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Teasing out T-box targets in early mesoderm

Fiona C. Wardle¹ and Virginia E. Papaioannou²

¹Department of Physiology, Development and Neuroscience, Downing Street, Cambridge, CB2 3DY, UK, fcw27@cam.ac.uk

²Department of Genetics and Development, College of Physicians and Surgeons of Columbia University, 701 W 168th St., New York, NY 10032, USA, vep1@columbia.edu

Summary

T-box transcription factor genes are widely conserved in metazoan development and widely involved in developmental processes. With the phase of T-box gene discovery winding down, the phase of transcriptional target discovery for T-box transcription factors is finally taking off and yielding rich rewards. Mutant phenotypes in mouse and zebrafish as well as morpholino studies in zebrafish have helped to link the T-box genes to a variety of signaling pathways through diverse target genes and feedback loops. Particularly in early mesoderm development, it is emerging that a network of T-box genes interact with Wnt/ β -catenin and Notch/Delta signaling pathways, among others, to control the important processes of mesoderm specification, somite segmentation and left/right body axis determination.

Introduction

Brachyury (*T*) is the gene most likely to come to mind in association with mesoderm due to the early chance discovery of a mutation in mice that eliminated the development of posterior mesoderm and notochord. But as the intricate choreography of mesoderm development has been genetically dissected, several other transcription factor genes in the same T-box family have emerged as important regulators of different aspects of mesoderm specification, differentiation and/or patterning. The overlapping temporal and spatial expression of T-box genes (Figure 1&2), as well as evidence from mutational analysis, hints at a complex network of interactions that is only just coming to light. To add to the complexity, individual genes have different roles in different tissues through regulation of diverse cell types. These roles are almost certainly mediated by context-specific protein-protein interactions on the promoters of a variety of downstream target genes.

Our review focuses on early mesoderm development in zebrafish and mouse, comprising specification of early mesoderm, somite patterning and left/right (L/R) body axis specification. Despite the apparent similarity of developmental mechanisms between the two species, the evolutionary distance between them is evident in the lack of complete correspondence of T-box genes in the genomes (Figure 3). The *Brachyury* gene has been highly conserved although a recent study has uncovered a second ortholog, *bra*, in zebrafish [1]. There are also two *Eomes* orthologs in zebrafish, presumably resulting from genome duplication. The mammalian

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Correspondence to: Virginia E. Papaioannou.

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Tbx6 gene has no ortholog in zebrafish, and in the same subfamily, two zebrafish genes, *tbx6* and *tbx16* (mutated in *spadetail*, *spt*) lack mammalian orthologs [2] but share similarities in expression and some aspects of function with *Tbx6*. Likewise, there is no ortholog of the zebrafish *tbx24* (mutated in *fused somites*, *fss*) in mammals [3] although the fish mutant phenotype shares similarities with mutations in mouse *Tbx6* and *Tbx18*.

T-box genes have come under close scrutiny and are the subject of several recent, detailed reviews [4,5], however, the discovery of transcriptional targets has lagged behind the understanding of their highly specific but diverse developmental roles. Here we will examine three developmental processes controlled by T-box genes to see how the two species have made use of a menu of related but not necessarily orthologous genes as the means to similar developmental ends. Additionally, we will examine the varied roles of multiple T-box genes, highlighting known and suspected downstream targets.

Early days – from epiblast to mesoderm

Eomesodermin

From the earliest stages of mesoderm specification, T-box genes play essential roles. The zebrafish genome contains two *Eomes* orthologues, *eomesa* and *eomesb* (Figure 3), although only *eomesa* has been characterized during embryonic development [6–9]. It is a maternal factor that plays a role in endoderm and dorsal mesoderm formation, and in controlling the cell movements of epiboly. In endoderm, *eomesa* directly regulates the expression of *sox32*, in combination with *gata5* and *bon* [6]. Its direct targets in dorsal mesoderm and during epiboly are unknown, although it is able to cell-autonomously induce the expression of *floating head* (*flh*), a notochord marker, and *mtx2*, a mix-type homeobox gene involved in epiboly [7,8].

In the mouse, *Eomes* is thought to be the earliest regulator of mesoderm formation in addition to playing an earlier role in trophectoderm. It is required for the formation of both embryonic and extraembryonic mesoderm [10]. In chimeric mutant embryos, which circumvent the trophectoderm defect, mutant cells of the epiblast appear to undergo an epithelial to mesenchymal transition but fail to emerge from the primitive streak as mesoderm. The expression of *T* and several other markers of primitive streak indicate that some early steps along the path toward mesoderm have been made, but further development of the primitive streak is blocked. Because mutant cells are capable of forming mesoderm derivatives in teratomas, it is thought that the primary defect is in morphogenetic movements through the primitive streak rather than a defect in mesoderm differentiation. The only gene so far identified as a potential downstream target of *Eomes* in mesoderm is *Mix-like* (*Mml*) [11], a homeodomain-containing gene of unknown function.

Brachyury (*T*)

T is perhaps the most studied of the T-box genes [4,5] but still attracts attention and generates surprises. It is commonly thought of as a pan-mesodermal regulator, and it is a prime example of a gene affecting diverse cell types. The zebrafish ortholog, *no tail* (*ntl*), is required for notochord and tail somite development and for the normal morphogenetic movements of mesodermal cells during gastrulation. It also appears to play additional, if redundant, roles in other mesodermal tissue, since double mutants display synergistic phenotypes. The zebrafish phenotype of *ntl* is much less severe than that of its ortholog, *T*, in mouse, which has been something of a puzzle until the recent discovery of a second *T* ortholog, *bra*. In compound mutants for *ntl* and *bra*, tail somites and all but the most anterior trunk somites are affected, a phenotype similar to mouse *T* mutants [1], suggesting that in zebrafish, *ntl* and *bra* together perform the function of *T* in mouse. *ntl* also regulates trunk somite development in combination with *tbx16*, *fibroblast growth factor 8* (*fgf8*) and nodal signaling [12–14]. Interestingly, unlike

its mammalian orthologue, zebrafish *ntl* does not show haploinsufficiency, since heterozygous mutants for *ntl* display no obvious phenotype. However, an allelic series exists for the *ntl* locus which produces proteins of different lengths with varying mutant phenotypes, suggesting different domains of the protein mediate different activities [15–18].

The activity of *ntl* in posterior somite development is mediated, at least in part, by its direct targets, *wnt8* and *wnt3a* [1]. Targets that regulate other *ntl* activities are as yet unknown, although several direct targets of *Xenopus Brachyury (Xbra)* have been identified, including *Xwnt11*, which mediates convergence during gastrulation, and *fgf4*, which acts in a positive feedback loop with *Xbra*. Likewise in zebrafish, *wnt11* regulates convergence of cells during gastrulation and is genetically downstream of *ntl* during late gastrula stages [19], suggesting that this molecular mechanism is conserved. Similarly, the FGF genes *fgf8* and *fgf24* regulate, and are regulated by *ntl* and both are required for posterior mesoderm development, suggesting these factors too may be direct targets of *ntl*.

In the mouse, *T* is known for its dose-sensitive, cell-autonomous requirement for specification of, and cell survival in, the notochord, as well as for its non-cell autonomous requirement for development of posterior mesoderm including posterior somites and allantois [4,5], where it is necessary for the maintenance and viability of the core of the allantois [20]. Although *T* was previously thought to mark the proximal epiblast prior to mesoderm and primitive streak formation [21], a new study puts its early expression domain in the extraembryonic ectoderm [22], overlapping with *Eomes* expression. Whether expression of either gene in this domain is required for mesoderm specification is unknown and the relationship between the two genes awaits analysis of compound mutants. There is a paucity of confirmed direct targets for *T*. *Tbx6* has been suggested as a potential direct target [23], however, *Tbx6* is expressed in *T* null mutants, showing that it is not necessary for *Tbx6* induction, although it may be required for maintenance of expression [24].

***Tbx6* subfamily of genes**

The two zebrafish *Tbx6* subfamily members, *tbx16* and *tbx6*, also act in the mesoderm of zebrafish both in combination with and independently of *ntl*. *tbx6* expression is dependent on the combination of *ntl* and *tbx16* expression since in double mutants *tbx6* expression fails to initiate, whilst *tbx16* expression depends on *ntl* for its maintenance during tail somite formation. Mutations in *tbx16* (*spt*) reveal it is necessary for formation of trunk somites and convergence of lateral marginal cells during gastrulation (Figure 1). However, as is the case with *ntl*, double mutants reveal redundant roles in the development of other mesodermal tissues including tail somites (with *ntl*), notochord (with *fgf8*) and cardiac muscle (with nodal signaling)[13,14, 25]. Some part of *tbx16*'s activity in trunk development may be mediated by *papc* and *msgn*, since both of these genes are down-regulated in *spt* mutants during trunk somite formation, but not during tail somite formation [25]. The role of *tbx6* is less well characterized as no mutant has yet been identified in this gene. However, *tbx6* appears to antagonize some part of *ntl* function, since both proteins are able to bind the canonical T-box binding half site (TCACACCT) in vitro, and it is tempting to speculate that *Tbx6* antagonizes *Ntl* function by competing for binding sites in target promoters in the embryo [26], although as yet such direct targets are unknown.

Mouse *Tbx6* is limited in its expression to the primitive streak and presomitic mesoderm (PMS; Figure 2) and thus is functionally downstream of both *Eomes* and *T*. Mutations of the gene affect the specification of somites whereas other mesoderm subdivisions develop normally. In the complete absence of the gene, posterior somites fail to form and instead, the presomitic mesoderm differentiates into ectopic neural tubes [27]. It is not known how this role in the specification of cell type is mediated, but involvement in the Notch/Delta signaling pathway

is implicated by the discovery of *Delta-like-1 (Dll1)*, a Notch ligand, as a *Tbx6* target gene [23,28].

Segmenting the mesoderm – formation of somites

A number of T-box genes are expressed in either the somites or in the unsegmented PSM that give rise to the somites (Figure 1B & Figure 2B). Several target genes have been identified and the hierarchical relationship and interactions among these genes is beginning to be illuminated. In addition to being functionally upstream in the sense that it is necessary for the formation of mesoderm, *Brachyury* and its orthologs may play a more direct role by regulating the expression of *Tbx6* in mouse and *tbx6* and *tbx16* in zebrafish, although it is not necessary for initiation of any of these genes [1,23,24,29,30]. Interestingly, *ntl* also acts in combination with *tbx16* to regulate the expression of *her1*, a downstream component of Notch signaling required for somite boundary formation [14,31]. At a slightly later stage in somite formation another T-box factor, *tbx24*, is needed for somite boundary formation through activation of rostrally expressed somite genes such as *mespb* which, like its mouse counterpart *Mesp2*, is also required for somite boundary formation [31]. It is notable that *tbx24* is expressed throughout the PSM, whereas the downstream targets show more restricted expression, suggesting another factor(s) must limit its activity to the rostral somite. Recent studies suggest Ripply1, a corepressor of the Groucho/TLE family, plays this role by interacting with Tbx24 and converting it to a repressor. In vitro, Tbx24 directly binds the *mespb* promoter and in conjunction with Ripply1 represses its activity, whilst in the embryo over-expression of *rippy1* inhibits *mespb* expression [32].

What appears to be achieved by a network of several T-box factors in zebrafish may be accomplished by *Tbx6* in mouse. *Tbx6* is not only required for the correct specification of paraxial, presomitic mesoderm, but also has a role in the correct segmentation of the somites. In null mutants, the anterior 6–8 somites form, but are abnormally segmented [27,33] whereas a hypomorphic allele, *Tbx6^{rv}*, causes abnormalities in somite epithelization and patterning resulting in fusion of ribs and vertebrae [34,35]. *Tbx6* seems to be at the center of a regulatory network controlling somite border formation in conjunction with the segmentation clock within the PSM. Four direct targets have been identified: In synergy with Wnt signaling, *Tbx6* regulates expression of *Msgn1*, a transcription factor necessary for PSM maturation and segmentation [36]; also in synergy with Wnt signaling, *Tbx6* controls PSM expression of *Dll1*, the ligand of Notch1, [23,28]. *Tbx6* in synergy with Notch signaling directly regulates *Mesp2* [37] which determines the segment boundary of somites; and subsequently, *Tbx6* together with *Mesp2* activates *Ripply2*, which, in a feedback loop, negatively regulates *Mesp2* thus controlling periodic expression of *Ripply2* [38]. Like *tbx24*, *Tbx6* is expressed throughout the PSM whereas the downstream target expression is limited to the anterior PSM in the position where somites are forming. Clearly, other factors must be present that limit the activation of these genes by *Tbx6* in the context of the wave front model of somite segmentation and it will be interesting to see whether Tbx6 too interacts with a Groucho-like repressor in this context.

Several other T-box genes are segmentally expressed in the somites and anterior PSM and, although it is speculated that they play a role in the initiation or maintenance of anterior/posterior polarity of the somites, only one of these genes has a known phenotypic effect. Mutation of *Tbx18* shows that it is required downstream of *Mesp2* and Notch/Delta signaling to maintain the integrity of the posterior compartment possibly by repressing *Dll1* and either indirectly or directly repressing *Uncx4.1* [39,40] in anterior somites. While it is tempting to speculate that the overlapping expression of the closely related *Tbx22* may share redundant repressor function with *Tbx18* [41], there is no direct evidence to that effect. In zebrafish *tbx18* is expressed in newly formed somites and pre-somitic mesoderm during early

somitogenesis, and later shows strong expression in the anterior and medial aspect of the more posterior somites [42] although its role or targets in the somitic mesoderm are not known at present. Similarly, no function has been assigned to the segmental expression of mouse *Tbx2* in the posterior compartment of some somites [43].

Asymmetric development – left/right axis specification

In the specification of the L/R body axis, asymmetric *Nodal* expression is the first molecular asymmetry evident and represents a conserved mechanism of symmetry breaking in all bilaterians. There is evidence in mammals that this asymmetric expression is brought about through signaling activity of motile cilia of the node [44]. Although a multitude of genes affect L/R axis determination, this is also a process in which several T-box genes have interwoven effects. Studies in zebrafish indicate multiple roles for *ntl* in L/R axis formation. *ntl* mutants show randomized laterality of organs, preceded by bilateral expression of *cyclops*, a Nodal ortholog, and other components of the Nodal signaling pathway [45]. Morpholino loss-of-function studies of *ntl* in specific cells indicate that effects on laterality are mediated through the ciliated cells of Kupffer's vesicle (KV), a structure analogous to the mammalian node and considered the organ of asymmetry in zebrafish. Ciliated cells are present in affected embryos, but fail to organize into the spherical KV. Independent of this effect, *ntl* also regulates expression of *left right dynein related-1* (*lrd1*), which is involved in ciliary motility [46,47], *polaris*, which is involved in intraflagellar transport [48], and the Nodal antagonist, *charon* [49]. These studies indicate that *ntl* could impinge on L/R determination through different pathways at several levels including ciliary motility, ciliogenesis, and morphogenesis of midline structures including KV.

Similarly, *T* mutant mouse embryos have randomized heart looping and fail to express *Nodal* around the node or in the lateral plate mesoderm. Thus there is a failure at the earliest stages of laterality determination, but whether this is due to midline abnormalities of the node or notochord, or to effects on mesodermal tissues lateral to the node, which are all affected by the *T* mutation, is unknown. It is known, however, that Wnt signaling controls *Dll1* expression in PSM in synergy with *T* and possibly by direct regulation of *Tbx6*, thus implicating all of these players in laterality determination [23,50,51]. Indeed, *Tbx6* also appears to be involved in L/R specification at several levels. *Tbx6* mutant embryos have randomized laterality and *Dll1*, a direct target, is downregulated around the node. In addition, there is a striking and apparently independent effect on the morphology and motility of nodal cilia, which are severely compromised even though the gene is not expressed in the node (Figure 2), and finally, Ca^{2+} signaling around the node is eliminated [52]. The closely related zebrafish gene *tbx16* also has an effect on laterality and, like *ntl* and *Tbx6*, appears to regulate L/R patterning on several different levels. Double mutants and specific knockdown of *tbx16* in dorsal forerunner cells (DFCs), the precursors to KV, show it acts in combination with *ntl* in first specifying KV fate, and then independently of *ntl* regulates KV lumen morphogenesis. *tbx16* also regulates the expression of *pkd2*, the gene coding for polycystin-2, the proposed Ca^{2+} ion channel associated with nodal cilia [48] and so may regulate L/R patterning at the level of cilia formation as well. Thus, the related genes *Tbx6* and *tbx16* both have effects on laterality and somitogenesis. *tbx16* is expressed in DFCs during gastrulation (Figure 1) and is then down regulated in KV [14,53] but similar lineage information for *Tbx6* is not available and it is unknown whether the node precursors ever express *Tbx6*.

Conclusions

T-box factors play a central role in the gene regulatory networks that orchestrate mesoderm development in the vertebrate embryo. At each stage of mesoderm specification and patterning T-box factors work in combination with each other and with signaling pathways to regulate

target gene expression that will ultimately lead to differentiation. Although for many years the only known direct targets of a T-box factor were those regulated by Xbra in the frog, in the last few years progress has been made in identifying targets of other T-box factors, such as mouse Tbx6 and zebrafish Ntl. It will be illuminating to determine whether the downstream target genes of T-box factors found in these species are conserved in other vertebrates and whether these targets play similar roles in mesoderm specification, somite patterning and L/R axis determination. But despite these advances, there are still many targets to be identified. What downstream targets, for instance, mediate Brachyury's crucial function in notochord differentiation? The advent of genomic technologies such as chromatin immunoprecipitation combined with microarrays or deep sequencing techniques will help elucidate the binding of these factors across the genome and advance our identification of T-box targets.

Another challenge for the future will be to understand how different T-box factors acting in the same tissue distinguish their over-lapping and distinct targets. An attractive possibility is that specificity of target recognition is achieved through interaction with other transcription factors. Related to this is the question of how T-box factors interact with signaling pathways involved in mesoderm development. Expression of T-box factors is regulated by several common developmental signaling pathways and the ligands of these pathways are known targets of T-box factors, as exemplified by the auto-regulatory loop of Brachyury with FGF. But do T-box factors and the downstream transcriptional effectors of these signaling pathways also intersect at common target promoters? We know, for instance, that Xbra can interact with Smad1 and regulate *Xvent2b* expression [54] and it will be interesting to determine whether this is a common theme, with T-box factors routinely interacting with the transcription factor effectors of signaling pathways.

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Figure 1. Expression of three T-box genes in zebrafish

Schematic representation of *ntl*, *tbx16* and *tbx6* expression domains showing overlapping and complementary expression patterns at (A) the start of gastrulation (50% epiboly) and during gastrulation (75% epiboly) and (B) at early somite stages. Initial expression of *ntl* is seen as early as 4 hours post-fertilization in the dorsal margin (not shown), and then expression expands to encompass the whole margin before the start of gastrulation. It is worth mentioning that *ntl* is expressed in cells at the margin that co-express *gata5* and will become endoderm [55]. As gastrulation proceeds, *ntl* expression remains in non-involved cells of the margin, the migrating dorsal forerunner cells, and the involution notochordal precursors on the dorsal side of the embryo, but is down-regulated in pre-chordal plate precursors and involution cells of the lateral and ventral margin (shown here at 75% epiboly). Expression is also seen in a small group of dorsal cells, the dorsal forerunner cells, where it overlaps with *tbx16* expression. At early somite stages expression is seen in the notochord, and in the tailbud progenitor zone and overlying cells. During early somite stages *ntl* expression is also seen in the lateral mesoderm (presumptive pronephros) where it overlaps with *tbx16* expression [14]. Initial expression of *tbx16* is seen after the mid-blastula transition and is ubiquitous (not shown). Expression is then gradually down-regulated in all cells but those at the margin by the start of gastrulation. As cells involute during gastrulation expression remains on the dorsal side in the pre-chordal plate precursors but is down-regulated in the notochordal precursors, whilst ventral and lateral

hypoblast cells continue to express *tbx16* after involution (shown here at 75% epiboly). At early somite stages, *tbx16* expression is seen in the tailbud progenitor and initiation zones, the posterior presomitic mesoderm (pPSM), adaxial cells, lateral mesoderm and prechordal plate. *tbx6* is initially expressed in ventral and ventral-lateral cells of the margin at early blastula stages (not shown). Expression then extends round the margin so that it is absent from only the dorsal cells of the margin by the start of gastrulation. During gastrulation expression remains in both involuted and non-involuted ventral and lateral marginal cells (shown here at 75% epiboly). At early somite stages expression is in the tailbud and the pPSM [49].

Figure 2. Expression of T-box genes during gastrulation (A) and somite formation (B) in the mouse embryo

A. *Eomes* is expressed in the extraembryonic ectoderm prior to primitive streak formation and transiently in the primitive streak. Expression persists in the chorion [56,57]. *T* is first expressed in a band in the distal extraembryonic ectoderm [22], partially overlapping with *Eomes*, and later in the primitive streak, node and core of the allantois [58]. *Tbx6* is first expressed in the primitive streak and newly formed paraxial mesoderm but does not appear in the node [24]. **B.** During somite formation, *T* continues to be expressed in the notochord and tailbud but is downregulated to undetectable levels in the PSM [59]. *Tbx6* is expressed throughout the presomitic mesoderm and tail bud but is downregulated as the somites form [24]. *Tbx18* is

expressed in the anterior compartment of somites as well as stripes corresponding to the anterior portion of two developing somites (s0 and s-1) in the PSM [60]. *Tbx22* shows a complementary expression pattern in the posterior portion of the forming somite (s0) and in the posterior portion of the newly formed somites, fading as the somites mature [41]. *Tbx2* is not expressed in the first 10–15 somites to form but at later stages is expressed in the PSM in the posterior aspect of the two forming somites and in the posterior compartment of 5–6 newly formed somites [43].

	Zebrafish	Mouse
Mesoderm Specification	<i>eomesa</i> <i>notail (ntl)</i> , <i>bra</i> <i>tbx16 (spadetail, spt)</i> <i>tbx6</i> -	<i>Eomesodermin (Eomes)</i> <i>Brachyury (T)</i> - - <i>Tbx6</i>
Somite Segmentation	<i>notail (ntl)</i> <i>tbx6</i> <i>tbx16 (spadetail, spt)</i> <i>tbx24 (fused somites, fss)</i> - <i>tbx18</i>	<i>Brachyury (T)</i> - - - <i>Tbx6</i> <i>Tbx18</i> <i>Tbx22</i> <i>Tbx2</i>
Left/right Axis Determination	<i>notail (ntl)</i> - <i>tbx16 (spadetail, spt)</i>	<i>Brachyury (T)</i> <i>Tbx6</i> -

Figure 3.

T-box genes involved in early mesoderm specification, somite segmentation, and left/right body axis determination in zebrafish and mouse. Orthologous genes are in the same row and a dash indicates that there is no ortholog in that species. Both *eomes* and *brachyury* have been duplicated in the zebrafish genome, resulting in two co-orthologous genes. Some genes are involved in all three processes and others are more limited in their roles. According to the rules of nomenclature, zebrafish gene names are lower case and the first letter of mouse gene names is capitalized. The first letter of both mouse and zebrafish proteins is capitalized.