Chapter 5

*XMeis3* is necessary for mesodermal *Hox* gene expression

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**Abstract**

Hox transcription factors provide positional information during patterning of the anteroposterior axis. Recently, an early expression sequence of Hox genes was found in *Xenopus* gastrulae mesoderm. Hox transcription factors can cooperatively bind with PBC-class co-factors, enhancing specificity and affinity for consensus binding sites. The nuclear localisation of these co-factors is regulated by the Meis-class of homeodomain proteins. During development of the zebrafish hindbrain, Meis3 has been shown to synergise with Hoxb1 in the autoregulation of Hoxb1. In *Xenopus* XMeis3 posteriorises the embryo upon ectopic expression. We investigated whether XMeis3 is involved in regulation of Hox gene expression in mesoderm during gastrulation. Here, we present evidence that XMeis3 is necessary for expression of Hoxd1, Hoxb4 and Hoxc6 in mesoderm during gastrulation. In addition, we show that XMeis3 function is necessary for the progression of gastrulation. Finally, we propose synergy between XMeis3 and Hoxd1 in Hoxd1 autoregulation during gastrulation.

**Introduction**

During the development of most animal species studied, Hox transcription factors specify positional information along the anterior to posterior axis (Bürglin *et al.*, 1991; Bürglin and Ruvkun, 1993; McGinnis and Krumlauf, 1992; Lawrence and Morata, 1994; Manak and Scott, 1994). Hox genes form a subfamily of the homeobox containing gene family, and are organised in four clusters, each located on different chromosomes. The homeobox encodes a DNA binding motif called the homeodomain. A strict control of the expression and function of these Hox genes is essential. It has been shown that Pbx family members, and their *Drosophila melanogaster* counterpart Extrathetic (Exd), function as cofactors for Hox proteins; they can enhance their binding specificity and affinity for target sequences on DNA (van Dijk and Murre, 1994; Knoepfler and Kamps, 1995; Chang *et al.*, 1995; Neuteboom and Murre, 1997; Ryoo and Mann, 1999). Pbx/Exd family members are part of a different subfamily of the homeodomain containing proteins, namely the TALE-class. This class is characterised by having a three amino acid loop extension between the first and second helices of their homeodomains (Bürglin, 1997). Cooperative binding of Hox and Pbx/Exd proteins can lead to transactivation while binding of the individual factors leads to repression on the same promoter elements (Pinsonneault *et al.*, 1997). When Hox proteins bind to DNA cooperatively with a Pbx/Exd family
member, the main protein-protein interaction consists of binding of the hexapeptide motif of the Hox protein to a pocket formed by the atypical homeodomain of PBC family members (Jabet et al., 1999; Passner et al., 1999; Piper et al., 1999). This pocket is composed of the three amino acid loop extension of the PBC homeodomain, residues in the third helix of the homeodomain, and a residue in the C-terminal helix of PBC homeodomains (Piper et al., 1999). The nuclear localisation of Pbx/Exd proteins is controlled by competing nuclear import and export signals (Abu-Saar et al., 1999). When members of the Meis family, or their Drosophila counterpart Homothorax (Hth), also members of the TALE-class of homeodomain proteins, are present in the cytoplasm they interact with Pbx/Exd family members in such a way that the nuclear export signal of the Pbx/Exd family member is shielded, resulting in a net influx of Pbx/Exd into the nucleus, influencing the function of Hox proteins present (Ryoo et al., 1999; Ryoo and Mann, 1999; Jaw et al., 2000). However, Pbx/Exd and Meis/Hth proteins are not used exclusively as cofactors for Hox proteins. The myogenic bHLH factors (Knoepfler et al., 1999) and Engrailed (Peltenburg and Murre, 1996) also depend on the activity of Pbx and Meis members for proper functioning. For Hox paralog group 1 members, autoregulation dependent on Pbx/Exd and Meis/Hth has been shown to function in neurectoderm of mouse embryos (Pöpperl et al., 1995; Ferretti et al., 2000), C. elegans (Streit et al., 2002) and in endoderm of Drosophila embryos (Ryoo et al., 1999; Marty et al., 2001). Binding of Hox and Pbx family members to bipartite Hox-Pbx binding sites is essential for autoregulation (Pöpperl et al., 1995; Grieder et al., 1997; Ryoo et al., 1999; Marty et al., 2001). Meis proteins have been shown to be indispensable as mediators of this process (Grieder et al., 1997; Ryoo et al., 1999; Marty et al., 2001).

In Xenopus, a member of the Meis family, XMeis3, is a posteriorising factor in neurectoderm of Xenopus laevis, and is required for hindbrain patterning (Salzberg et al., 1999; Dibner et al., 2001). In zebrafish embryos, similar functions have been reported for Meis3 and other Meis family members (Vlachakis et al., 2001; Waskiewicz et al., 2001; Choe et al., 2002). Expression of XMeis3 is reported as being initiated in a stripe in the neural plate of early-mid neurula embryos. During neurula and early-tailbud stages, expression is mainly localised to rhombomeres (r’s) 2, 3, and 4, and the anterior spinal cord, while posterior rhombomeres show some ventral expression (Salzberg et al., 1999). Expression of XMeis3 overlaps with neurectodermal expression of Hoxd1 (r4 and r5) (Kolm and Sive, 1995), Hoxb4 (r7, r8, and the anterior spinal cord) (Harvey and Melton, 1988), and Hoxc6 (anterior spinal cord) (Oliver et al., 1988; De Robertis et al., 1989).
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These overlaps are consistent with the idea that XMeis3 is involved in controlling the function of Hox proteins it is co-expressed with. These studies do, however, leave many questions unanswered. They pay little attention to when and where Meis cofactors actually interact with Hox proteins at different stages during the early AP patterning process. These details are likely to be crucial for understanding the mechanism at hand. Studies of vertebrate Hox expression and function have already made it clear that AP patterning depends on a specific early spatiotemporal sequence of Hox gene expression. Expression of each Hox gene is initiated in a specific domain in the gastrula embryo and then undergoes an establishment phase during which this expression domain changes to a gene specific AP zone in axial mesoderm and the neural plate and finally a maintenance phase during which this AP zone is consolidated. This sequence is employed universally in mammals, birds, fish and amphibians and shows generic features in these different species (Duboule and Dolle, 1989; Graham et al., 1989; Gaunt and Strachan, 1996; Deschamps et al., 1999; Wacker et al., submitted). A recent study analysed the early Hox expression patterns in Xenopus, and this revealed a spatiotemporally colinear initiation of expression of a sequence of Hox genes within a horseshoe-shaped domain of ventrolateral marginal zone mesoderm at different stages during gastrulation and then sequential dorsalisation of each Hox expression zone corresponding with its translation into a stable AP zone in axial mesoderm and the neural plate (Wacker et al., submitted). This sequence reflects timed interactions between a ventrolateral mesodermal Hox cascade and the Spemann organiser that are imperative for AP axis formation.

We set out to investigate whether expression of early Hox genes depends on the activity of XMeis3 and whether XMeis3 is involved in regulation of expression of these Hox genes in mesoderm during gastrulation. In order for XMeis3 to be able to regulate Hox expression in mesoderm they need to be co-expressed there. We performed whole mount in situ hybridisation to study the detailed early expression of XMeis3 and compared it to the early expression patterns of Hoxd1, Hoxb4, and Hoxc6 and found significant co-expression in lateral regions of marginal zone mesoderm, early during gastrulation. To gain further insight into the early function of XMeis3, we followed a gain- and a loss-of-function strategy. In the gain-of-function strategy synthetic XMeis3 mRNA was injected and expression of Hox genes was studied. These experiments showed that ectopic expression of XMeis3 during gastrulation is capable of inducing expression of the assayed Hox genes in mesoderm as well as in ectoderm. In the loss-of-function strategy we made use of an antisense morpholino oligonucleotide (reviewed in Heasman,
2002 and references therein) to inhibit the translation of \textit{XMeis3} mRNA (MO$_{XMeis3}$). Injection of MO$_{XMeis3}$ leads to a reduction in expression of \textit{Hoxd1}, \textit{Hoxb4}, and \textit{Hoxc6} in mesoderm and ectoderm during gastrulation, and to severe patterning defects. Finally we show synergy between \textit{Hoxd1} and \textit{XMeis3} and show that the mesodermal expression of \textit{Hoxd1} during early gastrulation is already dependent on \textit{XMeis3} mediated autoregulation.

\textbf{Results}

\textbf{The expression of XMeis3 overlaps with Hox gene expression in mesoderm}

To determine whether \textit{XMeis3} is co-expressed with \textit{Hox} genes in the mesoderm of gastrula embryos, whole mount \textit{in situ} hybridisations were performed for \textit{XMeis3}, \textit{Hoxd1}, \textit{Hoxb4}, and \textit{Hoxc6}. Expression of \textit{XMeis3} is initiated in a horseshoe-shaped domain in ventrolateral marginal zone mesoderm, before stage 11 expression is lost in the ventralmost tissue, resulting in two lateral expression domains on either side of the organiser in mesoderm of early gastrula stage embryos (Fig. 1A). Expression later in gastrulation becomes localised to mesoderm lateral to the midline and overlying ectoderm (Fig. 1B). Early expression of \textit{Hoxd1}, \textit{Hoxb4}, and \textit{Hoxc6} is initiated in ventrolateral mesoderm and each of these genes follows a similar spatiotemporal expression sequence (Wacker \textit{et al.}, submitted). During early phases of gastrulation mesodermal expression of \textit{Hoxd1} (Fig. 1C), \textit{Hoxb4} (Fig. 1E), and \textit{Hoxc6} (Fig. 1G) overlaps with expression of \textit{XMeis3} in the dorsolateral domains of these \textit{Hox} genes (compare Fig. 1A to 1C, 1E, and 1G). At the end of gastrulation the overlap between expression of \textit{Hoxd1} (Fig. 1D) and \textit{XMeis3} (Fig. 1B) in mesoderm is maintained, and newly initiated expression of both genes in the neurectoderm also overlaps. At the same time, the more posteriorly expressed \textit{Hoxb4} (Fig. 1F) and \textit{Hoxc6} (Fig. 1H) only partially overlap \textit{XMeis3} expression (Fig. 1B) in involuted mesoderm. \textit{Hoxb4} expression partially overlaps expression of \textit{XMeis3} in overlying ectoderm (compare Fig. 1F to 1B). These results show that there is indeed an overlap in expression of \textit{XMeis3} and of early \textit{Hox} genes in mesoderm during gastrulation, and that expression of \textit{XMeis3} and \textit{Hoxd1} also overlaps in ectoderm.
XMeis3 gain-of-function upregulates Hox gene expression in mesoderm and ectoderm

To investigate whether XMeis3 is capable of contributing to the regulation of Hox gene expression, 2 ng of synthetic mRNA containing the full-length coding region of XMeis3 was injected into the animal pole of embryos at the one-cell stage. The amount of 2 ng was chosen because this was shown to lead to posteriorisation of injected embryos (Salzberg et al., 1999). The effects on expression of Hoxd1, Hoxb4, Hoxc6, Xbra, and the posterior marker Xcad3 in gastrula stages were assayed by in situ hybridisation. The ectopic expression of Hoxd1 (Fig. 2A) in injected embryos is remarkable because it is found in the region harbouring the Spemann organiser, tissue that normally does not expresses Hox genes. The horseshoe-shaped domain of expression is also expanded and expression levels appear to be enhanced. Furthermore expression can be found in ectoderm of the animal cap and mesoderm underlying it, in the form of a streak of expression in contact with the expanded ring of expression around the blastopore (Fig 2A). Hoxb4 also shows ectopic expression in animal cap ectoderm and expansion of the endogenous expression domain (Fig. 2B), but no closure of the dorsal expression gap neither in organiser mesoderm nor in overlying ectoderm can be observed. Interestingly, induced expression of Hoxc6 can already be found in dorsal mesoderm at stage 10.25 (Fig. 2C), significantly earlier than endogenous initiation of expression (st11) and like ectopic Hoxd1 expression, this occurs in dorsal mesoderm. In later stages an expansion of the endogenous horseshoe-shaped expression domain is also found (data not shown). Expression of the mesodermal marker Xbra appears unaltered in injected embryos (Fig. 2D), suggesting that changes in Hox expression domains are not due to changes in induction of mesoderm, but rather to its patterning. The previously described posteriorising effect of XMeis3 on neurectoderm is confirmed by anterior expansion of expression of the posterior marker Xcad3 (Fig. 2E).

XMeis3 loss-of-function downregulates expression of Hox genes

To determine whether XMeis3 function is necessary for initiation and/or establishment of Hoxd1, Hoxb4, and Hoxc6 expression, an antisense morpholino oligonucleotide directed against XMeis3 mRNA (MOXMeis3) was injected into the animal hemisphere of embryos at the one-cell stage. XMeis3 loss-of-function leads to a loss of trunk structures and defects in axis specification, in a concentration dependent manner. When 12 ng MOXMeis3
was injected a loss of trunk structures and defects in head development and tail formation can be observed, while the anteriormost structure, the cement gland, remains present (Fig. 3B). When 24 ng MO$_{XMeis3}$ was injected, an enlargement of the cement gland was visible accompanied by a stronger loss of trunk structures (Fig. 3C) In half the injected embryos spina bifida’s are observed, suggesting that the embryos suffer from gastrulation problems. When 32 ng or more MO$_{XMeis3}$ were injected, the embryos arrested during gastrulation at stage 11 (Fig. 3D). Embryos injected with this high dose of MO$_{XMeis3}$ appear unaffected and posses normal looking blastopores until the moment of arrest. This is unlike what would be expected if the arrest was caused by toxicity of an injected agent, this would generally generate a much larger spread in stages at which embryos die or arrest, accompanied by irregular formation of the blastopore. Removal of the vitelline membrane revealed that cells have lost cell-cell contact, but appear round and intact. This suggests that the observed effect is the result of a strong knockdown of XMeis3 function and not an aspecific effect of MO$_{XMeis3}$. Injection of the same amount of a control morpholino (MO$^{cont}$), in sequence unrelated to MO$_{XMeis3}$, has no outward effects on embryos (data not shown). These findings support the idea that the gastrulation arrest phenotype is a true result of XMeis3 loss-of-function and that XMeis3 is required for patterning (a part of) the primary axis in Xenopus embryos.

To further test the specificity of the MO$_{XMeis3}$, 125 pg of synthetic XMeis3 mRNA, lacking most of the sequence that the MO$_{XMeis3}$ is complementary to, was co-injected with 32 ng MO$_{XMeis3}$ into the animal hemisphere of embryos at the one-cell stage (Fig. 3F). The exogenous XMeis3 was able to largely rescue the MO$_{XMeis3}$ phenotype (compare Fig. 3D to 3E, and 3F). In a small number of the co-injected embryos a full recovery of the axis can be observed, sometimes accompanied by a secondary axial outgrowth out of the primary axis, containing somites (Fig. 3G).

The effect of XMeis3 loss-of-function on Hox expression was studied by injecting 16 ng MO$_{XMeis3}$ into the animal hemisphere of embryos at the one-cell stage followed by in situ hybridisation. To be able to analyse marker expression in late gastrula stage embryos the arrest in gastrulation, observed after injection of a high amount of MO$_{XMeis3}$, was avoided, by the injection of 16 ng. The XMeis3 loss-of-function leads to downregulation of expression of Hoxd1 (Fig. 4A), Hoxb4 (Fig. 4B), and Hoxc6 (Fig. 4C), in mesoderm and ectoderm. This led to our conclusion that XMeis3 is necessary for Hox gene expression in marginal zone mesoderm, and neural plate ectoderm.
Synergy between Hoxd1 and XMeis3

Autoregulation is known to occur among labial type Hox genes in hindbrain ectoderm (Pöpperl et al., 1995; Struder et al., 1998), endoderm of Drosophila embryos (Grieder et al., 1997; Ryoo et al., 1999), and C. elegans (Streit et al., 2002). For a number of these cases it has been shown that this autoregulation is dependent on a Pbx/Hox bipartite binding site in their promoters (Pöpperl et al., 1995; Grieder et al., 1997; Ryoo et al., 1999; Streit et al., 2002). Because nuclear localisation of Pbx family members is dependent on the action of Meis family members and because XMeis3 loss-of-function led to a significant downregulation of Hoxd1 expression in mesoderm and ectoderm, we suspected that XMeis3 might be involved in Hoxd1 autoregulation. To test our idea that XMeis3 may mediate autoregulation of labial type Hox genes in Xenopus development, we co-injected relatively small amounts of synthetic mRNA for XMeis3 and Hoxd1 and also injected them separately using double the amount of mRNA. Small amounts of mRNA were used to be able to observe compound phenotypes in co-injected embryos. If a strong effect in embryos injected with only a single messenger was generated this would not have been possible. The embryos injected with only a single synthetic messenger show little or no phenotypic effects, while co-injected embryos show a significant retardation in head development (Fig. 5). This points towards a synergistic relation between Hoxd1 and XMeis3.

To further test this synergy, and to test whether XMeis3-mediated Hoxd1 autoregulation is involved in the establishment of Hoxd1 expression, we wished to investigate the necessity of Hoxd1 for maintaining Hoxd1 expression in mesoderm. If XMeis3 activity is needed in early gastrula mesoderm to enhance or alter the function of Hoxd1, then Hoxd1 loss-of-function should generate the same effect on Hoxd1 expression as XMeis3 loss-of-function. To test whether this is the case, 32 ng MO\textsuperscript{Hoxd1} (McNulty \textit{et al.}, manuscript in preparation) was injected into the equatorial region of the 2 blastomeres making up the presumptive left side of 4-cell stage embryos. The other half of the embryos served as an internal control. This results in a downregulation of expression of Hoxd1 in mesoderm on the injected side (Fig. 6A). To further test whether establishment of expression of Hoxd1 needs both Hoxd1 and XMeis3, sub optimal amounts of morpholinos against both messengers were co-injected and injected separately. Embryos were harvested at stage 11 and assayed for Hoxd1 expression (Fig 6B). Sub optimal morpholino amounts were used to allow different levels of reduction.
Figure 1. Expression of *XMeis3*, *Hoxd1*, *Hoxb4*, and *Hoxc6* during gastrulation. Embryos were analysed by whole-mount *in situ* hybridisation for expression of *XMeis3* (A and B), *Hoxd1* (C and D), *Hoxb4* (E and F), and *Hoxc6* (G and H). Whole mounts are shown on the left side of each panel, sections of these embryos are shown on the right side of each panel, in the inset, on the bottom right corner of every panel, the dotted line indicates the plane of sectioning. Embryos shown are at stage 11, vegetal views with dorsal up (A, C, E, and G) and at stage 13, dorsal views with anterior up (B, D, F, and H). *XMeis3* expression overlaps with dorsolateral expression of *Hoxd1*, *Hoxb4*, and *Hoxc6* in mesoderm at stage 11 (A, C, E, and G). *XMeis3* expression in ectoderm at stage 13 overlaps with expression of *Hoxd1* but not with expression of *Hoxb4* and *Hoxc6* (B, D, F, and H).
XMeis3 is necessary for mesodermal Hox gene expression

Figure 2. XMeis3 gain-of-function. Embryos were injected into the animal hemisphere of embryos at the one-cell stage with 2 ng synthetic mRNA containing the full-length coding region of XMeis3, and analysed by whole-mount in situ hybridisation. In each panel control embryos are shown on top, XMeis3 injected embryos are shown on the bottom. For A, B, and C, whole mounts are shown on the left side, sections of these embryos are shown on the right hand of each panel. The plane of sectioning is depicted by the dotted line in the insets. (A) Expression of Hoxd1, whole mounts are shown in dorsal view, with anterior to the top, at stage 10.5. Lateral expression of Hoxd1 in injected embryos is stronger and in a broader domain, the gap in expression on the dorsal mesoderm is closed and a streak of expression in dorsal mesoderm is observed. (B) Expression of Hoxb4, whole mounts are shown in lateral view, with dorsal to the left, at stage 11. Lateral expression of Hoxb4 is not affected by injection of XMeis3, the black arrow points to a patch of ectopic expression in ectoderm. (C) Expression of Hoxc6, whole mounts are shown in dorsal view, with anterior to the top, at stage 10.5. Injected embryos show ectopic expression of Hoxc6 in dorsal mesoderm, prior to initiation of endogenous expression of Hoxc6. (D) Expression of Xbra, embryos at stage 10.5 are shown in vegetal view with dorsal to the top. No change can be observed in the expression of the mesodermal marker Xbra as a result of injection of XMeis3. (E) Expression of Xcad3, embryos at stage 17 are shown in dorsal view with anterior to the top. The anterior expression boundary of the posterior marker Xcad3 is shifted to a more anterior position by injection of XMeis3.
in *Hoxd1* expression, thus allowing possible synergistic effects to be observed. A downregulation of *Hoxd1* expression in embryos injected with a single morpholino and an additional reduction by injection of both morpholinos is visible (Fig. 6B). This suggests that indeed there is a synergistic effect of *Hoxd1* and *XMeis3* on establishment of *Hoxd1* expression in marginal zone mesoderm during gastrulation.

**Discussion**

*XMeis3* expression overlaps early *Hox* expression

Much effort has been put into finding out details about the relation between *Hox* proteins and their cofactors Pbx/Exd and Meis/Hth. Although much has been accomplished, most of this work consists of *in vitro* binding studies. In *Xenopus* embryos, it has been shown that *XMeis3* has a function in hindbrain patterning (Salzberg *et al.*, 1999; Dibner *et al.*, 2001), these results are corroborated by recent reports concerned with Meis function in hindbrain formation in zebrafish embryos (Vlachakis *et al.*, 2001; Waskiewicz *et al.*, 2001; Choe *et al.*, 2002). We show here that *XMeis3* is expressed in marginal zone mesoderm significantly earlier than previously described (Salzberg *et al.*, 1999). We went on to show that an overlap in expression of *XMeis3* and early *Hox* genes is found in ventral and lateral mesoderm during gastrulation. In later phases of gastrulation the overlap is restricted to dorsolateral mesoderm. This co-localisation with early *Hox* genes suggests a role for *XMeis3* in the regulation of *Hox* gene expression in mesoderm during the early phases of gastrulation.

**Ectopic *XMeis3* enhances *Hox* expression in mesoderm**

By gain-of-function experiments we show that ectopic *XMeis3* is capable of inducing expression of *Hoxd1, Hoxb4,* and *Hoxc6,* expanding endogenous expression domains of these genes in mesoderm, and ectopically initiating expression in dorsal mesoderm. Interestingly, this induction of *Hox* expression by ectopic *XMeis3* can only be found as expansions of endogenous expression domains or in streaks of expression still in contact with the expanded endogenous domains of expression. This is most obvious for ectopic expression of *Hoxd1* in dorsal mesoderm, expanding into more animaly located mesoderm and ectoderm. This suggests that ectopic *XMeis3* only enhances the expression of the assayed *Hox* genes, requiring factors already present in their endogenous *Hox* expression domains rather than
Figure 3. Effects of XMeis3 loss-of-function on the phenotype and the rescue of MO\textsuperscript{XMeis3}.

Embryos at the one-cell stage were injected into the animal hemisphere with MO\textsuperscript{XMeis3} in amounts of 12 ng (B), 24 ng (C), and 36 ng (D), and allowed to develop until the control embryos (A) reached tadpole stages. The specificity of MO\textsuperscript{XMeis3} is shown by the rescue with XMeis3 synthetic mRNA. Embryos were injected with 32 ng of MO\textsuperscript{XMeis3} and 125 pg synthetic mRNA for XMeis3 and allowed to develop until the control embryos reached the tadpole stage (E). In the majority of the embryos a large part of the axis was rescued (F), in a small number of embryos the phenotype could even be reversed, not only is the axis fully rescued but the embryo shown in (G) even possesses additional trunk structures as shown by the presence of somites in the axis outgrowth.
Figure 4. XMeis3 loss-of-function. Embryos were injected at the one-cell stage with 16 ng of the MO
XMeis3, and analysed by whole mount in situ hybridisation at stage 10.5/11, shown on the left side of each panel, and at stage 12, shown at the right side of each panel. Injected embryos are shown at the bottom of each panel, untreated embryos are shown on top. Shown are vegetal views with dorsal to the top. Expression of Hoxd1 (A), Hoxb4 (B), and Hoxc6 (C) is downregulated in mesoderm of injected embryos at early gastrula stages. A reduction in ectodermal expression of the three studied Hox genes is observed in injected embryos at stage 12.

Figure 5. Synergistic effect between Hoxd1 and XMeis3 in ectopic expression. Embryos at the one-cell stage were injected into the animal hemisphere with either 100 pg Hoxd1 mRNA, 100 pg XMeis3 mRNA, or 50 pg of both mRNA’s. A single injection of 100 pg of either factor is not sufficient to induce a phenotypic effect. The combination of half the amount of Hoxd1 and XMeis3, results in posteriorisation, shown by a clear reduction of eye formation, and an anterior shift of the eye.
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inducing expression *de novo*. These patterns are consistent with our idea that *XMeis3* enhances Hox autoregulation in mesoderm of *Xenopus* embryos.

**XMeis3 is necessary for Hox expression in mesoderm and ectoderm**

The injection of MO<sup>XMeis3</sup> led to a downregulation of expression of all three *Hox* genes assayed. For *Hoxd1* and *Hoxb4* this held true for mesoderm and ectoderm, in the case of *Hoxc6*, mesodermal expression partially recovers during later phases of gastrulation, but ectodermal expression could not be observed. This indicates that *XMeis3* protein is necessary, in ventral and lateral mesoderm and in neurectoderm during gastrulation, for proper initiation and maintenance of *Hox* expression.

*XMeis3* loss-of-function using small amounts of MO<sup>XMeis3</sup> already led to a strong phenotype, indicating the necessity of *XMeis3* function in anteroposterior patterning. This phenotype corroborates the results of Dibner and co-workers (2001). The sudden arrest in gastrulation at stage 11, caused by injecting a high amount of MO<sup>XMeis3</sup> is very striking. We show by coinjecting a limited amount of *XMeis3* mRNA that the observed effect is not aspecific. The phenotype observed after injection of less morpholino, namely loss of trunk structures, head defects, and retarded tail formation described in this report and by Dibner and co-workers (2001), is therefore most likely a result of reduced *XMeis3* function, not a complete loss of it. We cannot be certain that the phenotype caused by injection of 32 ng MO<sup>XMeis3</sup> represents the complete loss-of-function phenotype, but it suggests the need for *XMeis3* in two processes during early development: the progression of gastrulation and the subsequent patterning of the hindbrain.

**Autoregulation by Hoxd1 is necessary for its establishment of expression in marginal zone mesoderm**

Autoregulation dependent on Pbx/Exd has been shown for Hox paralog group 1 and 4 members (Pöpperl et al., 1995; Gould et al., 1997; Ryoo et al., 1999; Ferretti et al., 2000; Marty et al., 2001; Streit et al., 2002), this suggests that the shown regulation of *Hox* expression by *XMeis3* could take place at the level of *Hox* autoregulation. Indeed, injection of MO<sup>Hoxd1</sup> led to a reduction in *Hoxd1* expression. This is most likely the result of a reduction in *Hoxd1* translation, leading to a reduced amount of *Hoxd1* protein. This reduction of *Hoxd1* protein levels subsequently led to an apparent reduction in *Hoxd1* transcription. This suggests that *Hoxd1* autoregulation is an essential step in the establishment, and not only the maintenance, of *Hoxd1* expression in
Figure 6. Synergistic effects in loss-of-function of Hoxd1 and XMeis3. (A) Embryos were injected with 32 ng of MO\textsuperscript{Hoxd1} into the lateral marginal zone on the left side of embryos, rendering the un-injected side an internal control. Embryos were allowed to develop until control stage 11 and assayed by \textit{in situ} hybridisation for expression of Hoxd1. Embryos are shown in vegetal view, with dorsal up. Expression of Hoxd1 is reduced on the left side of injected embryos (shown on the bottom of the panel). (B) To investigate whether there is synergy between Hoxd1 and XMeis3, 16 ng MO\textsuperscript{XMeis3} and 16 ng MO\textsuperscript{Hoxd1} were injected, together and separately, into the animal hemisphere of one-cell stage embryos, at stage 11 the embryos were harvested and assayed for expression of Hoxd1 by \textit{in situ} hybridisation. Embryos are shown in lateral view, with dorsal to the left. Injection of MO\textsuperscript{Hoxd1} and MO\textsuperscript{XMeis3} separately leads to a reduction in expression of Hoxd1, the co-injection leads to a reduction as compared to injection of either MO\textsuperscript{XMeis3} or MO\textsuperscript{Hoxd1} separately. This suggests that Hoxd1 and XMeis3 work synergistically in establishment of Hoxd1 expression in mesoderm during early gastrula stages.
mesoderm during gastrulation in *Xenopus* embryos. The observed reduction of *Hoxd1* expression could also be explained if binding of MO\(^{Hoxd1}\) to mRNA led to a reduction in stability of the messenger, however this potential effect has, to our knowledge, never been reported. The necessity for *Hoxd1* autoregulation in mesoderm is a remarkable discovery considering that vertebrate Meis family members have so far only been shown to be involved in Hox autoregulation in the hindbrain. Here we show that XMeis3 is an essential factor for establishment of stable *Hoxd1* expression in marginal zone mesoderm. A second noteworthy aspect is that apparently *Hoxd1* loss-of-function is not fully, if at all, rescued by the other labial type gene normally expressed during the early phases of gastrulation, *Hoxa1* and *Hoxb1* as would be expected from a viewpoint of redundancy. Either *Hoxa1* and *Hoxb1* are not capable of inducing the expression of *Hoxd1*, which seems unlikely taking into account the redundant function of these paralog group members (reviewed in Morrison, 1998, and references therein), or expression of *Hoxa1* and *Hoxb1* is also reduced or prevented by *Hoxd1* loss-of-function, this would suggest the necessity of *Hoxd1* to induce the two other labial homologous during gastrulation in *Xenopus* embryos. Additional experiments are needed to distinguish between the two possibilities but whatever the outcome, this sheds new light on the initiation and establishment of expression of the first *Hox* genes of the *Hox* cascade.

**Synergy between *Hoxd1* and XMeis3**

The synergistic effects we have observed in the gain-of-function experiment by injection of synthetic *XMeis3* and *Hoxd1* mRNA together show that indeed these two factors, when co-expressed can generate a phenotype that cannot be accomplished by injecting double the amount of either factor separately. These results corroborate the findings of Vlachakis and co-workers (2001), who have shown that in zebrafish embryos, Meis3, Pbx4, and *Hoxb1* synergise to promote hindbrain fate. The combined *Hoxd1* and *XMeis3* loss-of-function supports the suggested synergy, while sub optimal amounts of either morpholino against *Hoxd1* or *XMeis3* led to a reduction of the *Hoxd1* expression, the combination led to a significantly stronger reduction. This adds to the evidence for a synergistic relation between *Hoxd1* and XMeis3. Taken together our results show that XMeis3 is necessary in marginal zone mesoderm to establish the expression of early Hox genes. This XMeis3-mediated mesodermal *Hox* cascade is of vital importance for axis formation and AP patterning.
Materials and methods

Xenopus embryos and microinjections

Pigmented Xenopus laevis embryos were obtained by in vitro fertilisation, and after dejelling in a 2% cysteine solution (pH 8.0), cultured in 0.1x Marc’s Modified Ringers’s (MMR) (Sive et al., 2000), containing 50 µg/ml gentamycin at 14-21 °C. Embryos were injected in 1x MMR + 4% ficoll and afterwards transferred to 1x MMR + 1% Ficoll, and cultured in this medium for 1 to 7 hours, after which they were transferred and to 0.1x MMR in which they were cultured until harvesting. Staging of the embryos was performed according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Embryos at the one-cell stage were injected into the animal pole with synthetic mRNA dissolved in water. The synthetic capped mRNA was made using the Ambion mMessage mMachine Kit with CS2-XMeis3, or CS2-Hoxd1, linearised with NotI, as template. CS2-XMeis3 was constructed by cloning the full-length coding region of XMeis3, obtained by PCR using stage 15 cDNA as template and the following primers: f: 5’-gcgggatccatggcacaaaggtatgatgag, r: 5’–cgcctcgagcatgtagtgccactgcccctcc, containing a BamHI or a XhoI restriction site respectively, in the CS2+ vector (Rupp et al., 1994) using the restriction sites in the primers. CS2-Hoxd-1 contains the complete coding sequence of XHoxd1 in CS2+, kindly provided by W. Van den Akker.

Whole mount in situ hybridisation and antisense probes

Whole mount in situ hybridisations were performed according to Harland (1991), with minor modifications. The antisense RNA probes were generated by run off in vitro translation using DIG RNA labelling mix (Roche), and T7 or Sp6 RNA polymerase (Promega). The probes were generated using the following templates: Hoxd1: (Sive and Cheng, 1991), Hoxb4: a 708 bp fragment containing the complete Hoxb-4 ORF cloned in pGEMTE, Hoxc6: a 998 bp Hoxc-6 fragment in pGEM1 containing a part of the homeodomain and extending into the 3’ UTR, Xcad3: (Pownall et al., 1996); Xbra: pSP73Xbra (Smith et al., 1991).
XMeis3 is necessary for mesodermal Hox gene expression

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