T-Box Genes in Vertebrate Development

L.A. Naiche,[1] Zachary Harrelson,[1]
Robert G. Kelly,[1,2] and Virginia E. Papaioannou[1]

[1]Department of Genetics and Development, College of Physicians and Surgeons of Columbia University, New York, New York 10032; email: nla2@columbia.edu, zh42@columbia.edu, vep1@columbia.edu

[2]Developmental Biology Institute of Marseilles, Campus de Luminy Case 907, 13288 Marseille Cedex 9, France; email: kelly@ibdm.univ-mrs.fr

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Key Words

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Abstract

The myriad developmental roles served by the T-box family of transcription factor genes defy easy categorization. Present in all metazoans, the T-box genes are involved in early embryonic cell fate decisions, regulation of the development of extraembryonic structures, embryonic patterning, and many aspects of organogenesis. They are unusual in displaying dosage sensitivity in most instances. In humans, mutations in T-box genes are responsible for developmental dysmorphic syndromes, and several T-box genes have been implicated in neoplastic processes. T-box transcription factors function in many different signaling pathways, notably bone morphogenetic protein and fibroblast growth factor pathways. The few downstream target genes that have been identified indicate a wide range of downstream effectors.

Contents

INTRODUCTION

TBE: T-box binding element

The T-box family, defined by a common DNA binding domain known as the T-box, is evolutionarily ancient, probably arising in the common ancestor of metazoan organisms (2) (**Figure 1**). T-box genes first came to the attention of geneticists in 1927 with the discovery of a mutation, *Brachyury* (or *T*, for short-tail), which caused truncated tails in mice (30). In recent years, both spontaneous and induced mutations in T-box genes have demonstrated that these genes are important regulators of a wide range of tissues and organs during development, as well as major contributors to several human syndromes (**Table 1**). As this family was discovered quite recently, comparatively little is known about transcriptional regulatory capabilities and signaling interactions of its members. Nonetheless, its importance in an array of developing tissues has led to the rapid expansion of the field. In this review, we explore recent literature on T-box gene function, concentrating on mammalian development.

T-BOX TRANSCRIPTION FACTORS

T-Box Proteins and DNA Binding

The T-box DNA binding sequence, the T-site or T-box binding element (TBE), was first defined as the sequence with the highest affinity for Brachyury (57). Brachyury binds this palindromic sequence as a dimer (77), with each monomer of Brachyury binding half of the sequence, or T-half site (5′-AGGTGTGAAATT-3′). Extensive studies have demonstrated that all T-box proteins tested are capable of binding the T-half site as monomers (15, 49, 61, 77, 78, 97, 98), although some have different optimal target sequences (36, 65). Comparisons between T-box proteins have shown preference for different synthetic combinations of T-half sites in varying orientations, numbers, and spacing (27, 97), which may help create promoter specificity for target genes.

The crystal structures of both Brachyury and TBX3 T-domain homodimers bound to the canonical T-site have been elucidated (26, 71). Both T-box proteins make the same DNA contacts with the same amino acids, indicating strong conservation of the underlying DNA binding functions between T-box subfamilies. However, whereas the Brachyury dimer is stabilized by a hydrophobic patch and a salt bridge, TBX3 dimers are oriented differently on the DNA and are weakly connected. These differences in ternary structure probably underlie the differences in half site preference among different T-box family members.

Transcriptional Regulation

As well as binding DNA, T-box genes have been shown to regulate transcription. Activation domains have been mapped to the

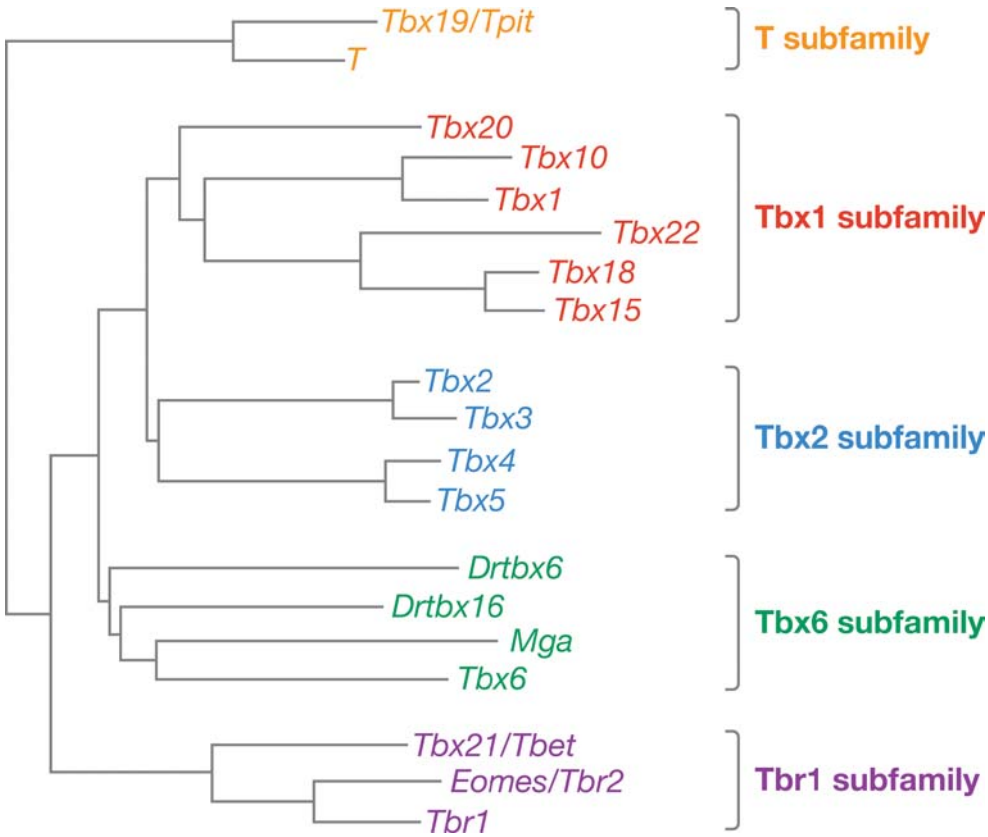

Figure 1

Schematic phylogenetic tree of the T-box gene family of vertebrates, based on the phylogenetic analysis in Reference (76) showing the relationship of genes in the five subfamilies indicated by brackets on the right. All of these genes are present in mammals with the exception of the zebrafish genes *Drtbx6* and *Drtbx16*, which do not have orthologs in mammals.

C-terminal domains of several T-box proteins (56, 98, 120). In some cases, the mechanism of activation is known: Tbx19 activates transcription by recruiting SRC/p160 coactivators to the promoter (68), while Tbr1 forms a complex with nucleosome assembly proteins (112). T-box proteins can also repress transcription, as has been shown for Tbx2 and Tbx3 (21, 41, 65). Some T-box genes contain both activation and repression domains in their C-terminal domains (56, 98) and Tbx2 has been reported to act in either fashion, depending on promoter context (78).

Interactions with Other Transcription Factors

Athough some T-box gene targets appear to be regulated by T-box proteins alone (60), there is a growing body of work demonstrating that target genes are controlled in combination with other transcription factors. Cooperative binding of promoters and synergistic upregulation of target gene expression is seen with T-box factors and homeodomain (13, 61, 98), GATA zinc finger (31, 35, 98), and LIM domain proteins (59). Frequently, these interactions enhance target gene activation, and

Table 1 Comparison of the effects of mutations in all known human and mouse T-box genes illustrating the prevalence of dosage sensitivity of the phenotypes[a]

Mouse gene; human gene	Human syndrome	Mouse heterozygous phenotype	Mouse homozygous phenotype
T ; *T*	Not known	Viable, short/no tail (30)	Embryonic lethal, failure of posterior mesoderm (45)
Tbx19 ; *TBX19 (TPIT)*	Recessive isolated ACTH deficiency (84)	Normal (84)	ACTH deficiency, pigment defects (84)
Tbx1; *TBX1*	DiGeorge, craniofacial, glandular, vascular, and heart abnormalities (4)	Viable, thymus and vascular abnormalities (52, 64)	Neonatal lethal; craniofacial, glandular, vascular, and heart abnormalities (52, 64)
Tbx10; *TBX10*	Not known	Susceptibility to cleft lip and palate (*Dancer*: ectopic gain-of-function) (17)	Cleft lip and palate (*Dancer*: ectopic gain-of-function) (17)
Tbx15; *TBX15*	Not known	Normal (19)	Craniofacial viable, malformations and pigment pattern alterations (*droopy ear*) (19)
Tbx18; *TBX18*	Not known	Normal (18)	Postnatal lethal, vertebral malformations (18)
Tbx20; *TBX20*	Not known	Heart contractile function defects (99)	Embryonic lethal, heart abnormalities (99)
Tbx22; *TBX22*	X-linked cleft palate with ankyloglossia (11)	Not known	Not known
Tbx2; *TBX2*	Not known	Normal (43)	Embryonic lethal, heart and limb abnormalities (43)
Tbx3; *TBX3*	Ulnar-mammary: hypoplastic mammary glands, abnormal external genitalia, limb abnormalities (5)	Hypoplastic mammary glands, abnormal external genitalia (28, 53)	Embryonic lethal, yolk sac, limb and mammary gland defects (28)
Tbx4; *TBX4*	Small patella (10)	Reduced allantois growth rate (72)	Embryonic lethal, allantois and hindlimb defects (72)
Tbx5; *TBX5*	Holt-Oram, heart and hand abnormalities (7)	Heart abnormalities, reduced viability (14)	Embryonic lethal, severe heart malformations (14)
Tbx6; *TBX6*	Not known	Normal (24)	Embryonic lethal, somite abnormalities (24)
Tbr1; *TBR1*	Not known	Normal (16, 46)	Olfactory bulb and cortical defects (16, 46)
Eomes; *EOMES*	Not known	Normal (89)	Embryonic lethal, trophoblast and mesoderm failure (89)
Tbx21; *TBX21 (TBET)*	Not known	Airway hyperresponsiveness, intermediate INF-γ levels in Th1 cells (32, 104)	Airway hyperresponsiveness, no Th1 cells (32, 104)

[a]Text colors indicate genes in different subfamilies as indicated in **Figure 1** (see text for additional references).

probably contribute to promoter specificity. Some of these interactions can be generalized to transcription factor subfamilies—both T subfamily (but not Tbx1 subfamily) proteins directly interact with all members of the Pitx family (but not the closely related Otx family) of homeodomain proteins (61). Some of these interactions are exquisitely specific—Tbx20 interacts with GATA5 but not the related transcription factor GATA4 (98). In an even more extreme case of specificity, LMP4 binds both of the most closely related vertebrate T-box

proteins, Tbx4 and Tbx5, but interacts with each via a different LIM domain repeat (59).

The observed in vitro interactions have biological relevance. Mutations in *TBX5* cause Holt-Oram syndrome (HOS), which results in multiple heart defects. While some patients have truncation mutations in *TBX5* that result in loss of DNA binding or activation, others have only point mutations. Analyses of such mutant proteins have shown that loss of interaction with cardiac transcription factor NKX2-5 is sufficient to cause disease, even when the mutant TBX5 is otherwise intact (31). Likewise, point mutations in NKX2–5, which ablate TBX5 binding, have been shown to cause heart disease in humans (35), further demonstrating the biological requirements for cooperative binding between T-box proteins and other factors.

Predicting T-Box Gene Function

Reliable prediction of T-box gene function is complicated by considerable functional lability. Depending on context, T-box proteins may homodimerize, heterodimerize, or cooperatively bind other transcription factors. Individual T-box proteins can exhibit either activation or repression activities, and single genes may do both depending on promoter context. In some cases, one T-box protein is capable of competing off another at a particular promoter (40, 41), making it difficult to predict which is relevant to a particular target gene in cells where the T-box genes are coexpressed.

Several labs have attempted to inhibit individual T-box gene function with putative dominant negative proteins, e.g., a truncated version containing only the T-box domain (29, 87, 90, 101, 106a). The action of these engineered proteins may be complex and may influence other T-box genes or targets, so caution must be used when interpreting such experiments. Although dominant negative proteins may be specific, in some cases a presumed dominant negative protein produces phenotypic consequences more severe (93) or different (90) from what is observed in the genetic null, indicating interference with other protein(s).

HOS: Holt-Oram syndrome

TE: trophectoderm

dpc: days post coitus

T-BOX GENES DURING EARLY DEVELOPMENT

T-box gene expression is widespread during embryonic development and has been noted in all stages, from the oocyte (12, 115) to the adult (102). In the early embryo, T-box genes are required for both evolutionarily ancient processes, such as gastrulation [recently reviewed by (94)] and comparatively recent developments such as uterine implantation and umbilicus formation.

Roles in Extraembryonic Tissues

The earliest demonstrated role of T-box genes in mammalian embryos is that of *Eomesodermin*. In the mouse embryo, the first lineage decision occurs when the outer cells of the morula differentiate into the trophectoderm (TE), which will form placental structures. TE dramatically upregulates *Eomesodermin*. In the absence of *Eomesodermin*, TE cells are defective and neither proliferate in vitro nor participate in uterine implantation in vivo (89), resulting in embryonic death shortly after implantation.

Brachyury is expressed in the caudal primitive streak and is responsible for posterior mesodermal development. *Brachyury* expression extends into the base of the allantois, an extraembryonic mesodermal outgrowth that will eventually become the umbilical cord. Deletion of *Brachyury* leads to stunted growth of the allantois; however, this is likely secondary to a more general defect in mesoderm production (8). In contrast, *Tbx4* is expressed in the allantois and has allantois-specific effects when mutated. Specifically, *Tbx4* is expressed at the site of origin of the allantois and continues to be expressed in the allantois and umbilical cord through at least 13.5 days post coitus (dpc). Embryos homozygous for a null mutation in *Tbx4*

BMP: bone morphogenetic protein

FGF: fibroblast growth factor

PSM: presomitic mesoderm

have multiple abnormalities in the allantois, including upregulated apoptosis, failure to undergo characteristic morphological changes, and failure to express known markers of allantois differentiation such as *Tbx2* and *VCAM1*. Loss of *Tbx4* also disrupts allantoic vasculogenesis after endothelial cells differentiate from the allantoic mesoderm, but before they coalesce into patent blood vessels (72). Little is known about the growth or patterning of the allantois, so it is difficult to firmly tie the diverse defects into known signaling pathways. Mutation of the bone morphogenetic protein (BMP) *Bmp4* in the epiblast causes similar allantois defects (33), but *Bmp4* is expressed normally in *Tbx4* mutants, suggesting that *Tbx4* may be a downstream effector of BMP signaling in this tissue.

Tbx3 is expressed in the yolk sac in both endoderm and mesoderm layers. Disruption of *Tbx3* results in an aberrant yolk sac with variably diminished vascular development and a highly apoptotic endoderm layer (28). It is unclear whether the primary defect is in one or both layers and it is possible that these defects are secondary to heart defects in the embryo (Z.H., R.G.K., V.E.P., unpublished).

T-Box Gene Function in Mesoderm

Many T-box genes serve important functions during mesoderm formation and patterning in the vertebrate gastrula: *T* and *Eomesodermin* in the mouse; *Xbra*, *Xeomesodermin*, and *XvegT* in *Xenopus*; *no tail*, *spadetail*, *eomesodermin*, and *tbx6* in zebrafish (75). These genes are critical for mesoderm formation at various axial levels and regulate such key factors as fibroblast growth factor (FGF) signaling and cell migration. However, T-box gene functions during gastrulation are recursive and overlapping, creating a complex web of developmental roles and signaling interactions. This field has been recently reviewed (94).

The role of T-box genes in patterning the embryonic mesoderm, however, is not restricted to gastrulation. Several T-box genes are involved in the patterning of nascent mesoderm following its ingression through the primitive streak. *Tbx6* is expressed in the paraxial, presomitic mesoderm (PSM) of the mouse embryo after its exit from the primitive streak (22a). Embryos homozygous for a null mutation in *Tbx6* die mid-gestation. Rostral somites are present but morphologically abnormal, indicating that *Tbx6* is not absolutely required for somite formation (24). However, the posterior embryo is progressively more affected: Caudal somites are replaced with ectopic paraxial neural tubes, and the tail bud, the ongoing source of new mesoderm, is aberrantly expanded. The *Tbx6* homozygous mutant phenotype lends itself to two nonexclusive interpretations. First, the presence of ectopic neural tubes in place of caudal somites suggests that *Tbx6* is required to promote a mesodermal fate in posterior paraxial tissue, and that in its absence a default neural program predominates. In vivo teratoma analysis revealed a conspicuous absence of skeletal muscle in tumors derived from *Tbx6* null tail bud cells, implying a potential requirement for myogenic specification or differentiation. In vitro differentiation assays, however, show the lack of an absolute requirement for *Tbx6* in myogenesis (22). A second potential explanation for the *Tbx6* mutant phenotype concerns the enlarged tail bud. Levels of cellular proliferation and programmed cell death are unaltered in *Tbx6* null tail buds (22), suggesting that *Tbx6* mutant mesoderm may fail to migrate out of the tail bud. Although this hypothesis is not incompatible with a role for *Tbx6* in mesodermal specification, the formation of ectopic neural tubes might result from an insufficient supply of mesoderm to the paraxial regions as a secondary defect of deficient migration from the tail bud.

Additional information on the role of *Tbx6* comes from transgenic rescue experiments and a naturally occurring *Tbx6* mutation called *rib-vertebrae* (*rv*) (113, 114). Homozygous *rv* mutant embryos, as well as null *Tbx6* mutant embryos rescued with a transgene that partially restores *Tbx6* expression, live past

mid-gestation only to display later defects in rib and vertebrae development. These abnormalities are preceded by reduced expression of markers of the anterior compartment of the somite, with reciprocally expanded expression of markers normally restricted to the posterior somite (114), showing that *Tbx6* is required to maintain the anterior compartment. *Tbx6* is therefore required not only to specify somite formation in the paraxial mesoderm, but also for somite patterning. Severity of the vertebral defects increases as levels of *Tbx6* are progressively reduced with *rv* and *Tbx6* null alleles, implicating dosage as an important aspect of *Tbx6* function in later somite development (113).

Similar to *Tbx6*, *Tbx18* is required to maintain the fate of the anterior compartment of somites. Homozygous *Tbx18* null mutant mice die perinatally and display a range of rib and vertebrae defects. Molecular analysis of these mutant embryos shows that, in the absence of *Tbx18*, anterior and posterior somite compartment specification occurs correctly in the PSM but fails to be maintained during somite maturation (18). Studies in zebrafish (9), chick (42a, 107), and mouse (58) show that *Tbx18* is expressed in the anterior region of somites. Although the mechanism through which this expression is achieved varies between species, all studies agree that *Tbx18* transcription is initiated in the PSM and progressively restricted to the anterior compartment as each somite matures. PSM injected with a *Tbx18* expression vector can induce the formation of somite boundaries when grafted into PSM caudal to where *Tbx18* is normally expressed, suggesting a role for the gene in somite segmentation (107).

tbx24 in zebrafish is a third T-box gene directly involved in the regulation of vertebrate somite development. *tbx24* does not cluster with any of the T-box gene subfamilies and there is no mammalian ortholog. Expression is first detected in paraxial mesoderm of the zebrafish gastrula and is later confined to the anterior and intermediate PSM. Morpholino-mediated *tbx24* knockdown experiments yield embryos with morphological and molecular evidence of disrupted somite segmentation. An early role in mesoderm specification is unlikely as mesodermal and neural markers are expressed normally in *tbx24* morphants. Rescue experiments show that the naturally occurring *fused somites* phenotype is caused by mutations in *tbx24* (74).

IFT: inflow tract

OFT: outflow tract

T-BOX GENES DURING ORGANOGENESIS

Complex Functions During Cardiogenesis

Many T-box genes are expressed in specific chambers or regions of the developing vertebrate heart, including *Tbx1*, *Tbx2*, *Tbx3*, *Tbx5*, *Tbx18*, and *Tbx20* (75). Despite overlapping expression patterns (**Figure 2**), experimental studies reveal that each gene has unique developmental functions. The identification of cardiac transcriptional binding partners with differential affinities for individual T-box factors has contributed to understanding how the combined regulatory influences of multiple, related and coexpressed genes generate unique downstream target gene expression patterns during organogenesis.

Targeted mutagenesis in mouse has revealed essential roles for *Tbx5*, *Tbx1*, *Tbx2*, and *Tbx20* in cardiac development. Mouse embryos homozygous for a null mutation in *Tbx5* exhibit abnormal development of posterior heart structures, including hypoplastic left ventricle, atria, and inflow tract (IFT) (15). These defects are accompanied by the reduced expression of critical cardiac genes, including *GATA4*, *MLC2v*, and *Irx4* (15). Ectopic expression of *Tbx5* in both chick and mouse also implicates *Tbx5* in the development and positioning of the interventricular septum (106). Homozygous loss of *Tbx1* produces abnormalities of anterior cardiac development, including shortening of the outflow tract (OFT), the absence of OFT septation, ventricular septal defects, and abnormal remodeling of the aortic arch arteries

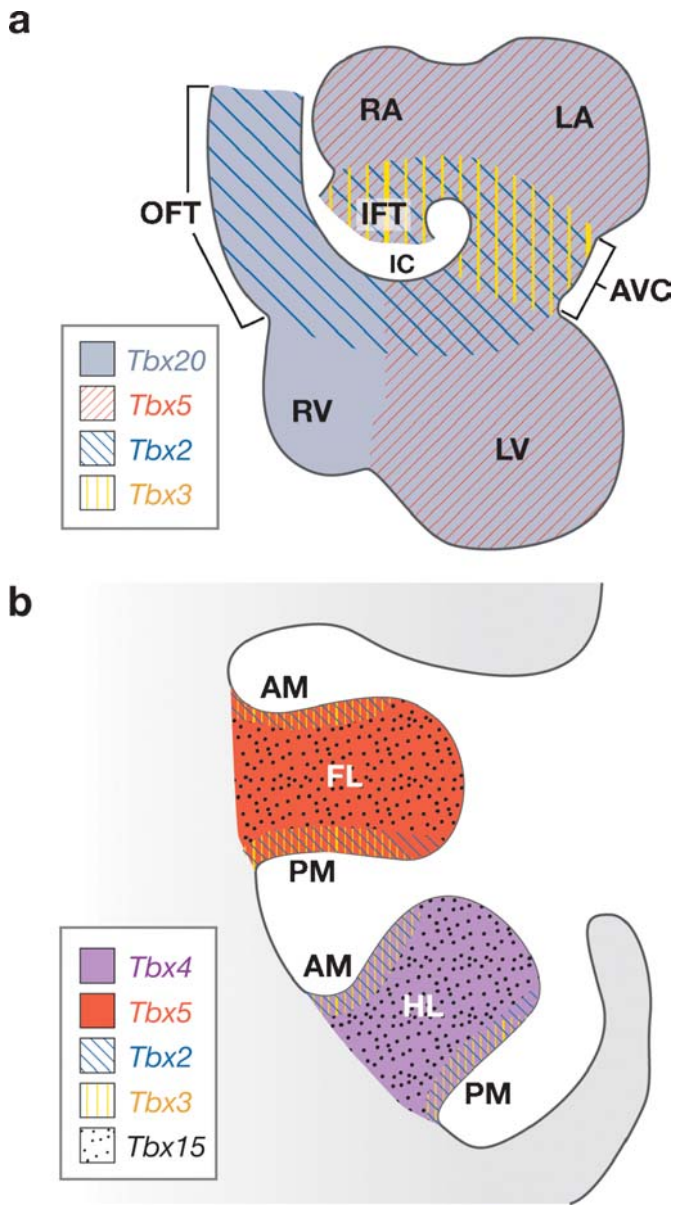

Figure 2

Diagrams of overlapping T-box gene expression in selected organs. (*A*) Cardiac T-box gene expression in 9.5 dpc–10.5 dpc embryos. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; AVC, atrioventricular canal; OFT, outflow tract; IFT, inflow tract; IC, inner curvature. (*B*) Limb T-box gene expression in 10.5–12.5 dpc mouse embryos. FL, forelimb; HL, hindlimb; AM, anterior margin; PM, posterior margin.

(52, 64, 109). Conversely, ectopic expression of *Tbx1* in the heart tube can lead to an elongated OFT (50). *Tbx1* mutants display reduced proliferation in the anterior heart field (AHF), which contributes to the OFT (116), demonstrating a role in proliferation of cardiac progenitor cells. Embryos homozygous for a null mutation in *Tbx2* reveal a role for the gene in repressing chamber differentiation in the atrioventricular canal (AVC) during functional specialization of the ventricular and atrial compartments. Several chamber-specific markers are ectopically expressed in the AVC of *Tbx2* homozygous mutants, including *Nppa* and *Cx40* (43). In a complementary experiment, the expression of these genes is undetectable when *Tbx2* is ectopically expressed throughout the heart tube (25). Many *Tbx2* homozygous null mutants also exhibit defects in OFT septation and remodeling of the aortic arch arteries (43). Homozygous null *Tbx20* mutants die midgestation due to defective hearts, which fail to loop and which display many morphological and molecular abnormalities, including widespread upregulation of *Tbx2*. The presence of two poorly developed chamber-like structures and the reduction of *Nppa* expression suggests that *Tbx20* plays a role during cardiac chamber formation in the early heart tube (18a, 96a, 99). Together, T-box genes therefore control development of most regions of the heart and regulate multiple aspects of cardiogenesis including chamber differentiation, suppression of differentiation in nonchamber regions, and cell proliferation.

As in the case of *Tbx4* in the allantois, T-box genes in the heart appear to be regulated by members of the BMP family. Bead implantation experiments in chick show that BMP2 can promote *Tbx2* and *Tbx3* expression (117). Chick explant cultures confirm that *Tbx2* and *Tbx3* expression can be enhanced by the presence of BMP2, whereas *Tbx2* message is reduced in the presence of the BMP inhibitor, noggin. Furthermore, cardiac *Tbx2* expression is greatly reduced in homozygous *Bmp2* mouse mutants (117). In a second pathway

common to several T-box genes [see (94) and next section], *Tbx1* regulates cardiac FGF signaling. *Fgf8* and *Fgf10* expression is reduced in the AHF of embryos with reduced or absent *Tbx1* expression (50, 116). A genetic interaction between *Tbx1* and *Fgf8* is also indicated by an increase in the frequency and severity of aortic arch artery remodeling defects in doubly heterozygous mutants compared to single heterozygotes (110). Recent work suggests that T-box genes also have the capacity to regulate their own expression. T-half sites in the *Tbx5* promoter were protected in fingerprinting assays after incubation with nuclear extracts, suggesting that T-box factors bind these endogenous sites. Furthermore, transfection of *Tbx5* activates a *Tbx5* expression reporter in cultured cell lines (100), supporting a potential auto-regulatory or T-box gene cross-regulatory loop.

The regulation of *Nppa* transcription during cardiac chamber formation provides an interesting example of how multiple inputs from T-box factors become integrated to generate a complex expression profile. The *Nppa* promoter contains Nkx binding elements (NKE) for the homeodomain factor and cardiac lineage marker Nkx2-5, in addition to several T-half sites (15, 41, 47). Biochemical studies show that Tbx5 and Nkx2-5 not only interact with each other (47), but also specifically and cooperatively bind the *Nppa* promoter to synergistically activate *Nppa*-reporter expression (15, 47). The success of this regulatory influence depends on intact NKEs and TBEs (15, 41). Tbx2, a demonstrated transcriptional repressor, is also capable of cooperatively binding the *Nppa* promoter with Nkx2-5. Tbx5-Nkx2-5–mediated activation of the *Nppa*-reporter is incrementally repressed by increasing amounts of Tbx2 (41). In the 9.5 dpc mouse embryo, *Tbx2* is expressed in the AVC (25, 41, 43) within a subdomain of the cardiac expression domain of *Tbx5* (23) (**Figure 2**). Considered together, this information has led to the hypothesis that *Nppa* expression is regulated by the competing interactions of Tbx5 and Tbx2 with Nkx2-5 and the T-half sites in the *Nppa* promoter (41). The progressive reduction of *Nppa* transcription in *Tbx5* heterozygous and homozygous null mutants (15) and the ectopic *Nppa* expression in the AVC of *Tbx2* homozygous mutants (43) support this hypothesis. In vitro reporter assays show that *Tbx3*, which is also expressed in the AVC of 9.5 dpc mouse embryos, can repress Tbx5-Nkx2-5–mediated activation of *Nppa* (48). Tbx20 can similarly interact with Nkx2–5 and synergistically activate reporter expression driven from a *Cx40* promoter fragment or a synthetic promoter containing only NKEs (98). Thus, Tbx5, Tbx2, Tbx3, and Tbx20 all potentially participate in the global regulation of *Nppa* expression, and their interactions are likely to control other aspects of cardiac development.

Cardiac development, like somitogenesis, is also sensitive to the dosage of T-box gene activity. Elimination of *Tbx5* leads to embryonic lethality at 9.5 dpc preceded by severe hypoplasia of posterior cardiac structures (15). Diminished *Tbx5* activity in heterozygous embryos leads to less severe cardiac abnormalities, including conduction and septal defects that contribute to compromised survival varying with genetic background. *Tbx5* heterozygous mutants exhibit a reduction in *Nppa* expression that is intermediate between wild-type and homozygous mutant embryos (15), demonstrating a correlation between gene dosage and target gene expression levels. Similar effects are observed in *Tbx1* mutant phenotypes. *Tbx1* heterozygous null mutants lack fourth arch arteries at 10.5 dpc, a defect less severe than that found in homozygous mutants (52, 64). Furthermore, combinations of null and hypomorphic mutations comprising an allelic series demonstrate differential sensitivity to *Tbx1* dosage. Small reductions in *Tbx1* dosage are sufficient to produce incompletely penetrant aortic arch artery defects. Progressively greater reductions in dosage increase the severity and frequency of arch artery remodeling defects and OFT septation anomalies (50). The range of developmental responses to varying

AHF: anterior heart field

AVC: atrioventricular canal

NKE: Nkx binding element

GENE DOSAGE

The first T-box gene mutation was discovered by virtue of a phenotype affecting the tail of heterozygous newborns concomitant with the failure of homozygous mice to survive to birth, demonstrating a semidominant mode of inheritance (30). All of the known autosomal human T-box gene syndromes, with the exception of the recessive isolated ACTH deficiency, occur in heterozygous individuals, whereas homozygotes have not been identified, presumably due to embryonic lethality. In mice, heterozygous effects have been identified for half of the genes mutated, but because these defects are sometimes quite subtle, further investigations may yet identify additional examples of dosage sensitivity. Mouse studies indicate that the full spectrum of tissues affected in homozygous mutants is not necessarily affected by haploinsufficiency. In comparing human and mouse phenotypes, there is a high degree of similarity in tissues affected and the nature of the mutant defects. However, the dose sensitivity may vary considerably—leading to examples of genes that have much more severe or extensive heterozygous phenotypes in humans than that observed in mice (**Table 1**).

T-box protein levels supports the idea that dosage-sensitivity is a common theme within the T-box gene family (see sidebar).

Limb Outgrowth and Patterning

Limb development has long been a major interest of T-box gene researchers. As early as 1996 it was noted that all four members of the Tbx2 subfamily are expressed during limb development in provocative expression domains (37) (**Figure 2**). *Tbx4* and *Tbx5* are expressed in similar patterns but in different limbs—the hindlimbs and forelimbs, respectively. Their expression initiates in the limb fields before morphological limb buds are apparent, and is maintained in their respective limbs until late in development. One of the only other genes known to be expressed specifically in one set of limbs from such an early stage is *Pitx1*, which is expressed only in the hindlimbs (62, 92), and which has been shown to cooperatively interact with another T-box gene, *Tbx19*, in the pituitary (see below). Thus, it was widely hypothesized that *Tbx4* and *Tbx5* conferred hindlimb and forelimb identities, possibly in cooperation with *Pitx1*. Ectopic expression studies in chick reinforced this hypothesis. Electroporation of *Tbx4* into the forelimb or *Tbx5* into the hindlimb produced a variety of malformations, some of which suggested transformation to opposing limb fates (88, 105). However, these experiments were complicated by the presence of the endogenous T-box gene activity. Electroporation of *Tbx4* or *Tbx5* into the limb where each is normally expressed also created severe abnormalities, further confounding analysis and again demonstrating the dosage sensitivity characteristic of this gene family.

Mutations in both *Tbx4* and *Tbx5* are associated with dominant limb defect syndromes in humans: Heterozygous mutations in *TBX4* cause small patella syndrome, characterized by minor hip, knee, and foot defects (10); heterozygous mutations in *TBX5* cause HOS, characterized by moderate-to-severe arm defects in addition to heart abnormalities (7). Targeted mutations in mouse have shown that *Tbx4* and *Tbx5* play similar, but not identical, roles in limb outgrowth. In *Tbx5* homozygous mutants, the forelimb field is defined and appropriate anterior/posterior and dorsoventral patterning is established (1). However, *Fgf10*, a gene critical for bud formation and outgrowth, is not expressed in the forelimb bud mesenchyme and a morphological bud is never observed. In *Tbx4* homozygous mutants, limb bud formation progresses somewhat further (72). The hindlimb bud of *Tbx4* mutant embryos is morphologically evident and mesenchymal and ectodermal FGF signaling is initiated. *Fgf10* is rapidly lost in the mesenchyme, however, and the limb does not progress beyond the bud stage. The mechanism behind the differences between *Tbx4* and *Tbx5* is not known.

The failure of limb outgrowth prevented assessment of forelimb/hindlimb identity in the *Tbx4* and *Tbx5* null embryos. A recent study using a conditional *Tbx5* allele in

combination with limb-specific recombinase transgenes has definitively negated the role of these genes in determining limb identity (70). *Tbx4* misexpressed in the forelimb field is capable of rescuing *Tbx5* homozygous mutant forelimb outgrowth, demonstrating functional overlap between these two genes. However, the *Tbx4*-rescued limbs are forelimb-like and show no morphological or molecular hindlimb characteristics. Conversely, misexpression of *Pitx1* with either *Tbx4* or *Tbx5* in the forelimb induces hindlimb-like morphology, indicating that *Pitx1* is indeed a regulator of fore- versus hindlimb identity, whereas *Tbx4* and *Tbx5* are not.

Tbx2 subfamily members *Tbx2* and *Tbx3* are also expressed in the developing limb in largely overlapping domains along the anterior and posterior margins of the limb mesenchyme (37, 38). In chick, *Tbx2* is restricted slightly more posteriorly than *Tbx3* in the posterior limb margin, and is also expressed in the apical ectodermal ridge (AER) at the distal limb margin. Overexpression and dominant negative studies in chick have shown that both *Tbx2* and *Tbx3* induce posterior digit identities, with *Tbx2* having the stronger posteriorizing activity (101). In the mouse and human, both the expression patterns and roles of *Tbx2* and *Tbx3* appear to be reversed relative to chick. In mouse, *Tbx3* is more restricted in the posterior margin and is expressed in the AER, while *Tbx2* is not in the AER (37). In contrast to the chick studies, genetic nulls of each gene in mouse indicate that *Tbx3* is required for development of the posterior margin of the limb (28), while *Tbx2* has only minor effects (43). In humans, *TBX3* is associated with ulnamammary syndrome (5), which causes defects in posterior limb formation, but no mutations have been identified for *TBX2*.

In addition to the Tbx2 subfamily genes, *Tbx15*, a member of the Tbx1 subfamily, is also expressed in limb mesenchyme in a pattern complementary to the expression domains of *Tbx2* and *Tbx3* (3, 96). Mice mutant for *Tbx15* are viable but have widespread minor defects in the skeletal elements of the limbs, characterized by abnormally short and thin bones with poor articulation. These defects may be caused by diminished proliferation in the early limb bud resulting in smaller precartilaginous mesenchymal condensations. Proliferation is reduced only in the core limb mesenchyme, where *Tbx15* is expressed, but is normal in the limb margins, suggesting that *Tbx2* and/or *Tbx3* may maintain proliferation levels in the latter tissues (see sidebar).

AER: apical ectodermal ridge

Multiple Craniofacial Effects

TBX1 has been identified as a major gene underlying DiGeorge or del22q11.2 syndrome in humans, a syndrome characterized by craniofacial and cardiovascular defects and heterozygous multigene deletions on chromosome 22 [reviewed in (4)]. In addition to cardiovascular defects, mice lacking *Tbx1* exhibit multiple craniofacial defects similar to DiGeorge patients, including defects in ear and mandible development, cleft palate, and defects in branchiomeric muscles (52, 55, 109). Mutations in the zebrafish homologue reveal that the role of *Tbx1* in craniofacial and pharyngeal development (82) is evolutionarily conserved.

Craniofacial development involves complex interactions between ectoderm, anterior mesoderm, endoderm, and neural crest-derived mesenchyme. *Tbx1* is expressed in both pharyngeal endoderm and anterior mesoderm (23) and has been shown to regulate growth factor expression in the pharyngeal region, providing an explanation for the hypoplasia of pharyngeal mesodermal derivatives in mutants (50, 110, 116). Potential targets of *Tbx1* in the pharyngeal region include *Fgf8*, *Fgf10*, and the forkhead transcription factor *Foxa2*, which in combination with *Shh* also acts upstream of *Tbx1*, mediating an autoregulatory loop (50, 110, 116, 118). Because *Tbx1* is expressed in anterior mesoderm rather than neural crest-derived mesenchyme, craniofacial defects affecting bone development observed in *Tbx1* homozygous mutant mice,

T-BOX GENES AND CANCER

Mounting evidence indicates a functional link between T-box genes, cellular proliferation/survival, and tumorigenesis, particularly breast tumors. A direct causal link between T-box genes and cancer, however, has yet to be unequivocally proven. *TBX2* is located within a human chromosomal segment, 17q22–24, which is frequently amplified in breast and pancreatic cancers (6, 51, 67). Several oncogenic candidates have been identified within this segment, including *TBX2*, which are frequently overexpressed in a subset of primary tumors and cancer cell lines (6, 51, 67). Unlike many of the other 17q22–24 candidates, *TBX2* is also amplified and overexpressed in a large fraction of *BRCA1*- and *BRCA2*-related breast tumors (95). *TBX2* and *TBX3* have been molecularly linked to the cell cycle machinery via the ARF-MDM2-p53 pathway. *TBX2* and *TBX3* can rescue a premature senescence phenotype in murine embryonic fibroblasts (20, 51). Recent work in melanoma cell lines has shown that a dominant negative Tbx2 is able to induce senescence (108). Both genes can also bind a putative TBE in the human $p14^{ARF}$ promoter and *TBX2* can repress a $p14^{ARF}$ expression reporter in vitro (65). *TBX2* is capable of repressing other transcripts from the *Cdkn* gene family, including $p15^{INK4b}$, $p16^{INK4a}$ (51), and p21 (83). There is no evidence, however, of $p15^{INK4b}$, $p16^{INK4a}$, ARF, or p21 overexpression in *Tbx2* homozygous null mutant embryos, and no evidence of a genetic interaction between *Tbx2* (or *Tbx3*) and p53 (43, 53). Nonetheless, the impaired proliferation of the mammary gland ductal tree in *Tbx3* heterozygous mice and the exacerbation of this phenotype in double heterozygous *Tbx2*; *Tbx3* mice (53) demonstrate the important contribution of these genes to normal mammary development and suggest that further work is required to determine how misregulation of *TBX2* or *TBX3* can contribute to mammary tumorigenesis.

such as micrognathia (small mandible), may be secondary to failure of mesodermal specification and/or mesodermal or endodermal-derived signaling to surrounding cell types.

Overexpression of human *TBX1* and three adjacent genes in transgenic mice leads to a spectrum of craniofacial and pharyngeal defects that overlaps with the *Tbx1* null phenotype (34). Dosage compensation using a *Tbx1* null allele reveals that the majority of defects, including cleft palate, thymic hypoplasia, and cardiovascular defects, are due specifically to overexpression of *Tbx1*, suggesting that closely regulated levels of Tbx1 are required for normal development and that either too much or too little can incur similar phenotypic defects (63) (see sidebar). However, inner ear defects and hearing loss observed in mice overexpressing *Tbx1* are not complemented by *Tbx1* null alleles, suggesting a different mechanism of *Tbx1* action in the otic epithelium. *Tbx1* specifies regional identity and fate boundary formation in the otic epithelium at early stages of inner ear development and acts cell autonomously to expand a population of cells that normally give rise to vestibular and auditory organs (86, 111). Overexpression of *Tbx1* leads to ectopic inner ear sensory organs, whereas loss leads to failure of sensory organ development and expanded neurogenesis. *Tbx1* therefore operates as a selector gene in the otocyst, controlling sensory versus neural cell fate (86).

Tbx1 also plays a central role in craniofacial muscle development. Branchiomeric skeletal muscles are derived from the mesodermal core of the pharyngeal arches. The most anterior, or mandibular, pharyngeal arch forms normally in the absence of *Tbx1* (52, 55, 109); however, the myogenic determination genes *Myf5* and *MyoD* fail to be activated, resulting in defects in jaw muscles (55). *Tbx1* regulates, but is not absolutely required for, myogenic specification in the mandibular arch since a low level of myogenic determination gene activation occurs sporadically in the absence of *Tbx1*, producing severely hypoplastic or unilateral jaw muscles. *Tbx1* therefore confers robustness on myogenic specification in the mandibular arch.

Another T-box gene, *TBX22*, is also required for palate development and mutations cause X-linked cleft palate and ankyloglossia (11, 69). The expression pattern of murine *Tbx22* is consistent with a role in the nasal septum during palatal shelf fusion, and in the tissue bridge between the tongue and floor of

the mouth (44). It is not known whether *Tbx22* interacts epistatically with *Tbx1* during palate development.

Cell Fate in the Pituitary

Tbx19 (119), also known as *Tpit*, is expressed in the two pro-opiomelanocortin (POMC)-expressing lineages of the pituitary gland, the adrenocorticotrophic hormone (ACTH) producing corticotrophs of the anterior lobe and the melanotrophs of the intermediate lobe. Loss-of-function mutations in human *TBX19* cause early onset ACTH deficiency. These mutations appear to be completely recessive as heterozygous humans and mice have no ACTH deficiency and heterozygous mice have normal numbers of POMC-expressing cells in their pituitaries (84, 85). *Tbx19* expression initiates prior to POMC expression during embryonic development and is also present in the adult pituitary. Although not required for lineage commitment of POMC-expressing cells, *Tbx19* is required for the terminal differentiation of corticotrophs and melanotrophs and for successful upregulation of POMC. *Tbx19* also represses gonadotroph differentiation, leading to the idea that this factor is responsible for alternative cell fates during pituitary development (61, 66, 85). *Tbx19* activates POMC transcription in cooperation with *Pitx1* (61, 66) through the recruitment of SRC/p160 coactivators to its cognate DNA target, the Tpit/Pitx regulatory element in the POMC promoter. This recruitment is mediated by direct binding between Tpit and SRC-2 and is upregulated by mitogen-activated protein kinase activity (68).

T-Box Genes in T Cells

Two T-box genes of the Tbr1 subfamily, *Eomesodermin* and *Tbx21* (also known as *T-bet* for T-box expressed in T cells), are important in the differentiation of both T and B cells of the immune system [reviewed by (39, 103)]. Adult mice with a null mutation in *Tbx21* show a complete lack of the T helper cell subset Th1, as well as effects on B cells and natural killer cells (39, 103, 104). Homozygous *Eomesodermin* mutants die in early gestation (see section on extraembryonic tissues), but heterozygous mutants display defects in $CD8^+$ T cells. There are indications that *Eomesodermin* and *Tbx21* work cooperatively in governing cellular immunity (79).

Tbx21 has a critical role in initiating Th1 development from naïve precursors both by inducing the Th1 terminal differentiation pathway and also by repressing the alternative Th2 differentiation pathway. Although it is not entirely clear how this effect is mediated, it very likely involves regulation of the *interferon-γ* locus, a definitive hallmark of the Th1 lineage, and may also involve chromatin remodeling (103). Because of this pivotal role in the differentiation of lymphoid cells, *Tbx21* is an important player in immune responses to infection and in autoimmune disease processes. For example, it has key roles in the pathogenesis of lupus (80), T-cell mediated colitis and Crohn's disease (73), and metastasis of primary prostate cancer (81). There is an association between type 1 diabetes and *Tbx21* polymorphisms in humans (54, 91). Mice with a null mutation in *Tbx21* show airway inflammatory features characteristic of asthma, whereas human asthma patients have reduced *TBX21* expression (32).

POMC: pro-opiomelanocortin

ACTH: adrenocorticotrophic hormone

SUMMARY POINTS

1. The T-box gene family, present in all metazoans, consists of 17 genes in mammals organized into 5 subfamilies.
2. T-box genes are expressed throughout development in dynamic patterns with both unique and overlapping areas of expression.

3. T-box proteins bind a common element, the T-site or TBE, but promoter specificity is provided by interactions with other transcription factors and different binding kinetics.
4. Mutations in T-box genes are responsible for developmental defects in many organisms, including several dominant or semidominant human developmental syndromes.
5. Dosage sensitivity to T-box proteins is frequently observed, with too much protein as disruptive as too little.
6. T-box factors function in a wide range of signaling pathways, but common themes put T-box genes downstream of BMP signaling and upstream of FGF signaling.

FUTURE DIRECTIONS

In vitro data have shown interactions between T-box proteins and other transcription factors in cooperative binding experiments. T-box proteins also have the potential to heterodimerize and are often expressed in overlapping patterns. A challenge for the future is to investigate the in vivo relevance of these interactions.

Little is known about how the complex expression profiles of T-box genes or the tightly regulated protein levels are achieved.

Because of the complex expression patterns of T-box genes, mutational analysis has not yet addressed all the possible developmental roles, and because early lethality often limits the exploration of later stages, new alleles, including conditional and hypomorphic alleles, will be needed.

While some developmental requirements for T-box genes have been demonstrated, relatively few downstream target genes effecting these functions have been identified.

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