PRIMER

The T-box gene family: emerging roles in development, stem cells and cancer

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ABSTRACT

The T-box family of transcription factors exhibits widespread involvement throughout development in all metazoans. T-box proteins are characterized by a DNA-binding motif known as the T-domain that binds DNA in a sequence-specific manner. In humans, mutations in many of the genes within the T-box family result in developmental syndromes, and there is increasing evidence to support a role for these factors in certain cancers. In addition, although early studies focused on the role of T-box factors in early embryogenesis, recent studies in mice have uncovered additional roles in unsuspected places, for example in adult stem cell populations. Here, I provide an overview of the key features of T-box transcription factors and highlight their roles and mechanisms of action during various stages of development and in stem/progenitor cell populations.

KEY WORDS: T-box genes, Tbx, Transcription factors

Introduction

Transcription factors of the T-box family play varied yet crucial roles throughout development. The founding member of the family, brachyury (from the Greek for short tail), was discovered following studies of a short-tailed mouse that harbored a mutation affecting tail length and embryonic development (Dobrovolskaïa-Zavadskaïa, 1927). The brachyury gene (which is also known as *T*) soon became a classic developmental gene studied in great detail for its pivotal role in the development of the notochord and posterior mesoderm; mutations in *T* cause embryonic lethality in homozygotes and short tails in heterozygotes (Papaioannou, 2001). The subsequent cloning and sequencing of *T* led to its classification as a DNA-binding protein and transcription factor (Herrmann et al., 1990; Kispert and Herrmann, 1993; Kispert et al., 1995) and, soon after, a family of related genes encoding proteins with transcriptional regulatory activity and linked by a similar DNA-binding motif was rapidly uncovered (Bollag et al., 1994).

Given the developmental roles of *T*, the T-box family genes were initially studied from a developmental biology perspective. Embryonic phenotypes were predicted on the basis of embryonic expression patterns; functions were investigated on the basis of the severe, usually embryonic lethal, phenotypes caused by ablation of the genes. In addition, most T-box gene mutations, like those in *T*, show dose sensitivity, thereby giving rise to dose-dependent effects in heterozygotes. These early studies led to the elucidation of the genetic basis of a number of human syndromes and defined some of the crucial embryonic roles for T-box factors (Table 1). These studies suggest that T-box genes, which are expressed in a highly

specific manner but can be widespread spatially and temporally, are involved in all of the major developmental signaling pathways, and accumulating evidence indicates that they act at multiple stages of development through multiple downstream target genes that can differ in different tissue contexts.

Although by no means a comprehensive review, this Primer covers the major features of the T-box family with an emphasis on recent discoveries of their roles during the development of vertebrates, primarily mouse and human, while recognizing some untapped areas for further study. Of the many aspects of development that involve T-box genes, several are highlighted to emphasize how multiple T-box factors can feed into a single system and also to illustrate how a single T-box factor can feed into multiple systems at different times and locations. A comprehensive review of the older literature and work on other species can be found in earlier reviews (e.g. Morley et al., 2009; Naiche et al., 2005; Papaioannou, 2001; Papaioannou and Silver, 1998; Showell et al., 2004; Smith, 1999; Takashima and Suzuki, 2013; Wardle and Papaioannou, 2008).

Defining and emerging features of the T-box family

Common to all T-box proteins is the DNA-binding motif, or T-box domain, which spans 180-200 amino acid residues and binds DNA in a sequence-specific manner. Phylogenetic relationships between T-box genes are reflected by homology within the region encoding this domain. On this basis, five subfamilies can be identified (Fig. 1): the T, Tbx1, Tbx2, Tbx6 and Tbr1 subfamilies. T-box proteins function as transcriptional repressors or activators and some, such as T and Tbx4, appear to have both activation and repression domains that may function in different cellular or promoter contexts (Kispert et al., 1995; Ouimette et al., 2010) (Fig. 2A). Unique among the T-box genes, *Mga* contains a single-exon T-box domain and a basic helix-loop-helix (bHLH) zipper domain (Hurlin et al., 1999), possibly representing a reverse transcription and reintegration event of the T-box domain into a bHLH zipper domain gene.

The T-box domain target sequence was first identified for the T protein as a consensus, near-palindromic sequence made up of two half sites to which the protein binds as a monomer (Kispert and Herrmann, 1993). It was subsequently found that other T-box proteins can bind the consensus half site (AGGTGTGAAA), which is called the T-box binding element (TBE), although the optimal target sequences vary, as do the preferences of different proteins for the number and spacing of TBEs. Crystallographic analysis of *Xenopus* Xbra bound to a palindromic sequence derived from the consensus sequence revealed that it binds as a dimer with a small protein-protein interface area and with the T-box domains contacting DNA in the major and minor grooves at the half sites (Müller and Herrmann, 1997) (Fig. 2B). However, the significance of dimerization was called into question by subsequent studies of TBX3 and TBX1 bound to the palindromic consensus sequence, as the dimerization interface in this context is arguably too small to constitute a biologically relevant protein interface, and the proteins

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Table 1. Major developmental effects of heterozygosity, homozygosity and tissue-specific mutation of T-box genes in mouse with the corresponding human developmental syndrome and phenotypes

T-box gene subfamily	Gene (alias)	Developmental effect of mutation in mice		Tissue-specific mutations: tissue affected	Human syndrome: phenotype[‡]	References
		Heterozygous*	Homozygous*			
T	*T*	Viable, short or no tail	Mid-gestation lethal, failure of notochord and posterior mesoderm development		Sacral agenesis, abnormal ossification of the vertebral bodies, persistent notochordal canal	Dobrovolskaïa-Zavadskaïa, 1927; Postma et al., 2014
	Tbx19 (*Tpit*)	Normal	Viable, ACTH deficiency, adrenal, gonadal and pigment defects		Recessive isolated adrenocortocotrophic hormone deficiency (IAD)[‡]	Pulichino et al., 2003
Tbx1	*Tbx1*	Viable, thymus and vascular abnormalities	Neonatal lethal, craniofacial, glandular, vascular and heart abnormalities	Pharyngeal mesoderm: OFT and thymus defects Pharyngeal endoderm: OFT defects Pharyngeal epithelium: pharyngeal arch artery, inner ear and thymus defects Endothelial cells: defects in lymphatic vessels Skin epithelium: decrease in HF-SC and thinning of hair	DiGeorge: craniofacial, glandular, vascular and heart abnormalities	Chen et al., 2012; Jerome and Papaioannou, 2001; Lindsay et al., 2001; reviewed by Papangeli and Scambler, 2013; Scambler, 2010
	Tbx10	Normal Ectopic gain-of-function *Dancer* mutation: viable, susceptibility to cleft lip and palate	Normal Ectopic gain-of-function *Dancer* mutation: viable, cleft lip and palate		Not known	Bush et al., 2004; Xue et al., 2010
	Tbx15	Normal	*Droopy ear*: viable, craniofacial and limb malformations, pigment pattern alterations		Cousin: craniofacial dysmorphism, hypoplasia of scapula and pelvis, short stature[‡]	Candille et al., 2004; Lausch et al., 2008
	Tbx18	Normal	Postnatal lethal, vertebral and ureteric malformations, defective venous return, defective sino-atrial node, defective pleuropericardial membranes	Otic mesenchyme: deafness	Not known	Airik et al., 2006; Bohnenpoll et al., 2013; Bussen et al., 2004; Greulich et al., 2011; Norden et al., 2012; Trowe et al., 2008
	Tbx20	Viable, heart contractile function defects, cardiomyopathy	Mid-gestation lethal, lack of heart chamber formation	AVC myocardium: constriction defects and lack of cushion mesenchyme Endocardium: endocardial cushion formation and valve elongation defects Adult cardiomyocytes: heart failure	Atrial septal defect 4 (ASD4); cardiomyopathy	Cai et al., 2011, 2013; Greulich et al., 2011; Kirk et al., 2007; Liu et al., 2008; Shen et al., 2011; Singh et al., 2005; Stennard et al., 2005
	Tbx22	Females unaffected	Reduced postnatal viability, submucosal cleft palate, ankyloglossia and choanal atresia		X-linked cleft palate with ankyloglossia (CPX): 1/3 female carriers affected[‡]	Braybrook et al., 2001; Pauws et al., 2009
Tbx2	*Tbx2*	Mammary gland branching decreased	Mid-gestation lethal, heart and limb abnormalities, cleft palate, lung hypoplasia	Myocardium: accessory atrioventricular bundles leading to pre-excitation of the ventricles	Not known	Aanhaanen et al., 2011; Harrelson et al., 2004; Jerome-Majewska et al., 2005; Ludtke et al., 2013; Zirzow et al., 2009

Continued

DEVELOPMENT

Table 1. Continued

| T-box gene subfamily	Gene (alias)	Developmental effect of mutation in mice		Tissue-specific mutations: tissue affected	Human syndrome: phenotype[‡]	References
		Heterozygous*	Homozygous*			
	Tbx3	Viable, hypoplastic mammary glands, abnormal external genitalia, atrial arrythmia, cleft palate (with Tbx2 reduction)	Mid-gestation lethal, yolk sac, limb and mammary gland defects, cardiac arrhythmia, liver hypoplasia, pituitary defects	Nascent atrioventricular bundle or nascent atrioventricular node: AV block. Adult heart: AV block	Ulnar-mammary: hypoplastic mammary glands, abnormal external genitalia, limb abnormalities, cardiac conduction defects	Bamshad et al., 1997; Davenport et al., 2003; Frank et al., 2012; Jerome-Majewska et al., 2005; Linden et al., 2009; Ludtke et al., 2009; Trowe et al., 2013; Zirzow et al., 2009
	Tbx4	Viable, reduced allantois growth rate	Mid-gestation lethal, allantois and hindlimb defects	Limb: required for initiation (not outgrowth), muscle and tendon morphogenesis. Lungs: reduced branching (with Tbx5 reduction)	Small patella: hindlimb abnormalities	Arora et al., 2012a,b; Bongers et al., 2004; Duboc and Logan, 2011; Naiche and Papaioannou, 2003
	Tbx5	Reduced postnatal viability, heart abnormalities	Mid-gestation lethal, severe heart malformations	Limb: required for initiation (not outgrowth), muscle and tendon morphogenesis. Lung/trachea: specification	Holt-Oram: heart and hand abnormalities	Arora et al., 2012a,b; Basson et al., 1997; Bruneau et al., 1999; Duboc and Logan, 2011
Tbx6	Tbx6	Viable, mild vertebral defects	Mid-gestation lethal, somite abnormalities, ectopic neural tubes, random left/right laterality. Hypomorph Tbx6[rv]: viable, rib and vertebrae fusions		Dominant spondylocostal dysostosis	Beckers et al., 2000; Chapman and Papaioannou, 1998; Hadjantonakis et al., 2008; Sparrow et al., 2013
	Mga	Normal	Peri-implantation lethal, failure of ICM		Not known	A. J. Washkowitz, C. Schall, K. Zhang, W. Wurst, T. Floss, J. Mager and V.E.P., unpublished
Tbr1	Tbr1	Impairment of amygdalar axonal projections, behavioral abnormalities	Neonatal lethal, olfactory bulb and cerebral cortex defects		Associated with autism spectrum disorder (ASD)	Bulfone et al., 1998; Hevner et al., 2001; Huang et al., 2014; O'Roak et al., 2012
	Eomes (Tbr2)	Normal	Early post-implantation lethal, trophoblast and mesoderm failure	Epiblast: defective EMT in primitive streak and failure of DE formation. VE: AVE failure	Microcephaly syndrome with polymicrogyria and agenesis of the corpus callosum[‡]	Arnold et al., 2008a; Baala et al., 2007; Nowotschin et al., 2013; Russ et al., 2000
	Tbx21 (Tbet)	Viable, airway hyperresponsiveness, intermediate INF-γ levels in Th1 cells	Viable, airway hyperresponsiveness, no Th1 cells		Asthma susceptibility	Akahoshi et al., 2005; Finotto et al., 2002; Szabo et al., 2002

*Heterozygous or homozygous loss of function unless otherwise noted.

[‡]Phenotypes given are in heterozygous humans with the exception of *TBX19* and *TBX15*, which are homozygous, *TBX22*, which is X-linked, and *EOMES*, which results from a homozygous loss of function due to a balanced translocation.

may be kept in register only by the DNA (Coll et al., 2002; El Omari et al., 2012). Furthermore, only half sites have been recognized in the promoters of target genes and, notably, it was shown that TBX5 binds as a monomer to a single half site in the promoter of its target gene *ANF* (*NPPA*) (Stirnimann et al., 2010) (Fig. 2B). For the growing number of target genes, further studies are needed to elucidate the DNA binding mechanism in the context of endogenous target gene promoters.

Target gene recognition and selectivity, as well as target gene regulation, can also be influenced by interactions with other proteins or co-factors. Several T-box proteins bind homeobox factors. For example, Tbx5 and Nkx2-5 interact with each other through their respective T-box domain and homeodomain and together they bind the *Anf* promoter in tandem and act synergistically to activate *Anf* during cardiomyocyte differentiation (Hiroi et al., 2001). Domains other than the T-box are also important for mediating interactions with co-factors. For example, in melanoma cells Tbx2 interacts with the tumor suppressor protein Rb1 through a domain that is immediately C-terminal to its T-box. This association increases the ability of Tbx2 to interact with its DNA recognition sequence in the promoter of $p21^{Cip1}$ (*Cdkn1a*), thereby enhancing the transcriptional repression function of Tbx2 (Vance et al., 2010).
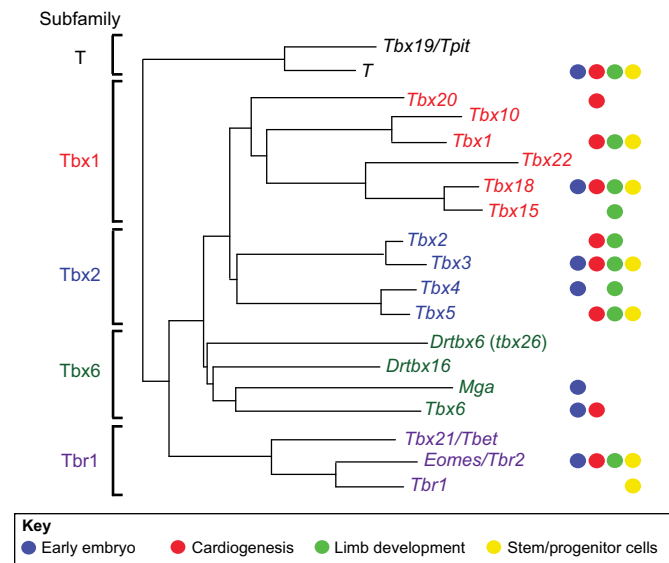
Fig. 1. Phylogenetic tree of the T-box gene family in vertebrates. Colored circles on the right indicate involvement of particular genes in the areas discussed in this Primer; additional roles are indicated in Table 1. All of the genes are represented in human and mouse with the exception of *Danio rerio* (*Dr*) *tbx16*, which is present in birds and frogs (*VegT*) but not mammals, and *Drtbx6*. The Tbx6 subfamily is divergent, rendering relationships between members less clear and complicating the assignment of orthology, although analysis of non-sequence information such as the exon-intron structure of a gene or the composition of a gene's genomic neighbors can eliminate ambiguities. Thus, the zebrafish gene *tbx24* (*fused somites*), rather than *tbx6*, is the ortholog of mammalian *Tbx6*, and it has been suggested that *Drtbx6* should be renamed *tbx26* (Ahn et al., 2012). There have also been some gene duplications, which are not indicated here, such as the duplication of *T* in *Xenopus* to form *Xbra* and *Xbra3* (Hayata et al., 1999). Common synonyms are indicated after the solidus. The phylogenetic tree is based on and modified from Naiche et al. (2005) and Papaioannou and Goldin (2008).

In addition to mediating transcriptional regulation, evidence for T-box protein-mediated gene regulation through epigenetic modifications is mounting. Tbx3, for example, was shown to regulate histone H3 lysine 27 (H3K27) methylation at its target gene *Gata6* during embryonic stem cell differentiation (Lu et al., 2011). In another study, two conserved domains within the T-box domain of *Tbet* (*Tbx21*) were shown to functionally recruit methyltransferase activity to target promoters to establish a permissive chromatin state, pointing to epigenetic regulation as an important component of T-box protein-mediated regulation of target genes (Miller et al., 2008).

Recently, a proteomic screen to detect Tbx3 interacting proteins identified a number of RNA-binding proteins and splicing factors. It was found that Tbx3 physically associates with mRNA and directly binds RNAs containing TBEs. Furthermore, it was found that the ability of RNA-binding proteins to influence splicing is Tbx3 dependent in some cases, implicating Tbx3 in the regulation of alternative splicing and providing additional complexity to the regulation of gene expression by Tbx3 (Kumar et al., 2014). Clearly, there is much more to be learned about T-box proteins and their co-factors to fully understand the target selectivity and transcriptional and post-transcriptional regulation that leads to their regional and temporal specificity in the regulation of target genes.

## Ancient origins and evolution of the T-box gene family

It has long been known that the T-box gene family is ancient in origin and present in all metazoans (Bollag et al., 1994), but the
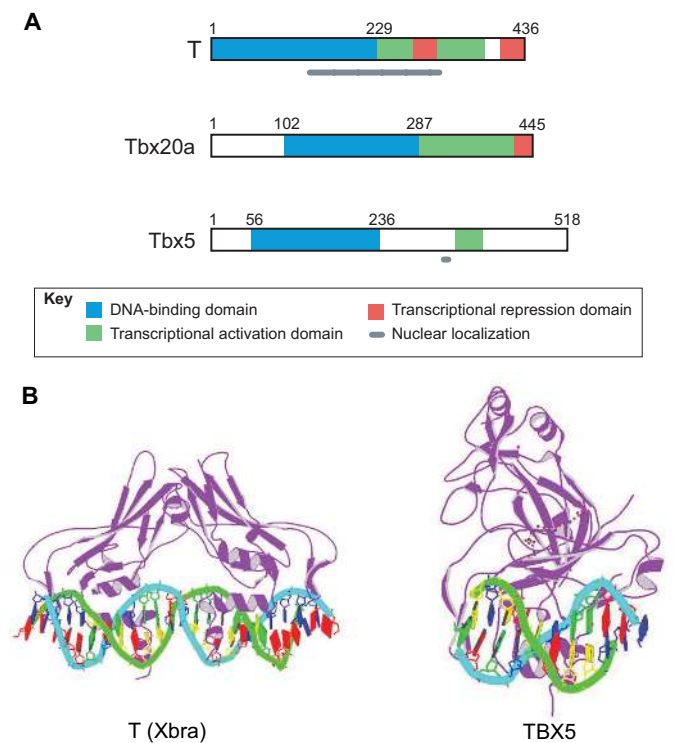
Fig. 2. Domain structure of T-box proteins and crystal structure of T-domains bound to DNA. (A) The domain structure of three example T-box proteins [T (Kispert, 1995), Tbx20a (Stennard et al., 2003) and Tbx5 (Zaragoza et al., 2004)] illustrating the location of the DNA-binding domain in the N-terminal portion and either a transcriptional activation domain or activation and repression domains in the C-terminal region. The domain involved in nuclear localization has not yet been determined for Tbx20. (B) Crystal structure of T-domains from *Xenopus laevis* T (Xbra) bound to a palindromic T-box binding element derived from the *in vitro* selected consensus sequence (Müller and Herrmann, 1997) and from human TBX5 bound to a natural T-box binding site from the *ANF* promoter (Stirnimann et al., 2010). Images are from the RCSB Protein Data Bank (www.rcsb.org): ID numbers 1XBR (Xbra) and 2X6V (TBX5).

increasing availability of sequenced genomes from diverse animal taxa has pushed back the origins of the family to unicellular organisms and fungi, in which one or two T-box genes, including *T*, have been identified (Degnan et al., 2009; Sebe-Pedros et al., 2013) (Fig. 3). Analysis of the genomes of bilaterians and representatives of the four basal metazoan phyla indicate that *T* is the most ancient member of the family and that the family expanded throughout metazoan evolution. Genes were added progressively by gene or genome duplication (Box 1) and were sometimes lost or gained in specific lineages. In present day vertebrates, the radiation of the T-box family resulted in genes that can be grouped into five subfamilies (Fig. 1), four of which were already present in the common ancestor of vertebrates and sponges (Sebe-Pedros et al., 2013) (Fig. 3).

Among vertebrates, there is usually a one-to-one correspondence of orthologs. For example, mouse and humans each have 17 orthologous T-box genes (Table 1). However, exceptions do occur, as exemplified by the existence of a paralogous gene pair in *Xenopus*, *Xbra* and *Xbra3*, which resulted from a *Xenopus*-specific gene duplication of the ancestral *T* gene (Hayata et al., 1999), and the *Xenopus* Tbx6 subfamily gene *XlTbx6r*, which is not present in other vertebrates (Callery et al., 2010). Furthermore, in zebrafish there are two additional Tbx6 family genes, which apparently have no orthologs in mammals (Ruvinsky et al., 2000a; Windner et al.,
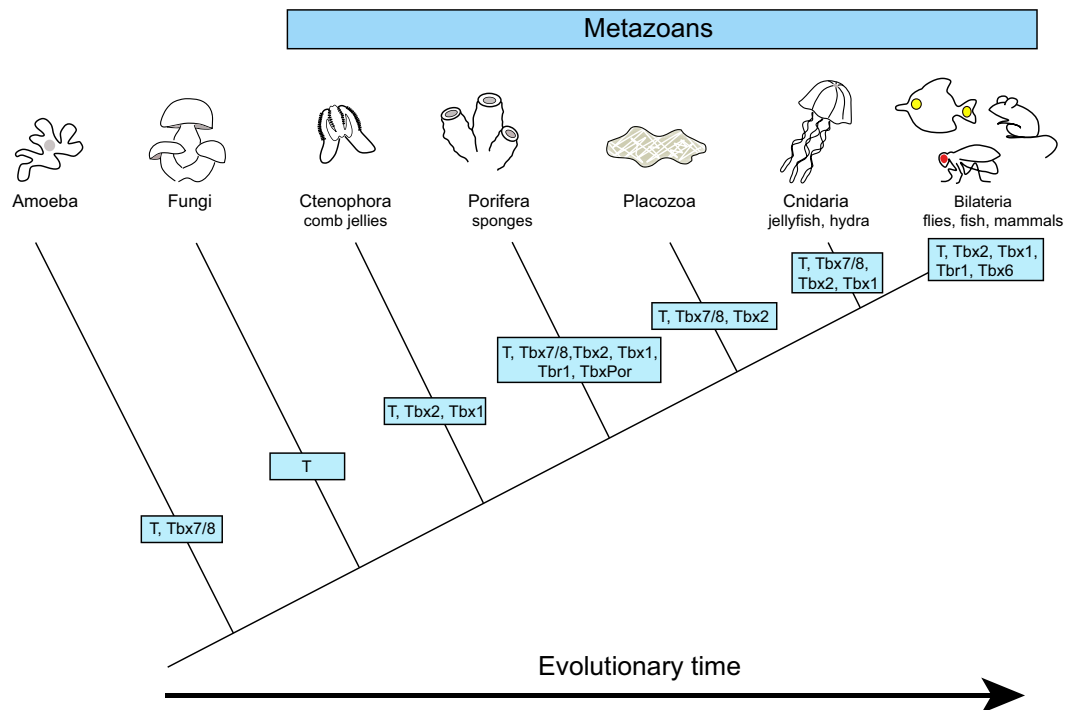
Fig. 3. Evolution of the T-box gene family. The subfamilies or classes of genes that have been identified in the different animal groups are indicated in the boxes. There has been a remarkable conservation of transcription factors between lineages that have been evolving independently since the last common ancestor to metazoans, and many of the T-box gene families have their origin at the base of the tree. However, diversification at the onset of metazoan evolution is evident. *T*, which is the most ancient T-box gene, is represented in unicellular organisms, as is *Tbx7/8*, a class not present in Bilateria. Sponges have a diverse set of T-box genes including several that have not been retained in Bilateria, whereas Tbx6 subfamily genes apparently arose in Bilateria. Note that this diagram represents one possible order of divergence of phyla (Degnan et al., 2009; Sebe-Pedros et al., 2013).

2012) (Fig. 1), possibly indicating loss of these genes in the mammalian lineage. As the genomes of more organisms are sequenced, the positive identification of orthologs in more distantly related taxa will improve.

T-box genes are scattered throughout the vertebrate genome with the exception of two parologous pairs, *Tbx2* and *Tbx3*, which are linked to *Tbx4* and *Tbx5*, respectively. This arrangement appears to have originated from a tandem duplication of a single ancestral gene followed by a duplication of the linked pair prior to the separation of bony fish from tetrapods (Agulnik et al., 1996).

Examples of T-box gene function during development

In the following sections, the involvement of T-box genes during cell fate specification and differentiation in the early embryo, during somite development and during heart and limb formation are highlighted to illustrate the complex and sometimes interactive or combinatorial roles that T-box genes play in a variety of developmental processes. In addition to these selected areas, the development of many other organs and tissues is known to be regulated by T-box genes but is not covered in detail here. T-box genes make major contributions to craniofacial development (*Tbx1*, *Tbx10*, *Tbx15*, *Tbx22*) and to development of the brain (*Tbr1*, *Eomes*), mammary gland (*Tbx2*, *Tbx3*), pituitary gland (*Tbx3*, Tbx19), thymus (*Tbx1*), liver (*Tbx3*), lung (*Tbx2*, *Tbx4*, *Tbx5*), pigmentation (*Tbx15*) and the immune system (*Tbx21*), among others (see Table 1 for references), and control processes that are also relevant to the development of cancers (Box 2).

Early development, gastrulation and somite patterning

A number of T-box genes are expressed in pre-implantation and early post-implantation embryos and play varied roles in embryo patterning and survival (Fig. 1 and Fig. 4). *Tbx3* is expressed in the inner cell mass (ICM), which is the pluripotent lineage of the pre-implantation blastocyst, and is later expressed in the extraembryonic endoderm of the developing yolk sac where it has a role in yolk sac development (Davenport et al., 2003). *Mga* is also expressed in the ICM and its derivative epiblast and is necessary for its survival after implantation (A. J. Washkowitz, C. Schall, K. Zhang, W. Wurst, T. Floss, J. Mager and V.E.P., unpublished) (Fig. 4). Trophectoderm (TE), which is the first tissue to differentiate, is required for the early interactions between the embryo and the uterus that establish implantation. The differentiation and proliferation of the TE beyond the blastocyst stage is critically dependent on *Eomes* (also known as *Tbr2*), which is expressed in the TE at the blastocyst stage (Fig. 4). *Eomes* loss results in lethality through failure of trophoblast stem cell maintenance after implantation (Ciruna and Rossant, 1999; Hancock et al., 1999; Russ et al., 2000; Strumpf et al., 2005). This is one of the earliest known roles for a T-box gene in mammals, but *Eomes* also has additional roles in the early patterning of the embryo, as revealed by tissue-specific deletion of the gene at later stages of development: loss of *Eomes* from its expression domain in the proximal posterior epiblast at the pre-streak stage blocks the epithelial-to-mesenchymal transition (EMT) and the migration of nascent mesoderm away from the primitive streak (Arnold et al., 2008a). Progenitors of the cardiac mesoderm, which are normally in the earliest cohort of cells to exit the streak, are not specified in the absence of *Eomes* and fail to express *Mesp1*, the master regulator of cardiovascular cell fate and a direct transcriptional target of Eomes (Costello et al., 2011). A few hours later, the specification and migration of definitive endoderm (DE) is also blocked (Arnold et al., 2008a). Specific loss of *Eomes* from the

Box 1. Evolution of the T-box family: insights from amphioxus

The bilaterian cephalochordate amphioxus is a close invertebrate relative of vertebrates and has therefore been used as a model organism to deduce information about the last common ancestor of vertebrates and invertebrates (Bertrand and Escriva, 2011). Phylogenetic analyses indicate that two whole-genome duplications occurred in the vertebrate lineage after the divergence of the cephalochordates, such that each amphioxus gene generally corresponds to two, or sometimes three, vertebrate genes (Dehal and Boore, 2005; Ruvinsky et al., 2000b). For example, a single amphioxus gene, *AmphiTbx1/10*, corresponds to two vertebrate genes, *Tbx1* and *Tbx10*, which presumably arose during one of the genome duplications. *AmphiTbx1/10* is expressed during gastrulation in ventral somites and the branchial arches (Mahadevan et al., 2004), corresponding to the expression of mouse *Tbx1* in the ventromedial somites and pharyngeal arches (Chapman et al., 1996). Mouse *Tbx10*, however, is expressed only in the developing hindbrain (Bush et al., 2003). Thus, the primordial function of *Tbx1/10* in chordates might have been branchial arch patterning and ventral somite specification, functions retained by the *Tbx1* gene while *Tbx10* lost its role in pharyngeal arch patterning and gained a novel role in hindbrain development. Another example is *AmphiTbx4/5*, which is the ortholog of vertebrate *Tbx4* and *Tbx5*, which both have roles in limb development and, in addition, *Tbx5* has a role in heart development (Duboc and Logan, 2011; Greulich et al., 2011). *AmphiTbx4/5* is transiently expressed in restricted, bilateral areas of ventral mesoderm that superficially resemble the restricted, limb field-specific expression of *Tbx4* and *Tbx5* in vertebrates. However, amphioxus has no limbs and the area of expression also coincides with presumptive heart precursors, suggesting that the original function of the gene was in heart specification and that it might have been co-opted for a novel role in limb outgrowth when paired limbs evolved in vertebrates (Horton et al., 2008). In this scenario, the two duplicated genes could have further evolved to take on limb-specific expression and functions. In an amazing feat of trans-subphylum transgenesis, an *AmphiTbx4/5* transgene was used to rescue limb outgrowth when heterologously expressed in the forelimb field of a mouse lacking *Tbx5*, indicating that, even across millions of years of evolution, the protein function has been conserved, suggesting that the acquisition of novel functions came about through changes in gene regulation (Minguillon et al., 2009).

visceral endoderm (VE) results in the primitive streak not being positioned properly and in failure of induction and migration of the anterior visceral endoderm (AVE), which is an essential component of axis patterning. This effect is mediated through direct transcriptional regulation of *Lhx1*, which is part of the gene regulatory network controlling AVE formation and function (Nowotschin et al., 2013). Thus, as the pattern of expression shifts with the progressive tissue rearrangements that characterize gastrulation (Fig. 4), Eomes adopts different, context-dependent roles in cell lineage specification and embryo patterning.

Like *Eomes*, *T* also affects the specification and migration of nascent mesoderm cells during gastrulation, in particular that of precursors of the node, notochord and posterior mesoderm. There are many mutant alleles of *T* in mice, all of which exhibit the hallmark loss of the notochord and posterior mesoderm but display differences in the extent of posterior development, with more severe alleles progressively affecting mesoderm at more anterior axial levels in heterozygotes as well as homozygotes (Kispert, 1995; Papaioannou, 2001; Showell et al., 2004). The notochord acts as an important signaling center during neural tube and somite patterning and its loss thus results in patterning defects. The morphology of the node is also compromised in *T* mutants, contributing, along with disruption of the midline, to abnormalities in right/left axis determination (Concepcion and Papaioannou, 2014; Conlon et al.,

1995; King et al., 1998). However, despite *T* being one of the best studied developmental genes, there is still a lot to learn about how it exerts its effects on mesodermal structures and the process of gastrulation. A screen to identify targets of zebrafish *ntl* (also known as *ta*), which is one of two zebrafish orthologs of *T* (Martin and Kimelman, 2008), identified a transcription factor gene, *flh*, the homolog of the mouse *Noto* gene, which is necessary for normal node and cilia formation. In addition, a large number of other genes involved in the formation of primary germ layers, mesoderm formation and mesoderm cell migration were identified by genome-wide binding site mapping as potential targets of *T* orthologs in zebrafish (Morley et al., 2009), *Xenopus* (Gentsch et al., 2013) and mouse (Lolas et al., 2014), indicating the existence of gene regulatory networks directed by brachyury and providing a treasure trove of candidate genes to elucidate the additional roles of this T-box gene.

T is expressed in the primitive streak and continues to be expressed in the core of the developing allantois, which is a midline, mesodermal structure derived from the posterior primitive streak that subsequently forms the umbilical cord (Fig. 4). In line with this, homozygous *T* mutants die due to failure of allantois development; in mutants, the core fails to survive and the vascular network fails to form (Inman and Downs, 2006). A different T-box gene, *Tbx4*, is similarly required for allantois growth, vascularization and fusion with the chorion, although in *Tbx4* mutants endothelial cells are formed but fail to undergo vascular remodeling (Arora et al., 2012a; Naiche and Papaioannou, 2003). A number of *Tbx4* candidate target genes that are involved in chorio-allantoic fusion and vascular remodeling have been identified, indicating that *Tbx4* affects a

Box 2. T-box genes and cancer

During development, T-box genes have roles in differentiation, proliferation, tissue integrity and the EMT, which are processes that are also relevant to the development of cancer and metastasis. Upregulation or downregulation of T-box genes has been associated with a variety of different types of cancer and may be causal in promoting neoplasia:

- Increased levels of TBX2 and TBX3 are associated with several types of cancer, including melanoma, pancreatic and mammary carcinoma, and the genes are expressed during normal development of the corresponding tissues (Abrahams et al., 2010; Begum and Papaioannou, 2011; Carreira et al., 1998; Douglas and Papaioannou, 2013; Rowley et al., 2004). Both genes repress the cyclin-dependent kinase inhibitors $p16^{INK4a}$ (*Cdkn2a*) and $p21^{Cip1}$ (*Cdkn1a*), which are involved in proliferation control, and in fibroblast cell lines TBX2 and TBX3 expression leads to bypass of senescence (Carlson et al., 2001; Jacobs et al., 2000). In mammary tumor cell lines, TBX2 is a powerful growth promoter and TBX3 drives migratory behavior (Peres et al., 2010) and promotes cancer stem-like cell phenotypes (Fillmore et al., 2010).

- Mutations or increased copy number of *T* are commonly found in the bone tumor chordoma and *T* expression is necessary for proliferation of chordoma cell lines. As the name implies, chordomas are thought to be of notochordal origin and one of the major roles of *T* is in the development of the notochord (Nibu et al., 2013). *T* has also been implicated in controlling EMT in some cancers such as adenoid cystic carcinoma and colorectal cancer (Sarkar et al., 2012; Shimoda et al., 2012).

- *Tbet* (*Tbx21*) and *Eomes* have indirect effects on metastasis as they are both involved in the normal development of natural killer (NK) T cells (Gordon et al., 2012) and, in their absence, adaptive antitumor immune responses are compromised by lower numbers of NK cells such that susceptibility to metastatic cancers is greatly increased (Lazarevic et al., 2013).
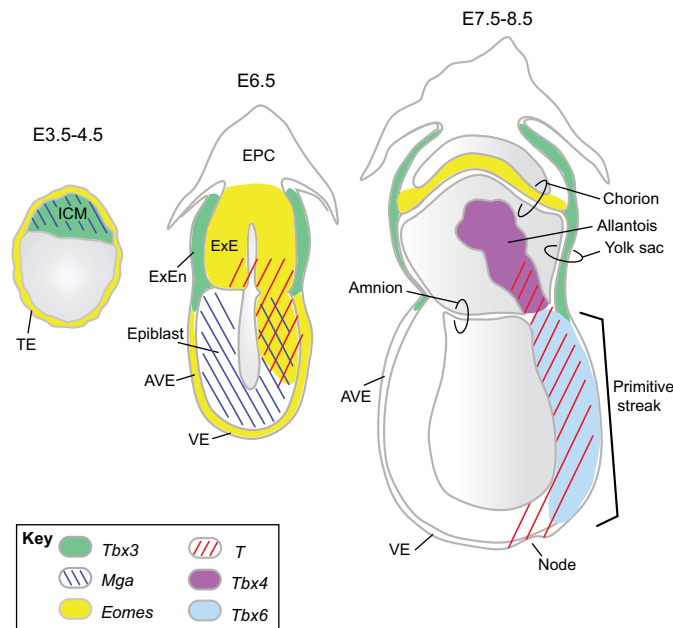
DEVELOPMENT

Fig. 4. Expression domains of T-box genes during mouse pre-implantation and early post-implantation embryo development. *Tbx3* is expressed in the inner cell mass of the pre-implantation embryo and later in the extraembryonic endoderm of the developing yolk sac. *Mga* is also expressed in the inner cell mass at pre-implantation stages and then in the epiblast of the post-implantation embryo. *Eomes* is expressed in the trophectoderm layer of the blastocyst and after implantation is expressed in the extraembryonic ectoderm and later the chorion, the visceral endoderm, including the anterior visceral endoderm, and the posterior proximal epiblast. *T* is expressed in the posterior epiblast and extraembryonic ectoderm and later in the core of the allantois, the primitive streak and node. *Tbx6* overlaps *T* in the primitive streak but is not expressed in the node. *Tbx4* is expressed in the allantois. AVE, anterior visceral endoderm; EPC, ectoplacental cone; ExE, extraembryonic ectoderm; ExEn, extraembryonic endoderm; ICM, inner cell mass; TE, trophectoderm; VE, visceral endoderm.

network of genes in the allantois (Arora et al., 2012a; Naiche et al., 2011; Naiche and Papaioannou, 2003).

In contrast to *T*, *Tbx6* is expressed in the presomitic mesoderm of the primitive streak and has several distinct effects on the specification and differentiation of the presomitic mesoderm, as well as on ciliogenesis in the node. *Tbx6* mutations result in disruption of the anterior-posterior polarity of somites and, in homozygous mutants for a loss-of-function allele, in the formation of ectopic neural tubes in place of the posterior somites, as well as disruption of left/right patterning (Chapman et al., 2003; Chapman and Papaioannou, 1998; Hadjantonakis et al., 2008; Watabe-Rudolph et al., 2002). As with other T-box genes, *Tbx6* is likely to be acting in different tissue contexts through different target genes, some of which have been identified. For example, the regulation of *Dll1*, which encodes a Notch1 ligand, may contribute to the left/right asymmetry defect of *Tbx6* mutants through perinodal Notch signaling, but, as yet, it is unknown how node ciliogenesis is regulated by Tbx6. In the presomitic mesoderm, Tbx6 normally represses the neural determinant gene *Sox2* through an indirect interaction with its N1 enhancer, thus influencing the neural versus mesodermal cell fate choice in axial stem cells (Takemoto et al., 2011). In its role in somite patterning, Tbx6 in conjunction with Wnt and Notch signaling lies at the center of a regulatory network that controls somite polarization and border formation through the direct targets *Dll1* (Hofmann et al., 2004; Watabe-Rudolph et al., 2002; White and Chapman, 2005), *Msgn1* (Nowotschin et al., 2012;

Wittler et al., 2007), which encodes a transcription factor involved in segmentation, *Mesp2* (Yasuhiko et al., 2006), which encodes a transcription factor essential for somite border formation and rostrocaudal patterning, and *Ripply2* (Dunty et al., 2008), which encodes a component of segment boundary formation. At least one other T-box gene, *Tbx18*, has a role in somite patterning. It is transiently expressed in the anterior half of newly formed somites and its expression is maintained in the anterior lateral sclerotome (Kraus et al., 2001). Tbx18 interacts with another transcription factor, Pax3, in regulating the gene expression program necessary for maintenance of anterior-posterior somite polarity (Bussen et al., 2004; Farin et al., 2008).

Cardiogenesis

T-box genes are also involved in all stages of heart development: the initial specification of the cardiac mesoderm, the regionalization of the primitive heart tube into chamber and non-chamber myocardium, the formation of the valves and septa that separate the chambers, the recruitment of second heart field (SHF) cells to the outflow tract (OFT), and formation of the cardiac conduction system (CCS). In addition, some T-box genes (*T* and *Tbx6*) indirectly affect the direction of heart looping through their effects on left/right axis formation, as discussed above. Importantly, several human developmental syndromes that include cardiac abnormalities are associated with mutations in T-box genes: *TBX1* (DiGeorge syndrome), *TBX3* (ulnar mammary syndrome), *TBX5* (Holt-Oram syndrome) and *TBX20* (atrial septal defect 4 and other abnormalities) (Table 1).

Similar to the situation in the early mesoderm, a number of T-box genes – *Eomes*, *Tbx1*, *Tbx2*, *Tbx3*, *Tbx5*, *Tbx18* and *Tbx20* – are expressed in the developing heart with both overlapping and unique areas of expression but with each gene playing at least some unique and essential role in cardiogenesis, as revealed by mutational analysis (reviewed by Greulich et al., 2011; Hariri et al., 2012) (Fig. 1 and Fig. 5). As noted above, *Eomes* is involved in the initial specification of cardiac mesoderm and, once the primitive heart tube has formed from the cardiac crescent, *Tbx5* and *Tbx20* both play early, non-redundant roles in the elaboration of the chambers of the linear heart tube. Homozygous mutant embryos for either gene die at mid-gestation with poorly developed chambers in which chamber myocardial genes are not activated (Bruneau et al., 1999; Stennard et al., 2005). The cardiac expression of *Tbx5* is primarily limited to the first heart field (FHF) derivatives in the posterior region of the heart. Through its interaction with co-factors such as Nkx2-5 and Gata4, Tbx5 is responsible for the activation of a set of chamber myocardial genes including *Nppa* and *Cx40* (*Gja5*) (Bruneau et al., 2001). Heterozygous mutations reveal later septation defects and defects in the CCS that are also characteristic of patients with Holt-Oram syndrome (Bruneau et al., 2001; Greulich et al., 2011; Takeuchi et al., 2003). Similarly, *Tbx20* activates a chamber myocardial program in derivatives of the FHF through direct targets such as *Nppa*, Nkx2-5 and *Mef2c*. Loss-of-function mutations of *Tbx20* result in poorly developed chambers and ectopic upregulation of *Tbx2*, a gene normally restricted to the atrioventricular canal (AVC) and OFT, throughout the heart tube (Greulich et al., 2011). *Tbx20*, acting via activation of *Bmp2*, also plays a role in the development and patterning of the non-chamber myocardium of the AVC and in the EMT that is required to form cushion mesenchyme (Cai et al., 2011). In the endocardium, *Tbx20* is required upstream of Wnt signaling for endocardial cushion formation and valve elongation (Cai et al., 2013). Studies with conditional alleles have also identified roles for *Tbx20* in the adult
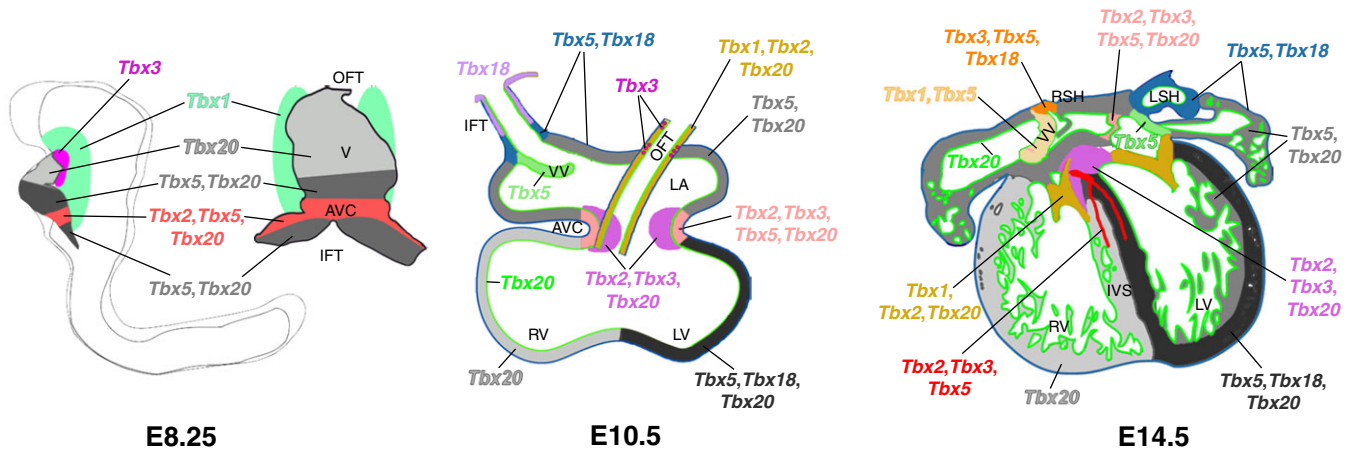
Fig. 5. T-box gene expression during heart development. T-box genes are expressed in complex, overlapping patterns throughout heart development. The E8.25 heart is shown in a left lateral view of the whole embryo and in a frontal view of the isolated heart and second heart field. Hearts at later stages are shown as schematic transverse sections. Color coding indicates expression of individual genes or combinations of genes in different regions. AVC, atrioventricular canal; IFT, inflow tract; IVS, interventricular septum; LA, left atrium; LSH, left sinus horn; LV, left ventricle; OFT, outflow tract; RSH, right sinus horn; RV, right ventricle; V, ventricle; VV, venous valves. Reprinted from Greulich et al. (2011) with permission.

heart, where it regulates the cardiac ion flux that is crucial for excitation-contraction functions in myocytes, a role that helps explain the involvement of this gene in adult cardiomyopathy (Shen et al., 2011).

In contrast to *Tbx5* and *Tbx20*, *Tbx2* and *Tbx3* are co-expressed in the non-chamber myocardium of the AVC and serve to suppress proliferation and chamber-specific gene expression, setting up a dichotomy between chamber and non-chamber myocardium (Christoffels et al., 2004). Both genes are involved in establishing the identity and function of the CCS and in inducing atrioventricular cushion development through *Bmp2*. Although mutations in each gene produce a distinct cardiac phenotype, indicating unique functions, they also have redundant roles in the AVC (Frank et al., 2012; Greulich et al., 2011; Singh et al., 2012). Similarly, during the development of the arterial pole of the heart, *Tbx2* and *Tbx3* play roles in SHF deployment and OFT alignment. Together with *Tbx1*, which is required for *Tbx2* and *Tbx3* expression in the pharyngeal mesenchyme and neural crest, they form a gene regulatory network for arterial pole development. Accordingly, loss of function of *Tbx1* and either *Tbx2* or *Tbx3* causes severe defects in OFT development (Mesbah et al., 2012). *Tbx1* is expressed throughout the pharyngeal region in endoderm, ectoderm and neural crest-derived mesenchyme and it affects many aspects of pharyngeal development, including the development of SHF, in which it drives elevated proliferation and delayed differentiation as SHF cells are added to the elongating arterial pole (Parisot et al., 2011). Loss-of-function mutations of *Tbx1* in mice result in severe OFT defects including truncus arteriosus and aortic arch artery defects, among other craniofacial and glandular abnormalities, similar to the defects seen in the DiGeorge syndrome in humans (Papangeli and Scambler, 2013; Scambler, 2010).

From the venous pole of the heart, the proepicardium spreads over the surface to form the epicardium, which then undergoes EMT, invades the underlying myocardium, and contributes to the smooth muscle cells of the coronary vasculature. In addition to its role in somite patterning, *Tbx18* is expressed in the proepicardium, the precursors of the myocardium of the sinus venosus, and the sino atrial node (SAN). Mutants for *Tbx18* die at birth with defects in the venous return of the heart including delayed myocardial differentiation and a severely reduced SAN. In addition, the

epicardium and coronary vessels have structural and functional defects that lead to defective vascular plexus remodeling in the coronary vasculature (Greulich et al., 2011; Wu et al., 2013).

Limb development

Many T-box genes, including *T*, *Eomes*, all the members of the Tbx2 subfamily and three members of the Tbx1 subfamily, are expressed in the developing limbs, and functional roles for most of these have been identified (for reviews see Duboc and Logan, 2011; King et al., 2006; Tanaka, 2013; Washkowitz et al., 2012) (Fig. 1 and Fig. 6). *T*, which is expressed in the subridge mesoderm beneath the apical ectodermal ridge (AER), plays a role in the regulation and maintenance of the AER (Liu et al., 2003). *Tbx1* is expressed in the muscle masses of the developing limbs and may play a role in limb myoblast differentiation (Dastjerdi et al., 2007), although no limb abnormalities have yet been identified in mice lacking *Tbx1*. Mutation of *Tbx15* results in mild skeletal abnormalities, notably in the scapular blade, a defect that is exacerbated in *Tbx15/Pax3* compound mutants (Farin et al., 2008). In humans, mutation of *TBX15* results in Cousin syndrome, which includes abnormalities of the limb girdles similar to the defects observed in the corresponding mouse mutant (Table 1). Mutation of the closely related *Tbx18*, which is expressed in the anterior lateral sclerotome and co-expressed with *Tbx15* in the core of the limb bud, results in no apparent limb defects, possibly indicating redundant functions, although defects in the scapular blade are apparent in *Tbx18/Pax3* compound mutants (Farin et al., 2008; King et al., 2006). *Tbx2* and *Tbx3* are both involved in digit development. They are expressed in overlapping regions at the margins of both fore- and hindlimbs and *Tbx3* is also expressed in the AER (Gibson-Brown et al., 1996). Tbx2 controls digit formation by repressing the Bmp antagonist *Grem1* in the posterior limb margin, thereby terminating Fgf4/Shh signaling in the posterior limb (Farin et al., 2013). In line with this, mutation of *Tbx2* results in mild polydactyly (Harrelson et al., 2004). Mutation of *Tbx3*, by contrast, results in more severe limb defects, with lack of development of the footplate and failure to form the posterior elements of the forelimb (Davenport et al., 2003). The situation is somewhat different in humans with mutation in *TBX3*, as only the forelimbs are affected in ulnar-mammary syndrome (Table 1) (King et al., 2006).
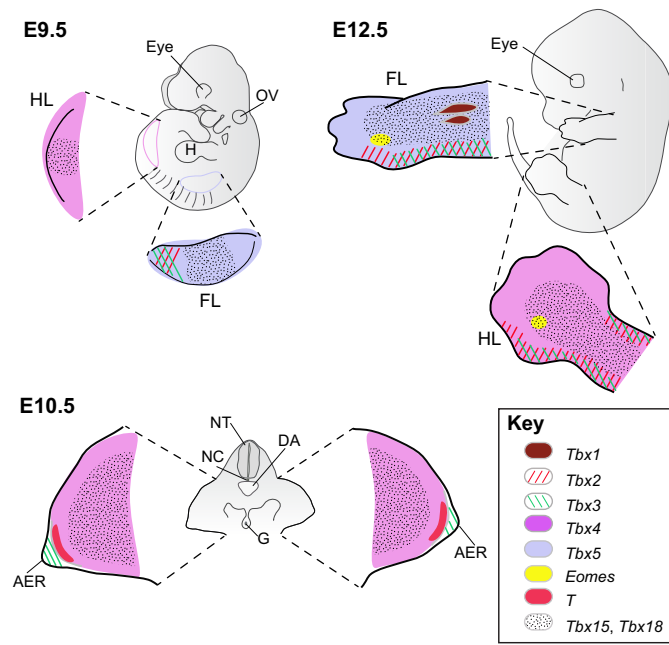
Fig. 6. T-box gene expression during limb development. T-box gene expression is present at the earliest stages of limb outgrowth when *Tbx4* and *Tbx5* are expressed in the hindlimb and forelimb buds, respectively. *Tbx2* and *Tbx3* are first expressed in the posterior margin of the forelimb bud and then in the anterior and posterior margins of both limbs, where they are largely overlapping. In addition, *Tbx3* is expressed in the apical ectodermal ridge and *T* is expressed in the mesoderm immediately subadjacent to the apical ectodermal ridge. *Tbx15* and *Tbx18* are largely overlapping in the core of the limbs, whereas *Tbx1* is expressed in the developing muscle masses. Finally, *Eomes* is expressed in a small domain of mesoderm at the base of the developing fourth digit, although no function has yet been assigned to this area of expression (Hancock et al., 1999). E9.5 and E12.5 are left lateral views and E10.5 is a transverse section through the hindlimbs. For clarity, expression in areas other than the limbs is not shown. AER, apical ectodermal ridge; DA, dorsal aorta; FL, forelimb; G, gut; H, heart; HL, hindlimb; NC, notochord; NT, neural tube; OV, otic vesicle.

The other two members of the Tbx2 subfamily, *Tbx5* and *Tbx4*, show limb expression that is primarily limited to the forelimb or hindlimb, respectively, and are involved in limb initiation and differentiation. In the earliest stages, *Tbx5* is at least partly responsible for the localized EMT of the somatopleure that initiates forelimb bud formation (Gros and Tabin, 2014), although it is not known whether *Tbx4* plays a similar role in the hindlimb region. Both genes regulate Fgf10 in the mesenchyme to establish the Fgf10/Fgf8 positive-feedback loop between mesenchyme and AER that drives limb outgrowth. However, the precise roles for these two genes differ slightly, as *Tbx4* is not exclusively required for the initiation of Fgf10 expression in the hindlimb, whereas *Tbx5* alone is necessary and sufficient to initiate Fgf10 expression in the forelimb. Conditional deletion mutations have shown that each gene is required during a brief period of limb initiation but is not required thereafter to maintain outgrowth, as the Fgf10/Fgf8 signaling loop is self-sustaining. Instead, both genes later play roles in muscle and tendon morphogenesis (Duboc and Logan, 2011; King et al., 2006; Naiche et al., 2005). It has already been mentioned that mutations in *TBX5* result in Holt-Oram syndrome, which is also known as heart-hand syndrome due to abnormalities in development of both the arm and hand. Similarly, mutation in *TBX4* results in the small patella syndrome, which includes a variety of defects in the hindlimbs (Table 1).

Because of their almost exclusive expression in either the fore- or hindlimb, *Tbx5* and *Tbx4* are also candidate determinants of limb type-specific patterning (Logan, 2003). Although ectopic misexpression studies in the chick lend support to this idea, gene deletion and replacement experiments in the mouse indicate that Tbx4 can direct limb development in a forelimb lacking Tbx5 and that the resulting limb has the molecular and morphological characteristics of a forelimb, indicating that Tbx4 does not direct hindlimb-specific morphologies and that Tbx5 is not required for forelimb-specific morphology (Minguillon et al., 2005). Complementary experiments have not been performed in the hindlimb and, although *Tbx4* can partially rescue hindlimb characteristics in a *Pitx1*-deficient hindlimb to a greater extent than *Tbx5*, there is no strong evidence to suggest that the two genes direct limb-specific morphologies (Duboc and Logan, 2011).

T-box genes and stem cell biology
Cells with the stem cell properties of self-renewal and differentiation potential exist either transiently during embryonic development or permanently in adult tissues, thereby providing a means of maintaining homeostasis by renewing or regenerating tissues. These cells may be pluripotent, giving rise to many cell types, or may have a more limited range of differentiation possibilities as progenitors of one or two differentiated cell types. Stem cell lines have been derived from early embryos and include ICM-derived pluripotent embryonic stem cells (ESCs) and epiblast-derived stem cells (EpiSCs), as well as trophoblast-restricted trophoblast stem cells (TSCs). It is also possible to induce stem cell properties by genetic or other manipulation of fibroblast cells *in vitro* to obtain induced pluripotent stem cells (iPSCs). Given their pivotal role in many developmental processes, it is perhaps not surprising that several T-box genes have been implicated either as stem cell factors that promote self-renewal or as differentiation factors that drive the differentiation of stem or progenitor cells (Takashima and Suzuki, 2013) (Fig. 1 and Fig. 7). As outlined below, complementary studies of T-box factors in stem cell lines *in vitro* and in stem/progenitor cell populations *in vivo* have been valuable for understanding the mechanisms of early development and adult tissue homeostasis, as well as furthering the goal of harnessing stem cells for use in regenerative medicine.

The pivotal role of Tbx3 in stem cell renewal and differentiation
Tbx3, which is expressed in the ICM of the pre-implantation embryo (Fig. 4) and in undifferentiated ESCs, has been identified as one of the core transcription factors in the regulatory circuitry of pluripotency that is required to maintain ESCs in a pluripotent state *in vitro*, promoting self-renewal and suppressing differentiation (Ivanova et al., 2006; Lu et al., 2011; Niwa et al., 2009). In the absence of leukemia inhibitory factor (LIF), Tbx3 is sufficient to support the self-renewal of mouse ESCs (Niwa et al., 2009) and it has also been shown to improve the quality of iPSCs, including their germline competence (Han et al., 2010) (Fig. 7A). The maintenance of pluripotency in ESCs involves an interconnected transcriptional network and parallel circuitry that integrates signaling pathways with the core transcription factors, and there is evidence to suggest that the loss of one regulator can be compensated by adjusting the expression of other components (Ivanova et al., 2006). This might explain why the loss of Tbx3, which plays such an important role in ESC maintenance *in vitro*, does not result in a lethal phenotype during peri-implantation development *in vivo* (Davenport et al., 2003).

During the lineage specification and differentiation that occurs *in vitro* following the removal of pluripotency-promoting factors
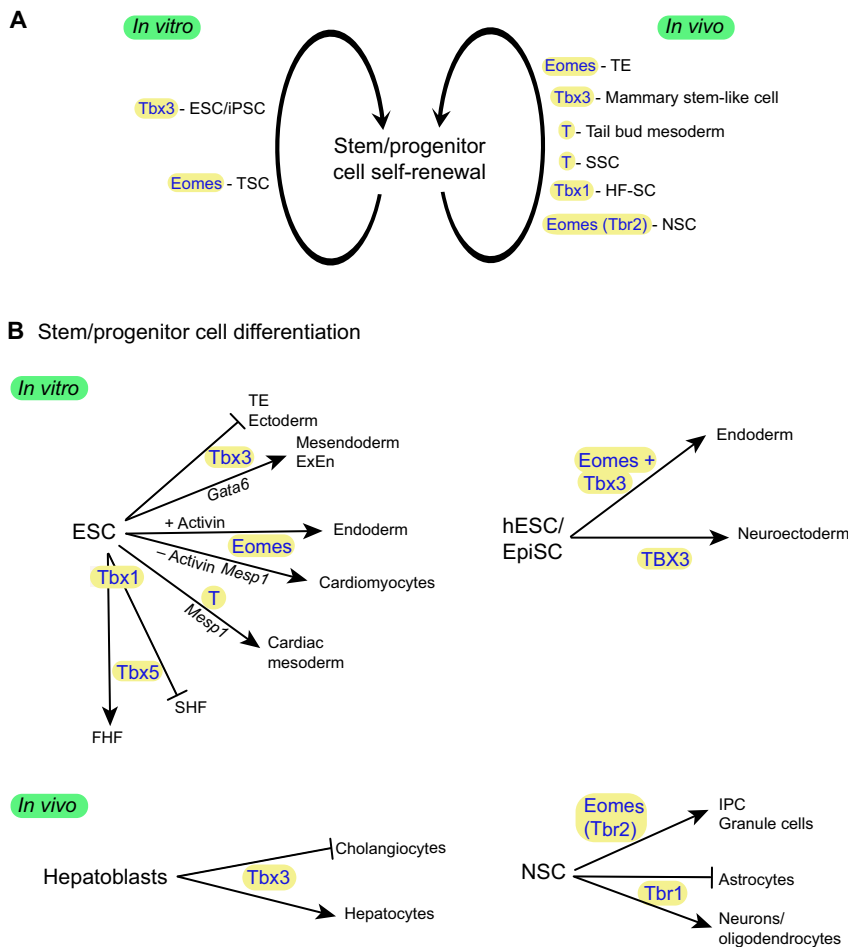
Fig. 7. Roles of T-box genes during stem/progenitor cell self-renewal and differentiation. (A) The self-renewal of stem cell or progenitor cell populations is affected by several T-box transcription factors (highlighted), both in the maintenance of cell lines *in vitro* (left) and in cell populations within the embryo or adult *in vivo* (right). (B) Studies of stem or progenitor cells have shown that T-box transcription factors are also important for driving the differentiation of specific lineages (arrows), sometimes while inhibiting alternative differentiation pathways, both *in vitro* and *in vivo*. Several direct target genes involved in the differentiation pathways are indicated along the arrow path. ESC, embryonic stem cell; EpiSC, epiblast stem cell; ExEn, extraembryonic endoderm; FHF, first heart field; hESC, human embryonic stem cell; HF-SC, hair follicle stem cell; IPC, intermediate progenitor cell; iPSC, induced pluripotent stem cell; NSC, neural stem cell; SHF, second heart field; SSC, spermatogonial stem cell; TE, trophectoderm; TSC, trophoblast stem cell.

from ESCs, Tbx3 takes on different, context-dependent roles (Fig. 7B). It is necessary for mesendoderm and extraembryonic endoderm (ExEn) differentiation, as well as for the suppression of TE and ectoderm differentiation (Lu et al., 2011; Weidgang et al., 2013). Mechanistically, Tbx3 functions through direct binding and epigenetic modification of histones on the promoter of *Gata6*, which encodes an essential regulator of ExEn (Lu et al., 2011). Overexpression of either of the two isoforms of Tbx3 in ESCs results in differentiation and the downregulation of the pluripotency-related transcription factor Nanog, although only the Tbx3+2a isoform directly binds the *Nanog* promoter (Zhao et al., 2014).

Human ESCs (hESCs) present a somewhat different picture, as TBX3 promotes neuroepithelial but not endoderm differentiation during hESC differentiation (Esmailpour and Huang, 2012), possibly reflecting the different states of human and mouse ESCs. However, TBX3 in conjunction with EOMES has been shown to play a non-transcriptional role in the differentiation of DE from hESCs and mouse EpiSCs; during the early steps of differentiation, Tbx3 recruits the histone demethylase Jmjd3 (Kdm6b) to the *Eomes* enhancer to alter chromatin structure, thereby allowing enhancer-promoter interaction and Jmjd2-mediated transcriptional activation of *Eomes*, a mechanism that is conserved in mouse and human ESCs (Teo et al., 2011; Kartikasari et al., 2013). Additional studies are clearly needed to clarify the complex and highly context-dependent functions of Tbx3 in ESCs and differentiation.

In addition to its role in pluripotent stem cells *in vitro*, Tbx3 has been implicated in several stem/progenitor cell populations *in vivo*, including in the liver (Fig. 7B). During liver development,

hepatoblasts are the bipotential progenitors of hepatocytes and cholangiocytes of the bile duct. *Tbx3* is expressed in the developing liver bud and its loss leads to a small liver with both fewer hepatocytes and increased differentiation of cholangiocytes. It is somewhat controversial whether Tbx3 acts through repression of $p19^{ARF}$ (*Cdkn2a*) to promote hepatoblast proliferation (Suzuki et al., 2008) or whether it maintains hepatocyte differentiation and suppresses cholangiocyte differentiation through control of regulatory genes for these two cell types (Ludtke et al., 2009). In another example, overexpression of Tbx3 in the mammary gland of mice causes hyperplasia and accelerated mammary gland development, and is associated with an increased number of cells identified as mammary stem-like cells. In this context, Tbx3 directly represses *Nfκbib*, an inhibitor of the NF-κB pathway that plays a role in cell proliferation (Liu et al., 2011).

Additional T-box genes in stem/progenitor cell populations
As discussed earlier, *Eomes* plays an essential role in TE survival in the pre-implantation embryo but it is also necessary for TSC derivation and self-renewal and for the induction of TSCs from human fibroblasts (Chen et al., 2013; Kidder and Palmer, 2010; Russ et al., 2000). Furthermore, during the differentiation of ESCs, Eomes can promote either endodermal fate or cardiovascular fate depending on high or low levels of Activin/Nodal signaling, respectively (Fig. 7B). The induction of cardiac mesodermal fate in the absence of added morphogens is at least partly controlled by direct regulation of the transcription factor Mesp1 (van den Ameele et al., 2012). *Mesp1* is also directly regulated by T, which appears to

be necessary for robust cardiovascular precursor cell differentiation from ESCs (David et al., 2011).

Differentiating ESCs have been used to investigate the role of T-box genes during heart development; for example, within the FHF and SHF lineages that arise from a common cardiovascular progenitor cell population. Loss of function of *Tbx1*, which is expressed in the multipotent progenitor cell population, results in premature differentiation, whereas gain of function results in reduced differentiation, indicating that *Tbx1* regulates the balance between proliferation and differentiation (Chen et al., 2009). *Tbx5*, which is expressed in the FHF and its derivatives, favors differentiation to the FHF lineage over the SHF lineage in differentiating ESCs, although it apparently cannot drive differentiation as it has no effect on ESC self-renewal; the effect of overexpression only becomes apparent in differentiating ESCs (Herrmann et al., 2011). Furthermore, Tbx5, in combination with several other factors, can reprogram postnatal fibroblasts directly into cardiomyocyte-like cells both *in vitro* and *in vivo* without passing through a stem/progenitor cell state (Fu et al., 2013; Ieda et al., 2010; Inagawa et al., 2012; Qian et al., 2012; Song et al., 2012).

Stem and progenitor cell populations within the intact embryo or adult are more difficult to investigate, although T-box genes are increasingly being identified as regulators of self-renewal or drivers of lineage-specific differentiation in various tissues *in vivo*. For example, in the developing tail of vertebrates, a self-renewing population of mesoderm progenitor cells resides in the tail bud and continuously differentiates throughout the process of somitogenesis (Dubrulle and Pourquie, 2004). In zebrafish, the *T* ortholog *ntl* has been implicated in maintaining this progenitor cell niche through the direct regulation of Wnt ligands and the retinoic acid degradation enzyme gene *cyp26a1* (Martin and Kimelman, 2010). In a different situation, T has been found to be required for the self-renewal of spermatogonial stem cells (SSCs) derived from mouse testes. In this context, *T* is directly regulated by Etv5, one of the transcription factors involved in regulating SSCs (Wu et al., 2011).

In another example, Tbx1 was identified as a factor affecting the self-renewal of hair follicle stem cells (HF-SCs) in an RNA interference screen to identify regulators of self-renewal capacity, possibly acting through repression of Bmp signaling. Loss of Tbx1 diminishes HF-SC renewal capacity resulting in eventual thinning of the hair (Chen et al., 2012).

Eomes, as well as *Tbr1*, also plays a role in the differentiation of neural stem cells (NSCs) in developing and adult brains. *Tbr1* regulates neuronal output from NSCs in the olfactory bulb by promoting the production of neurons and oligodendrocytes and inhibiting the formation of astrocytes (Mendez-Gomez et al., 2011). Similarly, although not directly affecting the self-renewal capacity of radial glial cells (RGCs) within the developing cerebral cortex, Eomes is a key determinant of the differentiation of intermediate progenitor cells (IPCs), which comprise the RGC daughter transit amplifying progenitor population (Arnold et al., 2008b; Sessa et al., 2008). The transition from NSC to IPC is regulated by miR-92a through post-transcriptional regulation of *Eomes* (Bian et al., 2013). *Eomes* is also a crucial regulator of granule cell formation from NSCs in the dentate gyrus, both during development and in the adult. In this case, *Eomes* is also thought to play a feedback role in regulating NSCs, as in its absence the NSC pool increases, possibly through regulation of the NSC factor Sox2 (Hodge et al., 2012).

Finally, it has been shown that *Tbx18* transduction into the adult guinea pig heart using an adenoviral vector results in the direct conversion of ventricular myocytes to pacemaker cells without the need for passage through a pluripotent state (Kapoor et al., 2013) – a case of direct reprogramming that could have implications for the use of T-box genes in directed differentiation.

Conclusions

There are many other organs and tissues in which T-box genes have major effects that have not been covered here (e.g. craniofacial development, mammary gland development, immune T cells, lung development; see Table 1), but from this sampling of some of the developmental processes controlled by T-box genes it should be clear that this ancient family of transcription factor genes has a multitude of diverse functions throughout development and that, throughout the evolution of metazoans, some ancient functions have been conserved while novel ones have evolved with the evolution of complexity. It is also evident that T-box genes are involved in all of the major developmental signaling pathways, and we have seen how multiple T-box genes with both unique and overlapping functions can affect the development of a single organ and how a single T-box gene can play roles in different tissues and/or at different times in development. This tissue and/or temporal specificity is accomplished in part by tight regulation of the expression of T-box genes, by the availability of co-factors, by the presence of other T-box proteins, and by the location and binding specificity of TBEs in different promoters. With the availability of conditional mutant alleles in mice, this complexity is gradually being elucidated and functions are being assigned to each area of expression, although there is still much work to be done. The regulation of T-box gene expression is an area ripe for exploration, as is the important issue of determining the battery of downstream targets affected in different developmental contexts. Finally, through the study of non-mammalian organisms, this highly conserved family offers a rich source of material that can further our understanding of the evolution of developmental mechanisms.

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