected wild-type Xhes6 (Figs. 8C, G; Table 3) but not with the ΔWRPW mutant (Fig. 8D, H; Table 3). Consistent with these observations, co-immunoprecipitation experiments using gastrula stage embryos expressing epitope-tagged Xgrg2 and Xhes6 or its mutant demonstrated that Xhes6 can physically interact with Xgrg2 (Fig. 8I). The DBM mutant also bound to Xgrg2 whilst the ΔWRPW mutant did not (Fig. 8I), indicating that the interaction is mediated via WRPW motif. Interestingly Xhes6 can interact with all forms of Xgrg2, irrespective of phosphorylation status. Similar results were obtained with Xgrg4 (data not shown). Overexpression experiments need to be interpreted with caution as the high levels of the overexpressed protein may lead to non-physiological interactions. However, our results are consistent with *Xhes6* relieving the repression of *XmyoD* induction resulting from Groucho overexpression by directly binding Groucho proteins. As Xhes6 is confined to the lateral and ventral mesoderm, this suggests that Xhes6 may function in a compartment-specific manner by regulating of endogenous Groucho proteins.

Hes6 is required for normal myogenesis in later development

The observation that hes6 is required for *XmyoD* induction in gastrulation raises the issue of whether there is a requirement for hes6 for *XmyoD* expression at later stages of development.

Table 3
Effect of wild-type and mutant Groucho proteins on *XmyoD* expression at gastrula stage

RNA	Increased (%)	Decreased (%)	Normal (%)	Total (*n*)
Uninjected	0	1 (0.6)	192 (99)	193
XGrg2	0	47 (34)	90 (66)	137
XGrg2AAA	0	88 (75)	30 (25)	118
XGrg2DDD	0	4 (6.3)	59 (94)	63
XGrg4	0	56 (34)	107 (66)	163
XGrg4AA	0	42 (56)	33 (44)	75
XGrg4DD	0	0	65 (100)	65
Xhes6	129 (82)	0	28 (18)	157
Xhes6+XGrg2AAA	33 (42)	10 (13)	35 (45)	78
Xhes6+XGrg4AA	27 (47)	0	30 (53)	57
ΔWRPW	0	0	63 (100)	63
ΔWRPW+XGrg2AAA	0	32 (71)	13 (29)	45
ΔWRPW+XGrg4AA	0	23 (45)	28 (55)	51

We examined this issue by analyzing the effects of MO directed against *Xhes6* on expression of *XmyoD* at neurula stage. MO-1 significantly reduced *XmyoD* induction, particularly in the posterior region of the embryos, whilst MO-2 produced a more modest effect (Supplementary Figs. 5A–E).

Finally, our observations raise the question of whether loss of *Xhes6* affects myogenesis at later stages of development. We therefore examined the phenotype of embryos injected with Xhes6 MO at tail bud stage. Morphant embryos exhibited a subtle phenotype of shortening of the tail bud, with some embryos exhibiting marked twisting of the body (Supplementary Figs. 6A–D). To assess whether loss of *Xhes6* had an effect on myogenic differentiation we examined the expression of *myosin heavy chain* (*mhc*), a marker of terminal muscle differentiation, at tail bud stage in embryos injected with Xhes6 MO (Supplementary Figs. 6E–H). We found that both Xhes6 MO produced a moderate reduction in the level *mhc* expression and a loss of the normal somite pattern (Supplementary Figs. 6I–J). We conclude that *Xhes6* is required not only for normal induction of *XmyoD* at gastrula and neurula stages, but also for normal muscle differentiation at later stages of development.

Discussion

Xhes6 co-expressed with *XmyoD* in early gastrula embryos, leading us to investigate whether Xhes6 plays a role in mesoderm induction and/or specification. We found that overexpression of Xhes6 alone results in modest induction of mesodermal markers in animal caps. Xhes6 cooperates with mesoderm-inducing pathways, acting downstream of the FGF target ERK and the activin target Smad2 to induce markers of early mesoderm and lateral and ventral specific mesoderm as well as *XmyoD* in animal caps. *In vivo*, however, the effects of loss of Xhes6 protein are restricted to mesoderm specification. Xhes6 is required for FGF-mediated induction of *XmyoD*, but not for induction of pan-mesodermal marker, *Xbra*. Significantly, all of these functions of Xhes6 require the Groucho binding WRPW domain of the protein. *Xenopus* Groucho, *Xgrg2* and *Xgrg4* are expressed in mesoderm and elsewhere. Strikingly, overexpression of Groucho proteins inhibits *XmyoD* expression, an inhibition that is alleviated by Xhes6 in a WRPW domain-dependent manner. Thus, Xhes6 acts downstream of and/or in parallel to mesoderm-inducing pathways and to regulate mesoderm specification by relieving Groucho-mediated inhibition of *XmyoD*.

Whilst Xhes6 requires the ability to bind Groucho to mediate its effects on *XmyoD* expression, DNA-binding is not required. This is consistent with previous studies and indicates that Xhes6 either acts by binding to a DNA-bound protein to regulate transcription, in a Groucho-dependent manner, or acts independent of DNA binding, by binding Groucho to prevent its recruitment by other transcription factors (Cossins et al., 2002; Jhas et al., 2006; Koyano-Nakagawa et al., 2000). An important issue is whether the results seen in this study are confined to *Xhes6*, or whether any *hes* family member would have similar effects. Strikingly, overexpression of Xhairy1 or Xhairy2 results in *decreased XmyoD* expression in gastrula stage embryos, the direct opposite of the phenotype produced by Xhes6 (Cui, 2005; Umbhauer et al., 2001). Furthermore, the inhibition of *XmyoD* expression does not require the Xhairy WRPW motif, suggesting that Xgrg proteins are not involved. These observations suggest that Xhes6 does indeed have a specific function, distinct from other hes proteins, in regulating mesodermal specification.

Here we have shown that the Xgrg protein family inhibits the induction of *XmyoD*. Although phosphorylation of Xgrg proteins by ERK attenuates their repressor activity (Table 3; Hasson et al., 2005), ERK activity alone is insufficient to relieve Xgrg-mediated repression of MAPK inducible genes during mesoderm specification. Xhes6 has no effect on phospho ERK levels (Supplementary Fig. 2A), so does not alter Groucho phosphorylation. We postulate that Xhes6 is required to augment the effects of ERK phosphorylation, binding Xgrg proteins directly to relieve Groucho-mediated inhibition of FGF targets such as *XmyoD* (Supplementary Fig. 4).

In addition to being required for *XmyoD* induction at gastrulation, Xhes6 is also required for *XmyoD* expression in the posterior region of neurula stage embryos (Supplementary Fig. 5). This is again consistent with Xhes6 acting via FGF signaling, as pharmacological inhibition of the FGF receptor has been shown to inhibit *XmyoD* induction in posterior, but not anterior explants of stage 15–19 embryos (Standley et al., 2002). The disruption of the tail bud seen in *Xhes6* morphant embryos is consistent with the high expression of *Xhes6* in the tail bud and resembles the posterior phenotype produced by FGF8 MO at tail bud stage (Supplementary Fig. 6; Cossins et al., 2002; Fletcher et al., 2006).

Finally, the lowering Xhes6 levels results in significant disruption of myogenesis, evidenced by the reduction of *mhc* expression in tail bud stage embryos (Supplementary Fig. 6). It remains to be determined whether this is due to the effects of Xhes6 on *XmyoD* expression, but this observation does indicate Xhes6 has a key role in myogenic differentiation.

Materials and methods

Isolation of full-length Xhes6 cDNA by 5′RACE

5′RACE PCR of cDNA from stage 11 *Xenopus* embryos was used to determine the sequence of the 5′UTR and translation initiation codon of *Xhes6*

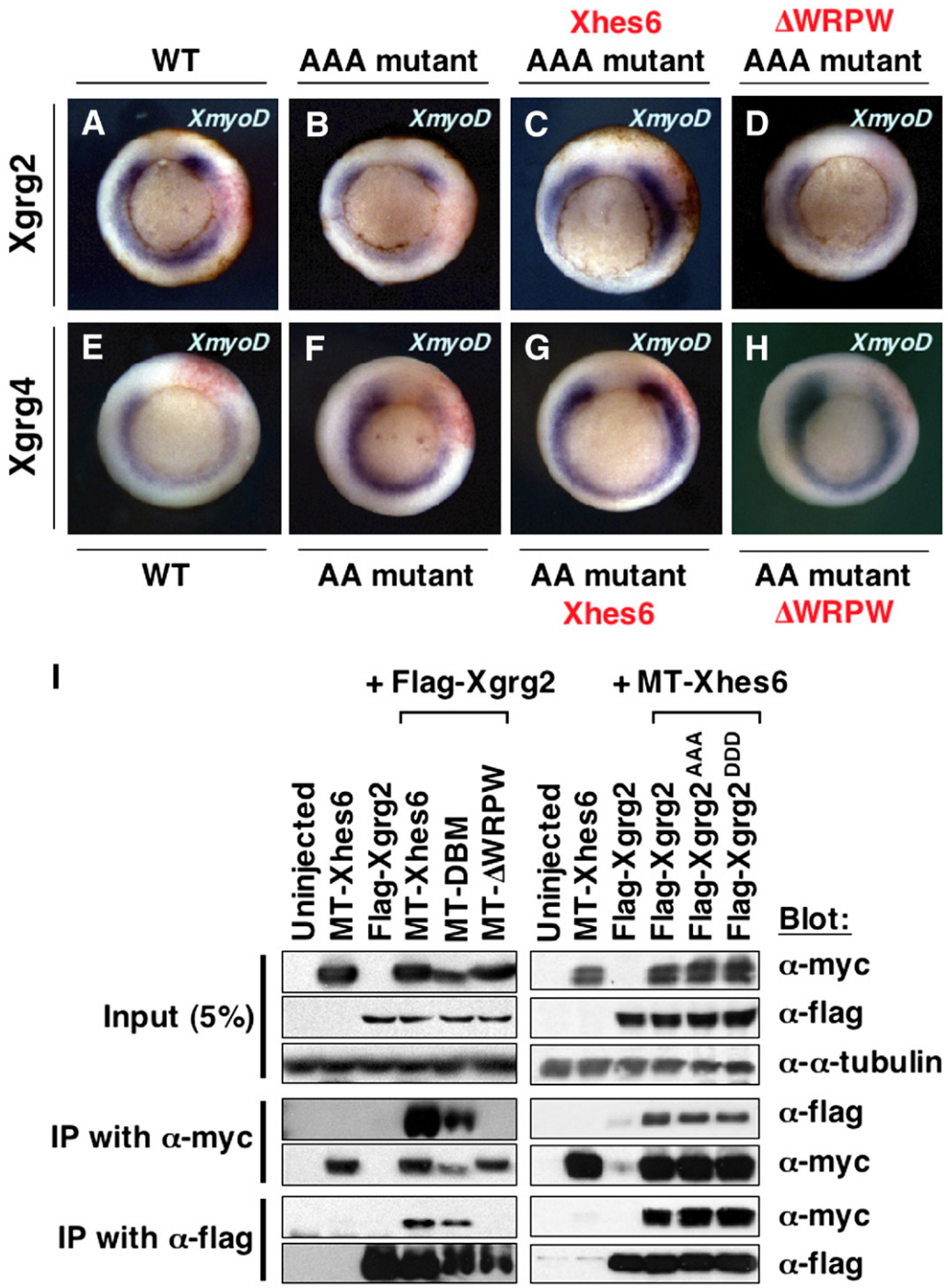


Fig. 8. Xhes6 interact with Xgrg2 and Xgrg4 and relieves inhibition. 1 ng flag-tagged Xgrg2 or Xgrg4 mRNA were injected alone or with 500 pg MT-Xhes6 or ΔWRPW to together with β-galactosidase mRNA (red staining) into marginal zone of a blastomere at 2-cell stage. (A–H) *In situ* hybridisation for *XmyoD* was performed on gastrula stage embryos. The side views of embryos were shown on the right (A–H). The arrow indicated the injected region. (I) Immunoprecipitations were carried out using whole lysate from gastrula embryos and the immunocomplex was analysed by western blotting.

(FirstChoice RLM–RACE kit, Ambion). The following gene-specific primers were used:

Outer, 5′GCTCAACGCGTTTCACAGTC3′;
Inner, 5′GGCGAATTCGCAGTTCCTGTAGGCTCTC3′.

RACE clones contained an additional 61–92 bp 5′ sequence compared with the original Xhes6 cDNA (Koyano-Nakagawa et al., 2000). The full-length sequence of Xhes6 is identical to that of the *Xenopus* image clone accession number BC130161.

Plasmid, mRNA and in situ probes

N-Terminally myc-tagged Xhe6, DBM and ΔWRPW constructs were constructed by subcloning of an *Eco*RI fragment from Xhes6, DBM or ΔWRPW into MT (Koyano-Nakagawa et al., 2000). For construction of

Xhes6HA, first Xhes6 cDNA excluding stop codon was amplified by PCR and subcloned into pCS2+. Following *Xho* I digestion, a synthesized fragment encoding an HA epitope was inserted. cDNAs encoding Xgrg2 and Xgrg4 were amplified by PCR from MGC clones (Geneservice) and inserted into pCS2+. Ala or Asp substitutions at S266/S294/S559 of Xgrg2 and S266/S293 of Xgrg4 were introduced by sequential site-directed mutagenesis. Following *Cla* I–*Xho* I digestion, a synthesized fragment encoding a flag epitope was inserted into all Xgrg2 and Xgrg4 constructs.

Plasmids used were pXFD/Xss, encoding a dominant negative FGF receptor (Amaya et al., 1991), MAPKK1$^{S217E/S221E}$, encoding an activated mutant of MEK1 (MEK$^{S\rightarrow E}$; Cowley et al., 1994), eFGF, (Lombardo and Slack, 1997), Xnr2 (Jones et al., 1995) and cytoplasmic β-galactosidase as a tracer. Capped mRNA was synthesized *in vitro* from linearised plasmids using the SP6 Message Machine kit (Ambion).

Antisense RNA *in situ* hybridisation probes were generated as described (Hopwood et al., 1989; Smith et al., 1991).

Xenopus embryos and injection of mRNA and morpholinos

Xenopus embryos obtained by hormone-induced laying were *in vitro* fertilized, dejellied in 2% cysteine pH 8.0, and washed in 0.1× MBS. Capped mRNAs were injected into embryos in 0.2× MBS supplemented with 4% Ficoll and 25 μg/ml gentamicin (Gibco). Amounts of injected mRNA and stages of embryos are as described in the figure legends.

Morpholinos against Xhes6 were:

Xhes6 MO-1, 5′TCTGCCTGCGCTCCATGTGATTGTT;
Standard control MO (STD-CTL), 5′CCT CTT ACC TCA GTT ACA ATT TAT A
Xhes6 MO-2, 5′CATGGTAGATGAATAAGGACACACG;
5-mis Xhes6 MO-2, 5′CATcGTAcATcAATAAcGACAgACG (Gene Tools LLC).

Animal cap assays

Animal caps were dissected from stage 9 embryos and explants were cultured in 0.7× MBS containing 0.1% BSA and 25 μg/ml gentamicin (Gibco). For growth factor stimulation, caps were incubated with 20 ng/ml of human FGF2 (bFGF), FGF4 or 1–5 ng/ml activin (R&D Systems). To inhibit the activin and MAP kinase signaling pathways, 20 μM SB431542 (Tocris) or 40 μM U0126 (Calbiochem) were added to the culture (Favata et al., 1998; Inman et al., 2002). To confirm the specificity and efficiency of the inhibitors, animal caps were dissected at stage 9 and treated with FGF or activin in the presence or absence of the MEK inhibitor, U0126 (U0) or SB431542 (SB), or an inhibitor of the activin receptor, ALK5. Activation of each signaling pathway was determined by Western blotting using antibodies against total and phosphorylated forms of ERK or Smad2.

Real-time PCR analysis

Total RNA was isolated from *Xenopus* embryos or animal caps using Qiagen RNeasy mini kit according to the manufacture's instructions. cDNA was synthesized by Superscript II (Invitrogen). Quantitative PCR using SYBR Green PCR mix (Qiagen) was performed on Corbett Research RotorGene PCR machine.

Primer sequences are as follows:

ODC, 5′GCCATTGTGAAGACTCTCTCCATTC3′ and
5′TTCGGGTGATTCCTTGCCAC3′ (Heasman et al., 2000);
Xbra, 5′TTCTGAAGGTGAGCATGTCG3′ and
5′GTTTGACTTTGCTAAAAGAGACAGG3′ (Sun et al., 1999);
XmyoD, 5′AGCTCCAACTGCTCCGACGGCATGAA3′ and
5′AGGAGAGAATCCAGTTGATGGAAACA3′ (Rupp and Weintraub, 1991);
chordin, 5′AACTGCCAGGACTGGATGGT3′ and
5′AACTGCCAGGACTGGATGGT3′ (XMMR);
Mix.1, 5′GCAGATGCCAGTTCAGCCAATG3′ and
5′TTTGTCCATAGGTTCCGCCCTG3′ (Xanthos et al., 2001);
Mix.2, 5′TGCAAGCCATCATTATTCTAGC3′ and
5′AGGAACCTCTGCCTCGAGACAT3′ (Xanthos et al., 2001);
Mixer, 5′CACCAGCCCAGCACTTAACC3′ and
5′CAATGTCACATCAACTGAAG3′ (Henry and Melton, 1998);
Xsox17α, 5′GCAAGATGCTTGGCAAGTCG3′ and
5′GCTGAAGTTCTCTAGACACA3′ (Xanthos et al., 2001);
Xwnt8, 5′CTGATGCCTTCAGTTCTGTGG3′ and
5′CTACCTGTTTGCATTGCTCGC3′ (Ding et al., 1998).

Whole-mount in situ hybridisation

Xenopus embryos were fixed for 1 h in MEMFA and stained for β-galactosidase (250 pg mRNA injected embryo) using Salmon Gal (Research Organics). Whole-mount *in situ* hybridisation was carried out as described (Shimamura et al., 1994) with Dig (Roche)-labelled antisense RNA probe.

Western blotting and immunoprecipitation

Total protein extracts were separated by SDS–PAGE and transferred to nitrocellulose (immobilon-P; Millipore) by standard methods. Primary antibodies are as follows: anti-c-myc (9E10) and anti-Smad2/3 (Santa Cruz Biotechnology, Inc.); anti-FLAG M2 HRP, anti-α-tubulin (Sigma-Aldrich) and anti-phospho-Smad2, anti-p44/p42 MAPK, anti-phospho-p44/p42 MAPK (Ser465/467), anti-HA and anti-Myc tag polyclonal (Cell Signalling Technology). For immunoprecipitation, *Xenopus* embryos were lysed at gastrula stage and whole lysate incubated with either anti-FLAG M2 affinity gel or Ezview Red anti-c-myc affinity gel (Sigma-Aldrich). Immunocomplex formation was analyzed by western blotting.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.09.011.

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