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# T-box Genes in Early Embryogenesis

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## Abstract

The T-box gene family, encoding related DNA-binding transcriptional regulators, plays an essential role in controlling many aspects of embryogenesis in a wide variety of organisms. The T-box genes exhibit diverse patterns of spatial and temporal expression in the developing embryo, and both genetic and molecular embryological studies have demonstrated their importance in regulating cell fate decisions that establish the early body plan, and in later processes underlying organogenesis. Despite these studies, little is known of either the regulation of the T-box genes or the identities of their transcriptional targets. The aim of this review is to examine the diverse yet conserved roles of several T-box genes in regulating early patterning in chordates and to discuss possible mechanisms through which this functional diversity might arise.

### Keywords

T-box; T-domain; Brachyury; Eomesodermin; VegT; spadetail; no tail; tbx6; mesoderm; transcription

## INTRODUCTION

Adult multicellular organisms typically contain a variety of different specialized cell types, their cooperative activity underpinning the function of the organism as a whole. The cellular diversity in the adult arises during embryogenesis, and one of the aims of modern developmental biology is to gain an understanding of the molecular mechanisms through which this process occurs. Genetic, biochemical, and molecular studies over the past two decades have identified numerous proteins whose function is to directly effect the specification and differentiation of specific cell types through the regulation of downstream gene transcription, by binding to regulatory elements in a sequence-specific manner. Sequence comparisons amongst these proteins highlight that many are members of families of related factors that have arisen through genetic events leading to the duplication of ancestral genes, followed by functional diversification.

The T-box genes encode a family of transcription factors sharing a characteristic sequence similarity within the DNA-binding domain (T-domain). To date, 18 different mammalian T-box genes have been identified, many of which have orthologues in a wide variety of multicellular organisms. The developmental functions of the T-box genes range from the specification of the primary germ layers by genes such as *VegT* and *Brachyury* (this review), to later roles in limb development (e.g., *Tbx4/5*; Rodriguez-Esteban et al., 1999; Takeuchi et al., 1999), and the specification of cell identity during organogenesis, e.g., *Tbx5* (Horb and Thomsen, 1999) and *Tbx20/hrT* (Szeto et al., 2002) in cardiac development, and *Tpit* in the specification of pituitary cell lineages (Lamolet et al., 2001). The biologically important roles of several members of this gene family are further emphasized by clinical studies demonstrating

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that mutations in T-box genes are associated with numerous disease states in humans, including congenital diseases such as the DiGeorge and Holt-Oram syndromes, and by the observation that T-box genes are amplified in a subset of cancers (for review, see Packham and Brook, 2003).

Collectively, studies on T-box genes have shown that many members have several properties that make them ideal candidates for studying early mesodermal patterning: the genes respond to mesodermal growth factors in the absence of protein synthesis; their response to mesoderm inducing factors is dose dependent; they are necessary and sufficient to specify mesodermal cell types; and their sequence, expression pattern, and function for any one member is evolutionarily conserved (for reviews, see Papaioannou and Silver, 1998; Smith, 1999; Papaioannou, 2001; Wilson and Conlon, 2002).

Although much is known about the requirement for T-box genes, little is known about how T-box genes select and regulate their downstream targets (Tada and Smith, 2001). This raises important questions concerning the determinants of functional diversity, questions that are relevant not only to the T-box genes but also to other families of developmentally important transcription factors, such as the homeobox (*Hox*) and *Sox* families. Paradoxically, the functional diversity within such families must be based largely upon variation in their choice of target genes, and yet the protein domains responsible for sequence-specific DNA-binding often exhibit little variation. Although studies of T-domain proteins and our understanding of similar transcriptional regulators offer some clues as to how variation in target gene specificity (and, therefore, developmental function) might be determined at the molecular level, many questions remain.

In the following discussion, we focus on the roles played by several T-box genes in regulating early embryogenesis in several chordate model systems and discuss both their upstream regulation and downstream transcriptional targets. Finally, we explore possible mechanisms, molecular or otherwise, that might enable their related products to perform unique developmental functions.

## *Brachyury* AND EARLY DEVELOPMENT IN THE MOUSE

The first T-box gene to be molecularly characterized was *Brachyury (T)* (Herrmann et al., 1990), from which the family takes its name. The phenotype of *Brachyury* mutant mice was first described by Dobrovolskaïa-Zavadskaïa, the most striking defect in heterozygous T mice being a truncated tail (Dobrovolskaïa-Zavadskaïa, 1927). Mice homozygous for mutations in *Brachyury* die shortly after gastrulation and display several mesodermal abnormalities, including complete loss of the posterior mesoderm (Gluecksohn-Schoenheimer, 1938, 1944). During normal mouse development, the three primary germ layers (ectoderm, mesoderm, and endoderm) become arranged through gastrulation movements in which the inner layer of cells (epiblast) in the cup-shaped embryo ingresses and moves between the remaining epiblast layer (future ectoderm) and the outer primitive endoderm layer. This gradual movement of presumptive mesoderm and endoderm cells, beginning in the posterior, creates a morphologically visible feature known as the primitive streak. As the embryo develops, mesodermal cells leave the streak, migrating laterally and anteriorly to occupy lateral and dorsal positions along the anteroposterior axis, and subsequently form axial and paraxial mesoderm structures such as the notochord and somites (Beddington, 1982; Tam and Beddington, 1992).

In $T^{-/-}$ embryos, the primitive streak is condensed and thick relative to wild-type littermates (Chesley, 1935; Gluecksohn-Schoenheimer, 1938, 1944; Gruneberg, 1958). By embryonic day eight (E8), the mesoderm: ectoderm ratio is elevated 15% in the posterior portions of mutant embryos while remaining normal in the anterior half (Yanagisawa et al., 1981). Because the

mitotic index of both the anterior and posterior portions of $T^{-/-}$ embryos appears normal, the primary defect appears to be an alteration in cell adhesion (Yanagisawa and Fujimoto, 1977; Yanagisawa et al., 1981). In support of such a hypothesis, measurements of mesodermal migration on an extracellular matrix show a reduction in the migration of mutant-derived mesodermal cells relative to wild-type (Hashimoto et al., 1987). A role for *Brachyury* in cell adhesion is further supported by in vivo analysis showing $T^{-/-}$ mutant embryonic stem (ES) cells are compromised in their ability to migrate away from the primitive streak and, therefore, unable to carry out the morphogenetic movements performed by their wild-type counterparts during gastrulation, leading to their accumulation in the primitive streak (Rashbass et al., 1991; Wilson et al., 1993, 1995; Wilson and Beddington, 1997). As shown in homozygous *T* mutant embryos, this accumulation eventually leads to loss by programmed cell death (Fig. 1; Conlon and Smith, 1999). Defects in mesoderm migration also affect extra-embryonic mesoderm, eventually affecting the formation of the allantois, which subsequently fails to contact the chorion. Thus, the mutant embryos lack a proper placental connection and this ultimately accounts for their death at approximately E10.5 (Gluecksohn-Schoenheimer, 1938, 1944; Wilson et al., 1993).

In addition to defects in the primitive streak, the notochord is absent in posterior portions of the embryo, and while the anterior portions contain notochordal precursor-like cells, these fail to undergo normal terminal differentiation (Chesley, 1935; Gruneberg, 1958; Yanagisawa, 1990). The notochord arises from a population of precursor cells contained within the node or organizer, a structure that appears anatomically abnormal in mutant embryos as early as embryonic day (E) 7.5. In addition to defects in the primitive streak and notochord, numerous other phenotypic abnormalities have been reported in $T^{-/-}$ embryos (Chesley, 1935; Gruneberg, 1958; Yanagisawa, 1990; Beddington et al., 1992; Herrmann and Kispert, 1994). However most of these, such as lack of posterior somites, appear to be secondary defects due, at least in part, to the absence of the normal patterning influence of the notochord (Herrmann, 1991; Conlon et al., 1995).

Chimeric and phenotypic analysis has shown that *Brachyury* acts cell-autonomously (Wilson et al., 1993); thus, the tissues directly affected by loss of *Brachyury* function are primarily those in which the gene is expressed. In wild-type embryos, expression is seen in the primitive streak at the onset of gastrulation and persists for a short time in both the newly formed mesoderm and in adjacent epiblast cells. As the future paraxial mesoderm cells migrate laterally, *Brachyury* is down-regulated to levels undetectable by in situ hybridization, while it continues to be strongly expressed in the notochord (Fig. 1; Wilkinson et al., 1990; Herrmann, 1991). Together, phenotypic and chimeric analysis of $T^{-/-}$ embryos strongly suggest that the *Brachyury* gene product is required during gastrulation for the proper specification of mesodermal identity in cells of the epiblast, just before their ingression through the primitive streak. This specification may be required to confer the correct migratory behaviour on these cells during gastrulation. Additionally, these mouse studies suggest that Brachyury may have a second function in maintaining the differentiated state in the notochord.

## FUNCTION OF *Brachyury* IN THE DEVELOPING MESODERM IS EVOLUTIONARILY CONSERVED

*Brachyury* orthologues have been identified in many organisms, including those commonly used in embryological and developmental genetic studies (Holland et al., 1995). This finding has allowed the role of *Brachyury* to be further examined through both molecular developmental techniques in the frog *Xenopus laevis* and through genetic analysis in zebrafish. The *Xenopus* homologue of *Brachyury*, *Xbra*, was cloned by Smith and coworkers (1991), based on sequence homology between the frog and mouse sequences. Analysis of its expression supported the idea that it represents a true orthologue of the mouse gene, displaying a high

degree of similarity in both sequence and expression pattern. Although low levels of maternal *Xbra* are detected in the unfertilized egg, the gene is expressed predominantly at the mid-blastula to neurula stages. In situ hybridization reveals expression throughout the dorsoventral axis of the prospective mesoderm around the equator of the embryo, although it is not possible to conclude that all prospective mesoderm cells express the gene (Smith et al., 1991). During gastrulation, *Xbra* expression is maintained in the developing notochord cells as they migrate anteriorly, producing a striking anteroposterior stripe of expression that persists into neurula stages. However, expression is down-regulated in non-notochord mesoderm cells as gastrulation proceeds. Expression continues in the prospective posterior and ventral mesoderm in late gastrulae and early neurulae, as a ring of *Xbra*-positive cells around the closing blastopore. This pattern of expression is clearly reminiscent of *Brachyury* expression in the epiblast of the mouse embryo before gastrulation, in the nascent posterior mesoderm of the primitive streak, and in the newly formed notochord, suggesting *Xbra* may play a similar role in the formation of mesoderm in mouse and frog (Smith et al., 1991). However, not all properties of *Brachyury* may be evolutionarily conserved. For example, Marcellini and coworkers have demonstrated recently that, while most orthologues of *Brachyury* can induce mesoderm, a second class of orthologues including those of *Drosophila* and ascidians can also induce endoderm (Marcellini et al. 2003).

*Xenopus* has several advantages as a model system for studying the very early steps in patterning the vertebrate embryo, including the ability to study protein function by over- and misexpression. As discussed, in the absence of *Brachyury* function gastrulation is disrupted as a consequence of a failure of the prospective mesoderm to ingress through the primitive streak. Although demonstrating a requirement for *Brachyury*, genetic analysis fails to distinguish between a role for *Brachyury* solely in regulating morphogenetic cell movements, versus a dual role in morphogenesis and the specification of mesodermal cell fate, specifically that of the notochord. To address this issue, Cunliffe and Smith examined the effect of *Xbra* misexpression on the fate of isolated animal pole explants (Cunliffe and Smith, 1992). Classic studies by Nieuwkoop demonstrated that the animal pole of the early *Xenopus* embryo gives rise to epidermal derivatives when cultured in isolation (Nieuwkoop, 1969). In stark contrast, misexpression of *Xbra* in animal cap tissue diverts the prospective ectoderm into the mesodermal lineage, and in particular ventral mesoderm (Fig. 2; Cunliffe and Smith, 1992). Thus, *Brachyury/Xbra* is both necessary and sufficient for mesoderm formation. Moreover, different doses of *Xbra* are capable of inducing different mesodermal cell types (O'Reilly et al., 1995). Somewhat surprisingly, notochord is never induced at any concentration. Nonetheless, notochord differentiation can occur when *Xbra* is coexpressed with *Pintallavis*, encoding an HNF3/fork-head family transcription factor normally coexpressed with *Xbra* in notochordal precursors (O'Reilly et al., 1995). Collectively, these studies show that, at least at the cellular level, Xbra can act synergistically with other transcription factors to specify defined cell fates. This occurs in parallel with its regulation of morphogenetic cell behaviors (Conlon and Smith, 1999).

Subsequent studies in zebrafish have shown that the role of *Brachyury* in morphogenesis and cell fate is evolutionarily conserved. Although, unlike the mouse, zebrafish heterozygous for mutations in the *Brachyury* orthologue *no tail* (*ntl*; Schulte-Merker et al., 1994) do not exhibit any obvious phenotypic defects, fish homozygous for *no tail* mutations have a phenotype that closely resembles mouse mutants, lacking posterior mesoderm and mature notochord (Halpern et al., 1993). As with mouse, the somite-derived myotomes in *no tail* mutants are disorganized in the trunk, failing to adopt their characteristic chevron shape seen in wild-type embryos, although they do contain differentiated muscle fibers. No defects were detected in the development of other mesodermal tissues and organs. Studies of gastrulation in *no tail* embryos revealed that the early cell movements occur normally. However, after involution, cells in *no tail* mutants exhibit a defect in convergence (although extension appears normal; Glickman et

al., 2003). The axial mesoderm cells fail to form a distinct notochord primordium and are instead scattered along the midline (Halpern et al., 1993). In chimeras generated by transplanting *no tail* mutant donor cells into wild-type host embryos, the *no tail* mutant cells do not contribute to the differentiated notochord while, in the converse experiment, wild-type cells differentiated as notochord regardless of the surrounding mutant cells (Halpern et al., 1993). These observations demonstrate both the cell-autonomous nature of the *no tail* phenotype and the requirement for the wild-type gene product for proper notochord differentiation and morphogenesis.

To what degree is *no tail* required for notochord cell identity in the zebrafish? In *no tail* homozygotes, the development of the central nervous system appears normal in the anterior regions of the embryo. The neural tube is patterned correctly along the anteroposterior and dorsoventral axes, and the floor plate (a morphologically and biochemically distinct group of cells in the ventral region of the neural tube) is present. The patterning of the neural tube is known to be partly dependent upon signals derived from the adjacent notochord, suggesting that, while morphogenesis of the notochord is severely disrupted in the *no tail* mutant, the source of many of these signals must still be present. Halpern and coworkers observed mesenchymal cells underlying the floor plate in mutant embryos and conducted clonal analysis that suggested that they shared a common lineage with the notochord cells of wild-type embryos (Halpern et al., 1993). This finding led to the hypothesis that the cells are residual notochord precursors that retain some notochord-like characteristics. Clearly, the results of these studies in the zebrafish confirm that, while *no tail*/*Brachyury* is not required for all aspects of notochord cell identity, it is an essential component of genetic pathways regulating morphogenesis and cell function.

## Brachyury FUNCTIONS AS A TRANSCRIPTION FACTOR

Brachyury has been shown to function at the molecular level as a classic transcriptional activator; the protein functions cell autonomously, is localized to the nucleus, binds DNA in a sequence-specific manner, and can regulate transcriptional levels of heterologous and downstream target genes in several different contexts (Kispert and Herrmann, 1993; Kispert et al., 1995; Conlon et al. 1996). Binding site selection assays conducted with the murine orthologue (Kispert and Herrmann, 1993) and later with the *Xenopus* orthologue of Brachyury (Conlon et al., 2001), established that it binds through the N-terminal region of the protein to a core DNA consensus sequence. Although crystallographic analysis of T-domain proteins has only been achieved for a truncated version of Xbra, and for Tbx3 (Müller and Herrmann, 1997; Coll et al., 2002), these studies clearly demonstrate that the T-domain represents a unique class of DNA binding motif in which the carboxy-terminal helix contacts and is embedded into an enlarged minor groove of DNA (Müller and Herrmann, 1997). Sequence comparisons across the family show varying degrees of identity within the DNA binding domain. However, specific residues within this region are completely conserved in all orthologues of a single family member (Smith, 1999; Papaioannou, 2001; Wilson and Conlon, 2002).

Kispert and coworkers (Kispert et al., 1995) and Conlon and coworkers (Conlon et al., 1996) went on to show that mouse, *Xenopus*, and zebrafish *Brachyury* orthologues are capable of activating transcription, and both groups used deletion analysis to map the regions both necessary and sufficient for activation in mouse (Kispert et al., 1995), *Xenopus*, and zebrafish (Conlon et al., 1996). In the case of mouse *Brachyury*, Kispert and coworkers identified two activation and two repressor domains, while experiments in *Xenopus* and zebrafish led to the identification of a single activation domain. The significance of these differences between orthologues is unclear, but in all three species, the full-length protein functions as a transcriptional activator, suggesting the endogenous role of Brachyury is to activate mesoderm-specific genes. Further support for an endogenous role for Brachyury as a transcriptional

activator comes from the observation that a deletion mutation in zebrafish *no tail* lacking only the sequence encoding the activation domain is phenotypically identical to null embryos. Moreover, when the activation domain of Xbra is replaced with a transcriptional repressor domain of the *Drosophila* protein Engrailed (Xbra-En$^R$) and introduced into *Xenopus* or zebrafish, the resultant embryos phenocopy *Brachyury*/*no tail* mutant mice and fish (see below; Conlon et al., 1996). Collectively, these studies suggest that Brachyury functions as a transcriptional activator and its sequence, expression, and molecular function is evolutionarily conserved.

## *Brachyury* DOWNSTREAM TARGETS

To understand how *Brachyury* functions at the molecular and cellular level, it is absolutely critical to isolate and characterize downstream genes. However, strikingly few targets of *Brachyury*, or any T-box gene, have been identified (Smith et al., 1997; Tada and Smith, 2001). Two approaches have been taken to identify direct targets of Brachyury, a candidate and a directed approach, i.e., functional screening. The candidate approach has identified *embryonic fibroblast growth factor* (*eFGF*) as a target, initially based on its coexpression with *Xbra* in the nascent mesoderm and developing notochord (Isaacs et al., 1992, 1994, 1995). Moreover, misexpression of the Xbra-En$^R$ fusion construct was shown to ablate expression of *eFGF* in the presumptive notochord, suggesting *eFGF* may be a notochordal target of Xbra (Casey et al., 1998). Based on these findings, Casey et al. cloned the *eFGF* promoter and demonstrated that *eFGF* can be activated through the monomeric binding of Xbra to two Brachyury consensus sites in its promoter (Casey et al., 1998). Thus, *eFGF* appears to be a direct target of Xbra.

Functional screens conducted in *Xenopus* and ascidians have identified all other known targets of Brachyury. In an elegant screen for the downstream targets of early functioning T-box genes, Tada and colleagues identified potential targets by injecting a hormone-inducible version of Xbra into *Xenopus* embryos, isolating animal caps derived from the injected embryos, and performing a subtractive screen between the Xbra-induced and un-induced tissue (Smith et al., 1997; Tada et al., 1997, 1998; Saka et al., 2000). This screen led to identification of several T-box targets, including four highly related homeobox genes *Bix1–4* (Tada et al., 1998). These genes have close similarity with *Mix.1* and *Mixer*, and all four are coexpressed with *Xbra* in the early mesoderm. Both *Bix1* and *Bix4*, like *Xbra*, can be induced in response to the mesoderm growth factors activin and BMP in the presence of protein synthesis inhibitors and are themselves sufficient to direct prospective ectoderm to form mesodermal tissue types (Tada et al., 1998; Casey et al., 1999). Moreover, the promoter of *Bix4* contains Brachyury consensus binding sites that are necessary for its proper tissue-specific expression (Casey et al., 1999). Although the identification of *Bix1* and *Bix4* has provided insights into the molecular pathway by which Brachyury functions, because both genes encode transcription factors with no clearly defined function in the mesodermal pathway, the precise cellular role for these genes remains to be established.

In addition to the *Bix* family, *Xwnt-11* was also isolated as a potential target of Xbra in the screen (Smith et al., 2000). Final confirmation of *Xwnt-11* as a target of Xbra awaits the cloning and characterization of its promoter; however, several findings are consistent with *Xwnt-11* acting directly downstream of *Xbra*. First, like *Brachyury, Xwnt-11* is required for convergent extension movements in both *Xenopus* and zebrafish. Interference with *Xwnt-11*, either through a dominant negative approach or genetic mutations in zebrafish, leads to phenotypes that bear a striking resemblance to those of *no tail* mutants or *Xbra-En$^R$*-injected fish and frogs. Second, *Xbra* and *Xwnt-11* show an almost identical expression pattern during early development and these expression patterns are conserved throughout vertebrate evolution. Third, expression of the Xbra-En$^R$ construct dramatically down-regulates expression of *Xwnt-11* in early gastrula

embryos. Fourth, induction of *Xwnt-11* by the hormone-inducible version of Xbra can occur in the presence of the protein synthesis inhibitor cycloheximide. Thus, induction appears to be direct and does not require intervening proteins. Finally, *Xwnt-11* can rescue the block in convergent extension movements in animal cap tissue derived from Xbra-En$^R$ embryos (Saka et al., 2000; Smith et al., 2000).

In *Ciona*, a subtractive hybridization screen revealed more than 500 cDNA clones up-regulated in embryos overexpressing a notochord-specific *Ci-Bra* construct (Takahashi et al., 1999a,b). Some of the 39 genes that are specifically (19) or predominantly (20) expressed in the notochord have been further characterized and fall into two categories: earlier expressed putative transcription factors or nuclear proteins, and later expressed genes implicated in cell adhesion, signal transduction, and cytoskeleton regulation, or encoding components of the extracellular matrix (for details, see Hotta et al., 1998, 1999, 2000). It has been shown previously that one of the identified genes, encoding the tropomyosin-like protein Ci-Trop, is directly regulated by Ci-Bra, possibly in conjunction with additional regulatory factors (Di Gregorio and Levine, 1999). Another gene of the second category, *Ciona intestinalis prickle* (*Ci-pk1/2*), a homologue of the *Drosophila* planar cell polarity gene *prickle* (Gubb et al., 1999; Mlodzik, 2000; Tree et al., 2002) has been identified recently in zebrafish and in *Xenopus* (Wallingford et al., 2002; Carreira-Barbosa et al., 2003; Takeuchi et al., 2003; Veeman et al., 2003). *prickle* expression in *Xenopus* gastrula stage embryos is reminiscent of *Xbra* expression and has been shown to be up-regulated by *Brachyury* overexpression in *Xenopus* animal cap cells and to function not only in convergent extension movements during *Xenopus* and zebrafish gastrulation but also in neuronal migration in zebrafish (Carreira-Barbosa et al., 2003; Takeuchi et al., 2003, Veeman et al., 2003).

## *Eomesodermin* AND EARLY MESODERM DEVELOPMENT

*Brachyury* is not the only member of the T-box gene family to play an essential role in the early patterning of vertebrate mesoderm. A screen designed to identify genes expressed in the prospective mesoderm of mid-gastrula stage *Xenopus* embryos led to the isolation of the T-box gene *Eomesodermin* (*Eomes*; Ryan et al., 1996). *Eomesodermin* expression precedes *Xbra* and, thus, is thought to be one of the earliest genes to be activated in the mesodermal lineage. Beginning at early gastrula, it is expressed in a dorsal to ventral gradient in the prospective mesoderm. In the dorsal region, expression is more widespread, and these cells express higher levels of the gene. Expression peaks at mid-gastrula and then rapidly declines such that, by mid-neurula, transcripts persist only at low levels in the dorsal mesoderm (Ryan et al., 1996). At later stages, *Eomesodermin* expression is induced in a small subset of cells in the central nervous system (Ryan et al., 1998).

The first clues for a role of *Eomesodermin* in mesodermal patterning came from misexpression experiments in *Xenopus*, which demonstrated that it is sufficient to divert prospective ectoderm cells into the mesodermal lineage, activating mesoderm-specific genes such as *Xbra*, *gsc*, and *Xwnt8* and leading to terminal differentiation of mesodermal cell types (e.g., muscle and notochord) (Fig. 2). As is the case with *Xbra*, mesoderm induction in response to *Eomesodermin* is dose-dependent, with high doses inducing muscle and lower doses frequently inducing both muscle and notochord. However, the activation of mesodermal genes displays a more complex dose-dependency: higher *Eomesodermin* levels induce elevated levels of dorsal mesodermal markers, such as *goosecoid* (*gsc*), but the induction of both panmesodermal markers, such as *Xbra*, and ventral mesodermal markers such as *XWnt8*, is reduced. It is presently unclear whether this effect is a direct result of increased *Eomesodermin* activity or if it is mediated by one or more secondary factors (Ryan et al., 1996).

Like *Brachyury*, *Eomesodermin* is essential for mesoderm formation. This requirement was first suggested by interference studies in *Xenopus* by using a heterologous fusion protein in which the Eomesodermin C-terminal region, thought to contain the transcriptional activation domain, was replaced by the transcriptional repression domain of the *Drosophila* Engrailed protein. Expression of this construct in early embryos leads to gastrulation arrest with the down-regulation of mesoderm markers (e.g., *muscle actin*) and concomitant up-regulation of neural markers (e.g., *XlF3* and *XlF6*; Ryan et al., 1996).

Like *Brachyury*, the sequence, expression, and function of *Eomesodermin* appears to be conserved throughout vertebrate evolution. In the mouse, for example, *Eomesodermin* is expressed in mesodermal tissues, including the prospective mesoderm of the epiblast layer, the primitive streak, and the nascent mesoderm, as well as in extra-embryonic tissues such as the trophoblasts (Ciruna and Rossant, 1999; Hancock et al., 1999; Russ et al., 2000). Genetic analysis of mutant mice shows that, like *Brachyury*, *Eomesodermin* is required cell autonomously within the tissues that normally express the gene. Although heterozygous animals are healthy and fertile, animals homozygous for mutations in *Eomesodermin* die shortly after implantation due to defects in the extra-embryonic trophoblast cell lineage. To address its role in mesodermal lineages at later stages of development, Russ and coworkers produced chimeric embryos between tetraploid host embryos and cells of the inner cell mass derived from *Eomesodermin* homozygous mutant embryos or, alternatively, homozygous mutant ES cells (Russ et al., 2000). Because tetraploid cells only contribute to extra-embryonic tissues, the resultant embryos contain extra-embryonic tissues that are predominantly wild-type (thus bypassing the early requirement for *Eomesodermin* in the trophectoderm), while the embryo proper is entirely derived from mutant cells. Chimeric embryos derived from such a procedure proceed to gastrulation but then die displaying gross mesodermal defects. Lineage tracing of the tissues shows that *Eomesodermin* mutant cells occupy a broad ectopic domain corresponding to the prospective mesoderm of the epiblast, but these cells fail to ingress and gradually accumulate in the primitive streak. Consequently, the mesoderm layer does not form (Russ et al., 2000).

Collectively, these studies are consistent with a pivotal role for *Eomesodermin* in mesoderm patterning, but is its role to direct the differentiation of mesodermal cell types (as suggested by misexpression studies in *Xenopus*) or, alternatively, to promote the migration of prospective mesoderm cells during gastrulation (as suggested by genetic studies in mouse)? Because teratomas derived from the injection of *Eomesodermin* mutant ES cells into syngeneic host mice contain terminally differentiated mesodermal cell types such as muscle, cartilage, and red blood cells, it would appear that differentiation of mesodermal cell types does not require *Eomesodermin* function per se (Russ et al., 2000). However, these studies cannot rule out an additional or transient endogenous role for *Eomesodermin* in specific aspects of mesodermal patterning. Unfortunately, no direct targets of *Eomesodermin* have been identified, although the *Mml* gene appears to be an attractive candidate. *Mml* is a member of the *Mix/Bix* family in mouse and is coexpressed with *Eomesodermin* in the primitive streak but is absent in *Eomesodermin* homozygous mutant embryos (Russ et al., 2000). This raises the possibility that members of the *Mix/Bix* family share a common relationship with the T-box family.

## *VegT* AND THE SPECIFICATION OF ENDODERM AND MESODERM IN *Xenopus*

Zygotic gene expression does not begin in *Xenopus* until the mid-blastula stage. In the absence of zygotic expression, early patterning events such as the induction of mesoderm and the establishment of the dorsal organizing center (“Spemann’s organizer”) are largely dependent upon the translation of maternally deposited messenger RNAs (for review see De Robertis et al., 2000). Several studies have demonstrated that regional differences within early *Xenopus*

embryos are based upon the differential localization of maternal mRNAs in the egg (for review see King et al., 1999). Their translation in turn leads to the synthesis of localized cytoplasmic determinants which, through cell division, gradually become differentially distributed amongst the blastomeres and subsequently play important roles in specifying cell identity. One such factor, VegT, encoded by a member of the T-box gene family, is essential for the correct specification of germ layers and early patterning of the *Xenopus* embryo.

*VegT* was identified independently by four groups as a result of a series of functional and expression screens in *Xenopus* and has been known by several names: *Xombi* (Lustig et al., 1996), *Antipodean* (Stennard et al., 1996), *VegT* (Zhang and King, 1996), and *Brat* (Horb and Thomsen, 1997). Sequence analysis of the corresponding four cDNAs showed they represented two alternate splice forms of the same gene (Stennard et al., 1999; for the remainder of the review, we will simply refer to the gene as *VegT* ). Preliminary analysis of VegT showed epitope-tagged versions of the protein to be localized to the nucleus and, when fused to the Gal4 DNA-binding domain, able to activate reporter gene expression in yeast. This finding led to the notion that VegT, like Brachyury and Eomesodermin, functions as a transcriptional activator in vivo (Zhang and King, 1996).

*VegT* is first expressed in *Xenopus* at high levels as a maternal mRNA in the oocyte and egg, where it is localized to the vegetal hemisphere. At early gastrula stages, zygotic expression is detected primarily throughout the prospective mesoderm. However, by late gastrula stages, *VegT* expression is excluded from the dorsal-most region of the embryo, the region that gives rise to the posterior notochord. After gastrulation, levels of *VegT* rapidly decline, becoming confined to a small subset of neurons in the CNS by early tadpole stages (Zhang and King, 1996; Lustig et al., 1996).

Clues to the endogenous role of *VegT* come from misexpression studies demonstrating that injection of *VegT* into the ventrovegetal blastomeres of early *Xenopus* embryos can induce a secondary axis (Zhang and King, 1996). However, lineage tracing shows the effect of *VegT* is not cell autonomous but, rather, reflects the ability of *VegT*-expressing cells to divert neighboring cells into the mesodermal lineage, possibly through the induction of endodermal tissue. In support of this hypothesis, *VegT* has been shown to induce the expression of both mesodermal and endodermal markers such as *Xwnt8*, *gsc*, *XMyoD*, *Xlhbox6*, *IFABP*, and *Xsox17* (Fig. 2; Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997; Zhang et al., 1998; Clements et al., 1999). The induction of endoderm markers, coupled with the localization of *VegT* mRNA to the vegetal pole and the ability of *VegT*-expressing cells to induce mesoderm, suggests maternal *VegT* may be a master regulator of endoderm differentiation, which in turn, leads to the induction of diffusible signals responsible for mesoderm induction in the overlying marginal zone. To define the role for *VegT* in the endoderm, Zhang and coworkers specifically depleted the maternal pool of mRNA in oocytes using antisense oligonucleotides (Zhang et al., 1998). This approach resulted in severe phenotypic abnormalities, including extensive defects in head development and reduction of gut endoderm, and a marked reduction in all endodermal, and consequently all mesodermal, gene expression. This reduction is also associated with a drastic shift in the spatial arrangement of the germ layers, with mesoderm markers and several ectoderm markers shifted from their normal sites of expression, in the equatorial region and animal pole, respectively, to the vegetal pole. The change in molecular properties is also associated with changes in cell behaviour, with vegetal pole explants from *VegT*-depleted embryos undergoing the convergence and extension movements normally associated with equatorial explants. Together these studies argue that maternal *VegT* is critical for the proper specification of endodermal differentiation in the vegetal pole of the *Xenopus* embryo, and support the conclusion that mesoderm induction by vegetal cells occurs after the mid-blastula transition, when *VegT* is able to regulate the transcription of downstream target genes (Zhang et al., 1998). However, its ability to induce

the ectopic expression of mesoderm markers in a cell-autonomous manner suggests that the zygotically expressed *VegT* isoform *Antipodean* may have a distinct and as yet unidentified role in specifying mesodermal cell fate within the marginal zone of the embryo.

Collectively, these studies indicate that the targets of *VegT* must include genes involved both in the specification of endoderm and the induction of mesoderm. What are the downstream targets of *VegT* that ultimately lead to the production of endoderm and the secretion of the mesoderm inducing signal(s)? Genes encoding the transforming growth factor beta (TGFβ) family signaling molecules Xnr1, Xnr2, Xnr4, and derrière have all been suggested to be direct targets of *VegT*. Although all four of these putative targets can induce mesoendoderm and are able to rescue both the phenotypic and molecular defects associated with *VegT*-depleted embryos (Kofron et al., 1999; Xanthos et al., 2002), only the *Xnr1* and *derrière* loci have been shown to contain VegT binding sites. However, in the case of *Xnr1* the binding sites do not appear to be required for its vegetal expression. Certainly, the best evidence in support of any endogenous endodermal *VegT* target is that for *derrière*. Unlike other candidate targets, derrière acts as a long-range mesoderm-inducing signaling molecule and can be both directly and indirectly regulated by *VegT* (White et al., 2002). Despite these studies, definitive experiments for any one candidate have yet to be reported.

In addition to diffusible growth factors, the transcription factor genes *Xsox17*, *Bix1*, and *Bix4* appear to be directly regulated by *VegT* in the endoderm (Casey et al., 1999; Clements and Woodland, 2003). Both *Bix1* and *Bix4* are targets of Xbra that are expressed in nascent mesoderm and are coexpressed with *VegT* within the endoderm. When misexpressed, both genes induce endodermal as well as mesodermal tissue. Both genes can be ectopically activated in response to *VegT*, and both contain VegT binding sites in their promoters (Casey et al., 1999; Tada et al., 1998). This finding is consistent with the possibility that the *Mix/Bix* family may be a common target of early acting T-box genes. These studies also suggest that *VegT* may regulate the formation of mesoendoderm in both a direct mechanism, by means of TGFβ signaling molecules such as derrière, and an indirect mechanism, through the regulation of a second set of transcription factor genes such as *Bix1* and *Bix4*.

## REGULATION OF *Brachyury*, *Eomesodermin*, AND *VegT* EXPRESSION

### Brachyury

The activities of *Brachyury*, *Eomesodermin*, and *VegT* in orchestrating the induction and formation of mesoderm within the developing embryo are dependent upon their proper temporal and spatial expression, and much of our understanding of the regulation of these genes comes from studies conducted in *Xenopus*. The initiation of zygotic expression of all three genes in the prospective mesoderm before gastrulation is dependent upon intercellular signalling. Secreted signals from vegetal cells have long been known to be responsible for the induction of mesoderm in the overlying cells of the marginal zone (Sudarwati and Nieuwkoop, 1971). This signalling capacity is evident in assays in which animal cap explants (prospective ectoderm) are induced to form mesoderm when cocultured in contact with vegetal explants. This type of animal cap assay has been used extensively in the search for endogenous mesoderm inducing factors and to investigate the events occurring downstream of these signals. Early experiments demonstrated that, in the presence of cycloheximide, *Xbra* expression was induced in animal caps as an immediate-early response to treatment with either of two candidate mesoderm-inducing factors: activin A (a TGFβ-family ligand) and basic FGF (Smith et al., 1991). Subsequent studies demonstrated that both TGFβ and FGF signalling are required for *Xbra* expression in the embryo (Amaya et al., 1993; Hemmati-Brivanlou and Melton, 1992). Of interest, expression of a dominant negative activin receptor blocks the ability of bFGF to induce *Xbra* expression in animal caps. Conversely, induction of *Xbra* expression by activin A is blocked by the dominant negative FGF receptor. However, because either factor can induce

*Xbra* in disaggregated animal cap cells, excluding any requirement for secondary autocrine signalling (Smith et al., 1991), it may be that, in the embryo, there is a requirement for both signalling cascades within the prospective mesoderm to render the cells competent to respond to either TGFβ or FGF signals, possibly through cytoplasmic cross-talk between the two pathways (LaBonne and Whitman, 1994).

While functional TGFβ and FGF signalling pathways are required for the initial induction of *Xbra* expression, FGF signalling appears to have an additional role in maintaining expression during subsequent development. For example, it was demonstrated that mesodermal explants in which the tissue remained intact maintain *Xbra* expression, but when mesodermal cells are disaggregated, which dilutes the effects of endogenous secreted growth factors such as FGF, *Xbra* expression is lost (Schulte-Merker and Smith, 1995). Moreover, this effect can be rescued by culturing the dispersed cells in the presence of FGF (Isaac et al., 1994; Schulte-Merker and Smith, 1995). As discussed above, *eFGF* is coexpressed with *Xbra* during gastrulation, and studies have shown that it functions to maintain *Xbra* expression as part of an autoregulatory loop (Isaacs et al., 1992, 1995; Casey et al., 1998). Support for this model comes from numerous studies. For example, eFGF or FGF2 can activate expression of *Xbra* in an immediate-early manner (in the absence of protein synthesis) through the RAS/RAF/MAPK signaling pathway (Smith et al., 1991, 1997; Isaacs et al., 1994; Schulte-Merker and Smith, 1995) and overexpression of active forms of RAS/RAF/MAPK signaling pathway components, such as $p21^{ras}$, MEK1, or MAPK itself, is also sufficient to activate expression of *Xbra* (Gotoh et al., 1995; LaBonne et al., 1995; Umbhauer et al., 1995). Conversely, inhibition of the FGF/RAS/RAF/MAPK signaling pathway, for example by a dominant negative RAS, RAF, or dominant negative FGF receptor, leads to inhibition of *Xbra* expression (Amaya et al., 1993; Umbhauer et al., 1995). In addition, *Xbra* expression is in turn required to maintain the FGF signaling pathway. For example, a dominant negative form of Xbra can abolish expression of *eFGF* (Conlon et al., 1996; Casey et al., 1998; Conlon and Smith, 1999). Subsequently, it was demonstrated that *eFGF* is a direct target of Xbra (Casey et al., 1998), and thus *eFGF* and *Xbra* function during gastrulation in an autoregulatory loop that is required to maintain their expression and is ultimately required for proper notochord formation. However, additional studies in *Xenopus*, mouse, and zebrafish suggest that expression of *eFGF* in other regions of the embryo may be independent of Brachyury function (and, therefore, of the autoregulatory loop).

Transgenic studies in mouse and frog have identified the minimal region both necessary and sufficient for the proper temporal and spatial expression of *Brachyury* in the nascent mesoderm. Moreover, in mouse, this promoter region is sufficient to rescue the short-tail phenotype of *Brachyury* heterozygous mutants (Stott et al., 1993; Clements et al., 1996) and in the case of the frog, the region also contains element(s) that confer dose-dependent transcriptional responses to both activin and FGF (see below; Latinkic et al., 1997; Lerchner et al., 2000) Sequence comparison of the two promoters shows strong conservation of an E-box and two canonical Lef1/Tcf1 binding sites, the latter being involved in transducing a subset of Wnt signals. In mouse, mutation of the Lef1/Tcf1 sites, but not the E-box, prevents expression of the reporter construct, suggestive of a role for Wnt signalling in *Brachyury* expression (Galceran et al., 2001). Consistent with this hypothesis, mouse embryos homozygous for mutations in *Wnt3a* fail to express *Brachyury* (Yamaguchi et al., 1999), and inhibition of Wnt signalling in frog, through the expression of a dominant negative Tcf (DN Xtcf-3), inhibits expression of *Xbra* in the early gastrula (Vonica and Gumbiner, 2002). These studies suggest a requirement for Wnt signalling in the induction of *Brachyury*. Because members of the Wnt family cannot induce mesoderm, the data suggest that other factors must contribute to *Brachyury* induction, with members of the TGF-β and FGF families being the most likely candidates.

Additional studies of the *Xbra* promoter have identified a 381-bp region within the minimal promoter that contains the element(s) that respond to both FGF and activin signals (Latinkic et al., 1997; Lerchner et al., 2000). In reporter constructs, this region confers a similar response to increasing doses of activin to that of the endogenous *Xbra* gene, with expression being actively suppressed at high doses. Binding sites for homeodomain proteins were identified within the promoter fragment, and three factors—Goosecoid, Mix.1, and Xotx2—were shown to bind to these sites and to have the capacity to repress *Xbra* expression (Latinkic et al., 1997). All three of these factors are present in the early embryo, and both *gsc* and *Mix.1* are strongly expressed in response to increasing activin levels. An additional regulatory element, a binding site for δEF1-family proteins, has also been identified and predicted to be involved in *Xbra* repression (Lerchner et al., 2000). Together, these regulatory regions are thought to play an essential role in restricting *Xbra* expression and, therefore, its function to the marginal zone of the gastrula. Surprisingly, this region of the *Xbra* promoter shares no apparent homology to the mouse promoter. One possible explanation for the lack of conservation is that the activin and FGF response sequences may have evolutionarily diverged while retaining their ability to respond to the two growth factors. Alternatively, it may be that mouse and *Xenopus* differ in the mechanisms that trigger gastrulation, but once under way, gastrulation in these species proceeds by a common molecular pathway (reviewed in Conlon and Beddington, 1995).

Although studies in both frog and mouse have led to the identification of elements controlling *Brachyury* expression in the nascent mesoderm, the elements controlling its expression in the notochord have yet to be found. However, clues to these elements are emerging from studies in ascidians. With their small genomes (*C. intestinalis* $1.6 \times 10^8$ bp/haploid; Simmen et al., 1998), well defined embryonic cell lineages, accessibility by electroporation, and minimal promoters usually located approximately 300-bp upstream of the transcription start site, ascidians provide an excellent system for analyzing T-box gene regulation. All three suggested ascidian *Brachyury* orthologs—*As-T* (*HrBra*) (*Halocynthia roretzi*), *Ci-Bra* (*Ciona intestinalis*), and *Cs-Bra* (*Ciona savignyi*)—are expressed exclusively in the primordial notochord after its induction at the 32-cell stage (Yasuo and Satoh, 1994; Corbo et al., 1997; Imai et al., 2000). Misexpression of either *As-T* or *Ci-Bra* transforms endodermal and neuronal lineages into notochord cells, demonstrating that these ascidian genes perform homologous functions to those of *Brachyury* orthologues in higher chordates. Each of the minimal promoters of *Ci-Bra* and *As-T* is able to drive notochord expression of reporter genes in both species, but their promoters differ in their regulative potential. In the 5′-flanking region of *As-T*, a simple distal module responsible for notochord expression and a proximal palindromic T-binding motif responsible for auto-activation of *As-T* have been identified (Takahashi et al., 1999b). The minimal promoter of *Ci-Bra* lacks a T-binding motif, but contains two regions responsible for positive regulation together with a binding site for the transcriptional repressor Snail (Ci-sna; Corbo et al., 1997, 1998). With the onset of zygotic transcription, *Ci-sna* is activated early during muscle specification at the 32-cell stage, and later in the developing tail muscle, restricting the expression domain of *Ci-Bra* to the notochord cells and, thus, establishing a muscle/notochord boundary (Erives et al., 1998; Fujiwara et al., 1998).

## Eomesodermin

The upstream regulation of *Eomesodermin* has been less extensively studied, but its regulation differs somewhat from that of *Brachyury*. Although experiments have shown that it is expressed as an immediate–early response to activin in animal cap assays, suggesting that it is likely to be downstream of TGFβ signaling in vivo, it is not induced by eFGF or Xwnt8 (Ryan et al., 1996). The *Xenopus Eomesodermin* promoter has been cloned and characterized by analysis of a deletion series, and by mutation of two of three binding sites for forkhead activin transducer-2 (FAST-2), a factor that functions to mediate transcription activation in response

to TGFβ signaling (Liu et al., 1999; Ryan et al., 2000). The presence of at least two FAST2 sites are found to be required to mediate the response to activin in reporter assays in vivo. Data from this study also suggests that, as appears to be the case for *Xbra*, the *Eomesodermin* promoter contains one or more un-defined elements responsible for mediating its repression in the endoderm of the early embryo (Ryan et al., 2000).

### VegT

Less is known of the upstream regulation of *VegT* than of either *Xbra* or *Eomesodermin*. Whereas early studies noted its induction in animal cap assays in response to the expression of TGFβ factors (such as activin- and nodal-related signals), FGFs, *Xbra*, and *Eomes*, it was not clear whether these represented direct regulatory interactions (Lustig et al., 1996; Stennard et al., 1996; Horb and Thomsen, 1997). It remains unclear whether *VegT* transcription is induced in the prospective mesoderm as a direct response to intercellular signals from vegetal cells. Further studies have examined the nature of the cross-regulatory interactions between *VegT*, *Xbra*, and *Eomesodermin. VegT* and *Eomesodermin* are able to regulate one another's expression, and that of *Xbra* (Lustig et al., 1996; Ryan et al., 1996; Stennard et al., 1996; Horb and Thomsen, 1997). However, *Xbra* appears only to induce *VegT*, not *Eomesodermin* (Lustig et al., 1996; Ryan et al., 1996). Experiments using a hormone-inducible form of *VegT* have shown that the expression of *Xbra* and *Eomesodermin* occurs as an indirect response to *VegT* function (Clements and Woodland, 2003).

Studies have shown that cross-regulatory interactions also exist between early T-box genes in *Ciona*. Of interest, the minimal enhancer of *Ci-sna*, which is sufficient to mediate Snail expression in the B4.1 derivative blastomeres from which the tail muscle develops, contains a conserved T-binding motif, raising the possibility that *Ci-Bra* might be regulated indirectly by a second early T-box gene acting by means of Snail. Erives and Levine identified the T-box gene *Ci-VegTR* (*VegT*-related), which is exclusively and maternally expressed in the vegetal region of the fertilized egg contributing to the myoplasm, and also demonstrated that a GST-Ci*VegT*R fusion construct is able to bind to the *Ci-sna* enhancer in vivo (Erives and Levine, 2000). These experiments thus identified a likely cross-regulatory interaction between *Ciona* homologues of *Brachyury* and *VegT*. Similar studies in the future may clarify the regulatory interactions between these genes and their importance in the induction and patterning of the mesoderm in *Xenopus*.

## T-box GENES REGULATE PARAXIAL MESODERM DEVELOPMENT

As discussed above, an essential function of *Brachyury* orthologues is to mediate the induction of mesoderm in response to FGF signalling. The notochord and the paraxial mesoderm of the tail, which form as a result of extensive cell movement, do not form in the absence of *Brachyury* function. This finding is evident in the "tailless" phenotypes of *T* mutant mice (Chesley, 1935), zebrafish *no tail* mutants (Halpern et al., 1993), and in *Xenopus* embryos expressing a dominant negative *Xbra* (*Xbra-EnR*; Conlon and Smith, 1999). However, the phenotypes that result from inhibition of FGF signalling in embryos are more severe than those of embryos lacking *Brachyury* function, exhibiting disruption of both tail and trunk mesoderm development (Amaya et al., 1991, 1993; Griffin et al., 1995, 1998). This additional effect on the trunk of the embryo indicates that one or more additional factors must mediate the inductive effects of FGF signaling in this region. The identity of one such factor has been uncovered through studies of the zebrafish mutant *spadetail*.

*spadetail* mutant embryos are severely deficient in trunk mesoderm (Kimmel et al., 1989). Tail development, however, is relatively unaffected, apart from the accumulation of mesoderm cells at the tail tip for which the mutant is named. Detailed analysis of the movement of cells during gastrulation revealed that, in *spadetail* mutants, the trunk paraxial mesoderm progenitors adopt

a different course, moving posteriorly toward the future tail rather than dorsally to form the segmental plate (and, eventually, the somites) of the trunk, as they do in wild-type embryos (Kimmel et al., 1989). Griffin and coworkers identified the *spadetail* (*spt*) locus as encoding a member of the T-box gene family most closely related in sequence to *VegT*, and further demonstrated that FGF signaling is required to maintain its expression in the embryo, thereby placing it downstream of this signaling pathway (Griffin et al., 1998).

The expression patterns of *spadetail* and *no tail* mirror, to some extent, their complementary roles as downstream effectors of FGF signaling in the trunk and tail. Both genes are initially expressed in the same cells, progenitors of mesoderm and endoderm, in the epiblast and hypoblast layers of the marginal zone of the early zebrafish gastrula (Fig. 3a; Griffin et al., 1998;Schulte-Merker et al., 1992). Analysis of single mutant phenotypes suggests that neither gene is required for proper cell behaviour in early gastrulation stages, as the epiblast cells still involute to form the hypoblast in both *no tail* and *spadetail* mutants (Kimmel et al., 1989;Halpern et al., 1993). However, this finding is due to a degree of redundancy early in development that is revealed by analysis of $ntl^{-};spt^{-}$ double mutants. Although embryos heterozygous for *no tail* loss-of-function mutations show no phenotypic defects, when combined with loss of *spadetail* function, an enhancement of the *spadetail* mutant phenotype occurs (Amacher et al., 2002;Goering et al., 2003). As gastrulation proceeds, the cells of the hypoblast begin to refine their expression of both genes, with *spadetail* becoming restricted primarily to the paraxial mesoderm progenitors and *no tail* restricted to the notochord (Schulte-Merker et al., 1992;Griffin et al., 1998). During these later stages, the functions of *no tail* and *spadetail* diverge, *spadetail* being required cell autonomously for the formation of the trunk paraxial mesoderm and *no tail* for notochord and tail paraxial mesoderm development (see above).

*spadetail* and *no tail* appear to belong to a regulatory network of mesodermal genes that includes a third member of the T-box family in zebrafish, *tbx6*. The regulatory hierarchy that exists between these genes (summarized in Fig. 3b) provides a possible explanation for the lack of a requirement for *spadetail* activity in the paraxial mesoderm of the tail, which develops almost normally in *spadetail* mutants. *tbx6* is closely related in sequence to *spadetail* and may conceivably perform a similar function in the paraxial mesoderm. The expression pattern of *tbx6* is similar to that of *spadetail*, i.e., in the trunk and tail paraxial mesoderm progenitors of the hypoblast layer (Fig. 3a; Hug et al., 1997). In *spadetail* mutant embryos, *tbx6* expression is greatly reduced in the paraxial mesoderm progenitors of the trunk but is still expressed in the paraxial mesoderm of the tail at later stages, when tail formation is under way. *tbx6* expression is absent in $spt^{-};ntl^{-}$ double mutants, suggesting that *no tail* and *spadetail* act synergistically to regulate *tbx6* in the trunk and tail paraxial mesoderm. *no tail* appears to compensate for loss of *spadetail* function, maintaining *tbx6* expression, and presumably its function, in the tail (although the converse is unlikely, as *spadetail* expression in the tail is dependent upon *no tail* function; Griffin et al., 1998).

Functional analysis of the zebrafish *tbx6* gene has been hampered by the lack of a corresponding mutant, but there is significant evidence in support of the view that *tbx6* and *spadetail* perform common functions in specifying paraxial mesoderm cell fate. When ectopically expressed in progenitors of the notochord, as occurs in zebrafish *floating head* mutants, *spadetail* promotes a switch to muscle (i.e., paraxial mesoderm) cell fate (Halpern et al., 1995). This ability of *spadetail* to specify muscle is strikingly similar to that of *Tbx6* in the mouse and in *Xenopus* (Chapman and Papaioannou, 1998; Uchiyama et al., 2001). *Tbx6* is expressed in the primitive streak of the early mouse gastrula and, slightly later, in the paraxial mesoderm (Chapman et al., 1996). Its expression is then down-regulated as the tissue undergoes an epithelial to mesenchymal transition to form somites. Its role in somitogenesis was apparent in gene targeting experiments that demonstrated that animals homozygous for a disrupted *Tbx6* allele

lack all paraxial mesoderm posterior to the forelimb (Chapman and Papaioannou, 1998). Astonishingly, the paraxial mesoderm is replaced by two ectopic neural tubes that appear to be correctly patterned along the dorsoventral axis. Somite formation can be rescued in these mutants with a *Tbx6* transgene that is expressed at the correct time and place, but at lower levels than those observed in heterozygous mutant embryos (White et al., 2003). Although somites form at the correct time in the rescued mutants, the rostrocaudal patterning of the somites is defective, ultimately resulting in the fusion of the rib and vertebra, a phenotype also seen in the *rib-vertebrae* (*rv*) mutant in which the promoter of the *Tbx6* locus is disrupted (Watabe-Rudolph et al., 2002; White et al., 2003). Taken together, these results show that *Tbx6* is necessary for both the specification and the patterning of newly forming somites in the mouse. While it is worth noting that zebrafish *tbx6* may not represent the true orthologue of the murine and *Xenopus Tbx6* genes, these findings strongly suggest that the closely related *spadetail* and *tbx6/Tbx6*-like genes share a common functionality in regulating the development of the paraxial mesoderm.

## A CONSERVED GENETIC PATHWAY FOR PARAXIAL MESODERM VS. NOTOCHORD DEVELOPMENT?

Although in the past emphasis has been placed upon the complementary roles of *spadetail* and *no tail* in controlling the development of the trunk and the tail, respectively, they could equally be interpreted as being regulators of paraxial versus axial mesoderm fate. As mentioned above, in *Ciona*, this cell fate decision seems to involve an indirect repression of *Brachyury* expression in the future paraxial mesoderm by *Ci-VegTR*. This occurs by means of the transcriptional repressor Snail, whose activity establishes the boundary between the notochord and muscle (Fujiwara et al., 1998). Do *VegT* or-thologues in other organisms actively repress notochord differentiation in the paraxial mesoderm by indirectly repressing *Brachyury*? In zebrafish and *Xenopus* gastrulae, the expression patterns of the two genes become mutually exclusive as the differentiation of paraxial mesoderm and notochord takes place. *no tail/Xbra* is down-regulated in the future paraxial mesoderm, while *spadetail*/*VegT* is lost from the future notochord. Similarly, *snail* orthologues are coexpressed with *spadetail*/*VegT* in the paraxial mesoderm, where they are essential regulators of the epithelial to mesenchymal transition occurring during somitogenesis but are not expressed in the notochord (Thisse et al., 1993; Carver et al., 2001). The conservation of their tissue-specific expression patterns is consistent with the idea that the regulatory interactions between these three genes in the ascidian embryo may have been conserved in higher chordates. In zebrafish, *spadetail* expression is excluded from the notochord through the activity of the homeodomain transcriptional repressor encoded by the *floating head* locus, which in turn is expressed by notochord precursors as a response to the inhibition of BMP and Wnt signals in the dorsal mesoderm. When *floating head* function is lost in the axial mesoderm cells fated to form the notochord, so too is *no tail* expression, perhaps as a result of the ectopic expression of *spadetail* and *snail1* (Halpern et al., 1995). In *spadetail* mutants, *no tail* expression persists in the presomitic mesoderm arising from the tail bud region, while in wild-type embryos its expression is rapidly extinguished as the presomitic mesoderm forms (Griffin and Kimelman, 2002). These observations support the idea that the genetic program seen in *Ciona* may indeed have been conserved in zebrafish, regulating the decision between notochord and paraxial mesoderm fate. The *Xenopus* orthologue of *floating head*, *Xnot*, is similarly expressed in response to BMP/Wnt inhibition (Yasuo and Lemaire, 2001). It remains to be seen whether *Xnot* acts to repress *VegT* in the future notochord in *Xenopus* (just as *floating head* represses *spadetail* in zebrafish), and whether *VegT* in turn indirectly represses *Xbra* in the paraxial mesoderm by means of an activation of *snail* (*Xsna*) expression.

The relatively normal development of the tail of zebrafish *spadetail* mutants suggests that one or more additional factors compensate for the loss of *spadetail* function in the posterior of the

embryo, acting at a genetic level to restrict *no tail* expression to the future axial mesoderm. *Tbx6* is a likely candidate for such a factor, but it is not known whether it shares the apparent ability of *spadetail* to indirectly repress *no tail* expression in the paraxial mesoderm. In the early gastrula, *no tail*, *spadetail* and *tbx6* are coexpressed in the ventrolateral cells of the epiblast (the "germ ring"), fated to give rise to the paraxial mesoderm. This finding raises an important question, namely, what prevents *no tail* from inappropriately specifying axial mesoderm fate in these cells? A recent study has shown that expression of Tbx6, or even the Tbx6 DNA-binding domain alone, in regions of *no tail* expression produces similar phenotypes to those resulting from the expression of the No Tail antagonist Ntl-EnR-myc (Goering et al., 2003). Furthermore, the activity of a Tbx6-myc fusion mimicked that of the Ntl-EnR-myc construct, antagonizing No Tail activity when coexpressed in animal cap assays. Thus, Tbx6 may act at the protein level to prevent the activation of dorsal-specific genes by No Tail, rendering it unable to specify axial mesoderm fate while more general aspects of its ability to specify early mesoderm remain intact.

## WHAT IS THE MOLECULAR BASIS FOR FUNCTIONAL DIVERSITY?

### Does Specificity Reside in the T-domain?

The preceding discussion has focused on the roles of T-box genes in governing cell fate decisions and morphogenesis in early embryos and on the upstream regulation of these genes by early patterning signals. Clearly, there are both similarities and differences between the functions of these related factors. In *Xenopus*, *Xbra*, *Eomesodermin*, and *VegT* have all been shown to activate mesoderm-specific genes in isolated animal pole tissue, but the type of mesoderm that forms in response to these factors in tissue explants is qualitatively and quantitatively distinct. In particular, *Xbra* induces posterior mesodermal cell types and activates genes typically expressed in the posterior mesoderm (e.g., *Bix4*, *Xwnt11*), while *VegT* and *Eomesodermin* can induce virtually the entire spectrum of mesodermal cell types and mesodermal genes (e.g., *gsc*, *chordin*, *Xwnt8*) (Conlon et al., 2001).

What determines these functional differences? Ultimately, these factors must differ to some degree in their choice of direct transcriptional targets. Brachyury, Eomesodermin, and VegT have all been shown to function as transcriptional activators, and this activity resides primarily in regions C-terminal to the T-domain, while the T-domain itself is responsible for DNA binding (Conlon et al., 2001). A structural study of the complex formed between DNA and the T-domain of Xbra suggests that Brachyury binds as a dimer to a palindromic sequence (Müller and Herrmann, 1997). This palindromic consensus sequence or "T-site" was identified by PCR-based binding site selection. The same technique has been used to identify consensus sequences bound preferentially by Eomesodermin and VegT (Conlon et al., 2001). The core motif or "half site" of the selected sequences -TCA CACCT- was the same for all three factors, although they differed in their preference for certain flanking nucleotides. In these binding site selection experiments, all three factors often showed a greater affinity for DNA sequences consisting of two half sites. However, each protein exhibited a different preference for certain orientations and spacing of the half sites in electrophoretic mobility shift assays (EMSA). For example, Xbra can bind to two half sites arranged head-to-head (TCACAC CTAGGTGTGA) while Eomesodermin cannot. Conversely, Eomesodermin can bind to two core motifs arranged head-to-tail (TCACAC CTaaatTCACACCT) while Xbra cannot (Conlon et al., 2001). It is possible that differences such as these in part underlie the different effects of the different T-domain proteins. However, no palindromic or "double site" has been identified in the promoter of any well-characterized downstream target of Xbra, Eomes, or VegT (Tada and Smith, 2001).

Efforts to identify endogenous targets for transcriptional regulation by early T-domain factors have focused both on candidate genes, such as *eFGF* (in the case of Xbra), and on screening for transcripts up-regulated in response to T-domain factors. Analysis of the *eFGF* genomic

locus identified two T-sites, including a 10-bp nonpalindromic T-binding motif located approximately 1 kb upstream of the transcription start site (Casey et al., 1999). This sequence was sufficient to bind Xbra in an EMSA assay and to drive expression of a reporter gene in vivo in response to Xbra activity. This finding suggests that close pairing of half-sites, as palindromes or otherwise, in the regulatory regions of target genes is not required for binding and transcriptional regulation by T-domain factors in vivo. This is further supported by studies of the promoters of other T-domain target genes. The promoter of *Bix4* contains three half-sites separated by 15-bp and 9-bp sequences and is a target of both Xbra and VegT. Both proteins are able to bind to individual half sites in vitro, but, interestingly, they differ in their affinity for particular sites (Tada et al., 1998). In vivo, individual sites are sufficient to drive region-specific expression of a reporter gene in the *Xenopus* embryo, with an Xbra-only target site driving expression in the mesoderm while a VegT/Xbra target site is able to drive both endodermal and mesodermal expression. The promoters of the VegT target genes *Xnr1* and *derrière* similarly contain half-sites that, in the case of *derrière*, are sufficient to bind VegT protein (White et al., 2002).

Where present, multiple binding sites (half-sites) for T-domain proteins in the promoters of their direct transcriptional targets appear to enhance the level of gene transcription, rather than to mediate an all-or-nothing response to protein dimers. For example, deletion of either the distal or proximal T-site within the *eFGF* promoter was shown to result in a significant reduction in, but not complete loss of, the expression of a CAT reporter gene in oocyte assays (Casey et al., 1998). Similar observations have been made in studies of the *Bix4* promoter (Casey et al., 1999).

Binding site selection data and analysis of the promoters of T-box target genes indicate that the functional differences between the early T-domain factors are in part due to quantitative variation in their affinities for particular binding site sequences. While they recognize the same core sequence, each exhibits a characteristic preference for certain sites, perhaps due to subtle variation in the nucleotide sequences flanking the core of the T-site and to corresponding variations in the T-domain of each factor. In vivo assays have shown that fusion proteins consisting of a T-domain fused to the VP16 transactivation domain display very similar inducing activities to those of the native T-domain protein (Conlon et al., 2001). This finding suggests that the choice of target genes is dictated primarily by the T-domain, not by the amino-terminal or carboxy-terminal regions of the protein. Comparison of the T-domain sequences of Xbra, Eomes, and VegT at regions predicted to contact DNA identified a nonconservative variation in a single amino acid residue, lysine 149 of Xbra (Fig. 4). In Eomes and VegT, an asparagine residue occupies this position. When mutated to lysine in Eomes or VegT, the inducing activities of these proteins more closely resemble that of Xbra (Conlon et al., 2001). This lends further support to the idea that the sequence of the T-domain is a major factor responsible for determining functional specificity, and that diversity within the T-domain family is founded upon subtle sequence variations between T-domains.

## Interactions With Other Proteins May Contribute to Specificity

The function of T-domain proteins is also likely to be influenced by their ability to participate in protein–protein interactions with other transcriptional regulators. Genes are often subject to combinatorial regulation, their regulatory regions containing binding sites for more than one transcription factor, and for the RNA polymerase II transcriptional machinery (for review see Levine and Tjian, 2003). A specific interaction with a second DNA-bound protein may stabilize the binding of a T-domain protein to a T-site, and exclude other T-domain family members that do not share this interaction from participating in the regulation of a particular gene. This may be a second key factor in generating functional diversity within the T-domain family and is likely to depend upon the highly variable regions outside the T-domain. There are very few

known interaction partners for T-domain proteins, but studies with the T-box family member TBX5 have shown it to interact directly with the homeobox containing protein NKX2-5 and the zinc finger protein GATA4. All three proteins are expressed during cardiogenesis, and pairwise coexpression of TBX5 with either NKX2-5 or GATA4 results in synergistic activation of expression from the *Nppa/ANF* promoter, which contains binding sites for all three factors (Hiroi et al., 2001; Garg et al., 2003). Indirect support for the existence of Brachyury-interacting proteins comes from two sets of genetic studies with *Brachyury* mouse mutations that both suggest the existence of Brachyury-interacting partners (MacMurray and Shin, 1988; Harrison et al., 2000). These studies, coupled with the observation that Brachyury, Eomesodermin, and VegT, as well as Brachyury and Tbx6, are coexpressed during periods of development at which they function, is suggestive of a functional role for heterodimerization or protein–protein interactions, but to date, there is no report of direct protein–protein interactions between Brachyury, Eomesodermin, VegT, and Tbx6.

Protein–protein interactions also appear to influence the regulatory characteristics of T-domain proteins. The murine Mga transcription factor contains a T-domain and interacts directly with the basic helix-loop-helix leucine zipper protein Max, which in turn binds to E-box DNA sequences. While Mga alone represses the expression of reporter genes containing T-sites, Mga–Max heterodimers function as transcriptional activators of reporters containing T-sites, E-boxes, or both (Hurlin et al., 1999).

## FUTURE DIRECTIONS: PATHWAYS TO CELLULAR DIFFERENTIATION AND MORPHOGENESIS

Although there is a wealth of data highlighting the involvement of T-box genes in regulating developmental processes in the early embryo, little is known of the complex genetic pathways through which the genes exert their effects. Future work will no doubt include continuation of candidate and functional screening approaches that have so far been successful in identifying some of the downstream targets of early T-box genes, particularly those of *Brachyury* and *VegT*. Microarrays offer an alternative to the generation of subtracted libraries as a means of conducting functional screens and may accelerate the identification of downstream targets. The increasing availability of genome sequences for several model organisms will also aid this process by allowing not only large-scale *in silico* searches for potential T-domain binding sites but also the identification of evolutionarily conserved sites through cross-species comparisons of the regulatory regions of putative target genes. The same approach may also further our understanding of the upstream regulation of the T-box genes themselves, giving insight into the signals that determine where and when these genes are expressed.

Another focus for future work is likely to be the functional characterization of the highly variable regions outside the T-domain. The identification of additional loss-of-function alleles of early T-box genes in organisms amenable to genetic analysis would be a valuable means of uncovering important regions of the corresponding proteins, and would provide a basis for studying the function of these regions in vivo. Such studies would complement in vitro analyses of interactions between T-domain factors and other proteins involved in mediating both their target gene specificity and their transcriptional regulation activities.

Finally, we end this review where we began—with the control of embryogenesis by T-box genes. There is still a lot to be learnt of the developmental roles of the T-box genes we have discussed. For example, what is the significance of the apparent functional differences between genes such as *Brachyury*, *Eomesodermin*, and *VegT*? To what extent do they perform unique functions in specifying mesodermal cell types? It is hoped that future embryological studies coupled with the identification of the genetic and molecular pathways in which T-box genes

are involved will provide answers to questions such as these, deepening our understanding of the T-box family and their control of cellular differentiation in early embryogenesis.

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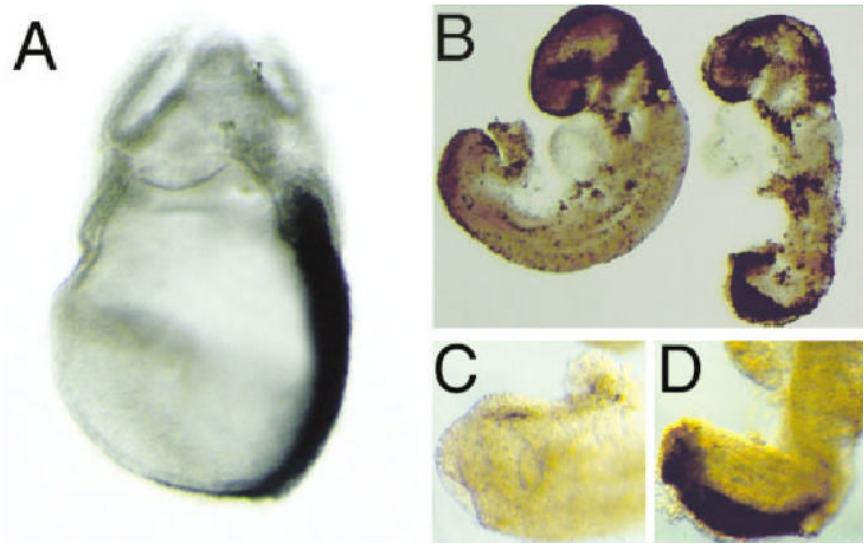


**Fig. 1.**

*Brachyury* is expressed in, and required for, posterior and axial mesoderm. A: Whole-mount in situ hybridization of an embryonic day 8 mouse embryo contained within its extra-embryonic tissue. Note strong expression of *Brachyury* in the primitive streak, the future posterior region of the embryo, and in the prospective notochord cells, along the ventral side of the embryo. Anterior is shown to the left, posterior to the right. B: Wild-type (left) and *Brachyury* homozygous mutant (right) littermates after whole-mount TUNEL staining to visualize programmed cell death. C,D: Posterior tissue shown at higher magnification of the wild-type embryo (C) and homozygous mutant (D).

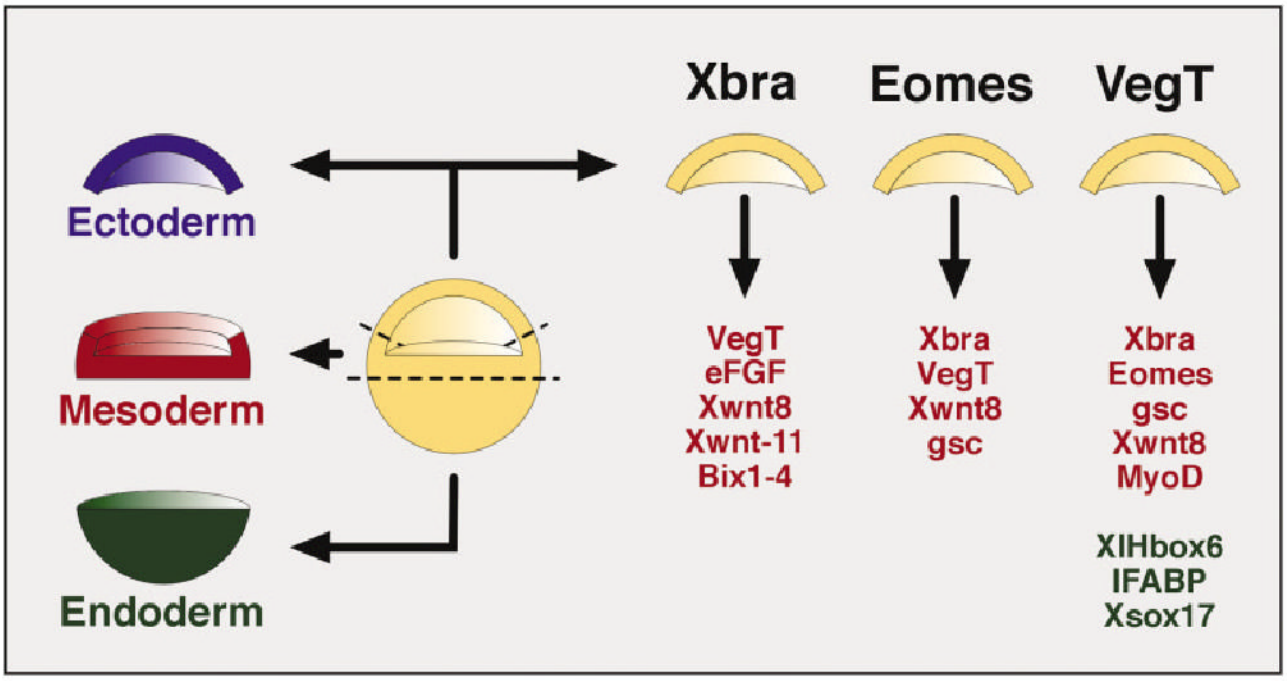


**Fig. 2.**

The inducing activities of T-box genes in *Xenopus* animal cap assays. Explants from the animal pole ("animal cap"), marginal zone, and vegetal pole differentiate into ectodermal, mesodermal, and endodermal cell types when cultured in isolation. Misexpression of T-box genes in animal caps induces expression of specific subsets of mesodermal (red) and endodermal (green) genes and promotes differentiation into corresponding cell types.

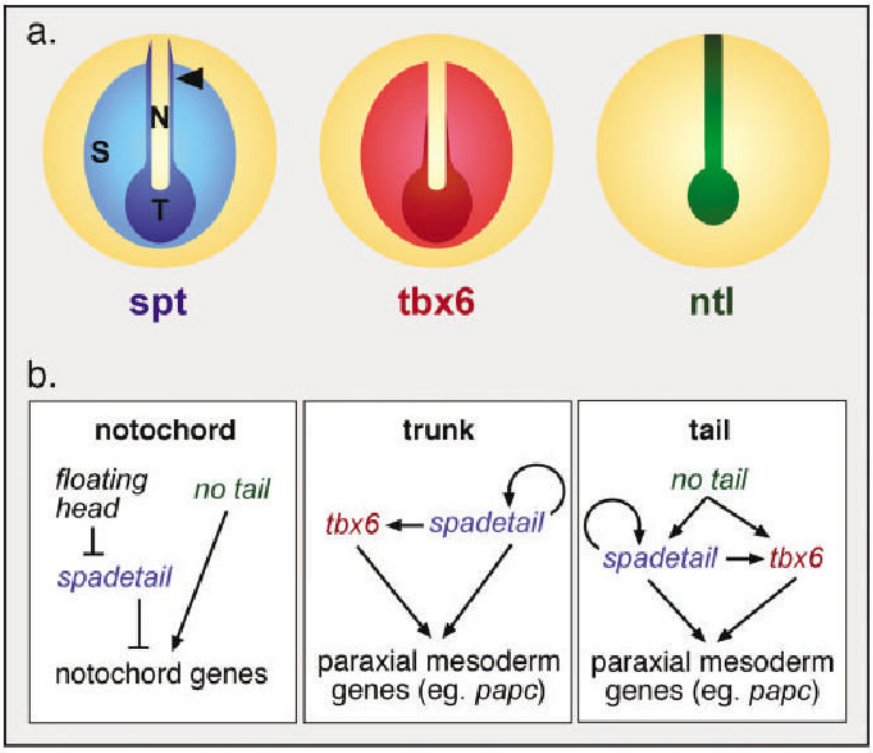


**Fig. 3.**

T-box genes regulate axial and paraxial mesoderm development in zebrafish. a: Diagrammatic representation of T-box gene expression patterns in the epiblast and hypoblast of zebrafish gastrulae. Dorsal views are shown. Expression of *spadetail* (spt; blue) and *tbx6* (tbx6; red) overlaps in the segmental plate (S; presomitic paraxial mesoderm) and in the tail bud (T). *no tail* (ntl; green) is expressed in the notochord (N) and in the tail bud, where it is coexpressed with *spadetail* and *tbx6*. Note the *spadetail*-expressing adaxial cells (arrowhead, see Griffin et al., 1998). b: Genetic pathways governing cell fate in the axial mesoderm (i.e., notochord) and paraxial mesoderm of the zebrafish trunk and tail.

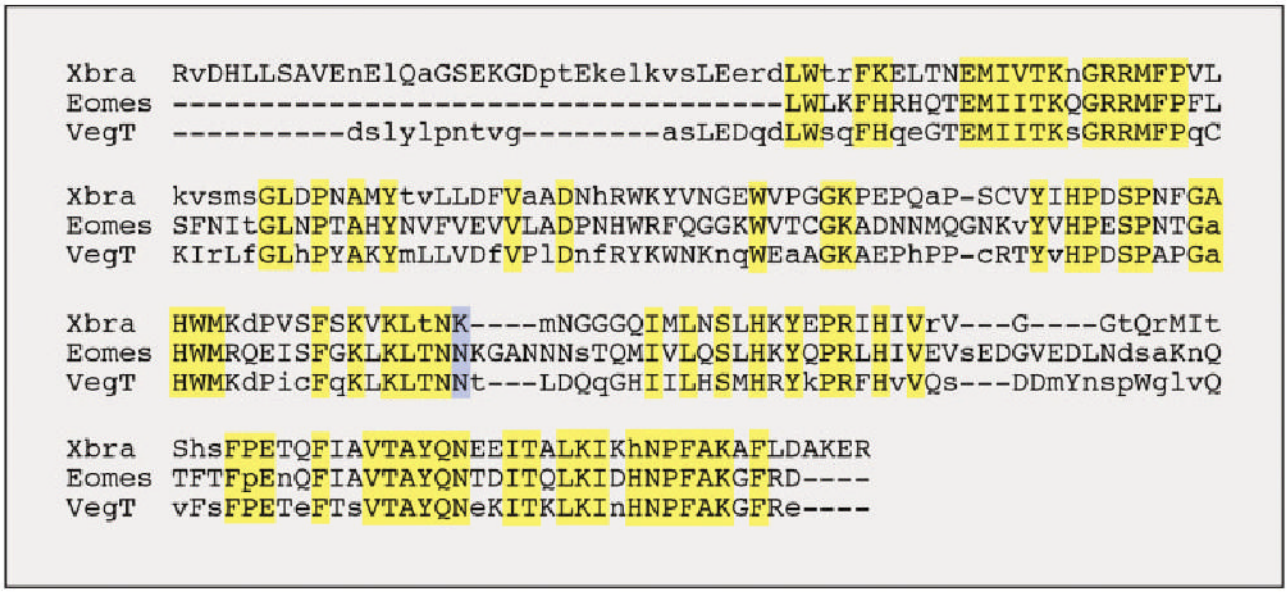


**Fig. 4.**

Sequence alignment of the T-domains of *Xenopus* Xbra, Eomesodermin (Eomes), and VegT. Regions of sequence identity are shown for each protein (upper case), based on comparisons between the *Xenopus* proteins and their orthologues in zebrafish (Xbra, VegT), mouse (Xbra, Eomes), and human (Eomes). Regions of sequence identity between the three T-domains are highlighted in yellow. Amino acid residue K149 of Xbra, contributing to functional specificity (Conlon et al., 2001), is also highlighted (in blue) along with the corresponding residues in Eomesodermin and VegT.