Transcriptional Repression by the T-box Proteins Tbx18 and Tbx15 Depends on Groucho Corepressors**

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T-box (Tbx) genes encode a family of transcription factors that regulate a variety of developmental processes. The unifying and designating feature of all family members is a highly conserved region of 180 amino acid residues that confers DNA binding. To date, a limited number of direct target genes are known that all contain one or several T half-sites in their upstream promoter sequences. These elements are based on the consensus sequence 5′-AGGTGTA-3′ that was initially identified in vitro as a DNA binding site for Brachyury (T), the founder of this gene family (1). Brachyury proteins preferentially bind as dimers to a palindromic repeat of two anti-parallel T half-sites. Orientation and spacing of T half-sites are likely to affect DNA binding of specific T-box proteins (1–3). Target gene specificity is additionally controlled by protein interaction partners. T-box proteins have been shown to bind to several classes of transcription factors including homeodomain and zinc finger proteins conferring synergism in binding to adjacent DNA binding elements (4–10).

Exhaustive searches have identified T-box genes in all metazoans ranging from hydra to humans. Mammalian genomes are known to harbor 17 family members that have been divided into five major subfamilies based on sequence conservation of the T-box. Loss-of-function studies in the mouse have revealed functional requirements of T-box genes in a diverse array of developmental processes in the post-implantation embryo including formation and patterning of the mesoderm and organogenesis (11). Notably, mutations in a number of T-box genes have been identified as underlying causes for human congenital disorders (12).

Tbx18 and Tbx15 encode a closely related pair of T-box proteins that, together with Tbx22, form a subgroup of the Tbx1 subfamily in vertebrates. Mice carrying a null allele of Tbx18 die shortly after birth due to severe malformations of the axial skeleton, a phenotype that was traced back to the function of Tbx18 in maintaining anterior-posterior somite polarity (13). Additionally, Tbx18 regulates the condensation of mesenchymal cells around the distal ureter stalk. Newborn Tbx18−/− mice display a prominent hydroureret and hydronephrosis phenotype due to the lack of the smooth muscle layer of the ureter (14). Finally, Tbx18 is essential for the formation of the sinus horns from the mesenchyme of the pericardial wall and for their myocardial differentiation (15).

Mice homozygous for a targeted null allele of Tbx15 or for the spontaneous mutation droopy ear, in which Tbx15 is deleted, exhibit defects in skin pigmentation and in the skeleton. These phenotypic changes reveal a role for Tbx15 in dorso-ventral patterning of the mouse coat and in mesenchymal aggregation that precedes endochondral bone formation (16, 17).

Loss-of-function analyses of Tbx15 and Tbx18 in mice and conservation of expression in other vertebrates has revealed the importance of this closely related pair of T-box transcription factors in an impressive number of patterning and differentiation processes during vertebrate development. In contrast, our current understanding of the molecular mechanisms underlying these phenotypes is scarce since neither protein interaction partners nor transcriptional targets for either factor have been identified. As a step toward this goal we initiated a molecular analysis of Tbx18 and Tbx15 proteins. We here characterize the subcellular localization, DNA binding specificities, protein interactions, and transcriptional properties and their structural prerequisites in the two proteins. We provide evidence that com-

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petition with activating T-box proteins constitutes a possible mode of regulation of the promoters for Nppa (natriuretic peptide precursor type a) and Dlll (Delta-like 1) in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Expression Constructs—cDNA fragments encoding full-length Tbx18 and Tbx15 and subregions thereof (see Fig. 1A) were PCR-amplified from mouse cDNA AF306666 and NM_009323, respectively. Full-length (aa 1–436) and T-box region (aa 41–225) of Brachyury were amplified from mouse cDNA NM_009309. cDNA fragments encoding full-length protein (aa 1–767) and the WD40 domain (aa 445–767) of human TLE3 were amplified from the human cDNA BC043247 (kind gift from S. Stifani). For in vitro expression of proteins, cDNA fragments were cloned with C-terminal myc or HA tags in the vector pSP64 (Promega) that was modified to contain a globin leader/cDNA/globin trailer cassette was shuttled into HindIII and EcoRI sites of pcDNA3 (Invitrogen). The Tbx18-VP16 fusion construct was generated by introduction of the Herpes simplex VP16 activator fragment (aa 419–490, kind gift of D. Kessler) in front of the stop codon of Tbx18. GAL4 fusions of Tbx18 and Tbx15 were generated by subcloning the coding sequences into the yeast-two hybrid vector pGBK7T (Clontech) 3’ of the DNA binding domain of GAL4 (aa 1–147) followed by the SV40 nuclear localization signal (NLS). cDNA fragments encoding GAL4 fusion proteins were released with HindIII (5’) and SalI (3’) and directionally cloned into the HindIII and Xhol sites of pcDNA3. Expression plasmids encoding Gata4.HA, Nkx2–5.FLAG, Tbx5.HA, and Tbx2 cloned into pCS2-lof were a kind gift from V. Christoffels, and expression constructs were generated as N-terminal glutathione S-transferase (GST) fusions in pGEX-4T3 (Amersham Biosciences). All plasmids were sequenced, and expression was tested on Western blot. Details on cloning strategies and primer sequences are available upon request.

Site-directed mutagenesis was performed as cloning (20). Mutagenesis primers for the deletion of NLS sequences were 5’-GAGAAGACAGAACACGCTTCAATTAATCACGGAAGA-GGCGCCGGG-3’ (Tbx18) and 5’-GCCTTGATCGGCTCAAA-TATCGATTGGAGAAGAGGCGGT-3’ (Tbx15), and primers for the generation of point mutants in the eh1 motif were 5’-CTAAGCGCTCAAGCCGCACGCATATTATGTCGAGGCGA CTGATCCCGG-3’ (Tbx18) and 5’-GAGCTCCCGGACACATGC ATGATCACTGTTGAAGCTGGATGCGG-3’ (Tbx15).

Random Binding Site Selection and EMSA—Proteins used for binding site selection and electrophoretic mobility shift assay (EMSA) were generated from pSP64 expression constructs using the SP6-coupled Tnt rabbit reticulocyte lysate (Promega) according to the supplier’s instructions. The binding site selection was essentially carried out as described (1). After four rounds, the gel-eluted PCR products were subcloned in pBluescript and sequenced. Oligonucleotides used in EMSA were: BS.pF, 5’-GATCCGGTTTCCACCTAGGTGTAA-GGA-3’; BS.pR, 5’-GATCTCTCTTACACCTAGGTGTGAA ACGCC-3’; BS.invF, 5’-GATCCGGAGTGTTGAAATTTTAC CACCTGGA-3’; BS.invR, 5’-GATCTCAGGTGGAATTTC TCAACCTCCG-3’; BS.dirF, 5’-GATCCGGAGTGTTGAA GGTGTGAAGAAGA-3’; and BS.dirR, 5’-GATCTCCTTTTACA CACCTACACACTCCG-3’. Oligonucleotides were boiled for 5 min and cooled slowly down to room temperature to anneal. Double-stranded fragments were end-labeled with T4-PNk (New England Biolabs) in the presence of [γ-32P]ATP. Binding reactions for gel shift assays contained 2–5 μl of in vitro transcribed myc-tagged protein in a total volume of 20 μl of Nonidet P-40 buffer (5 mM Tris, pH 7.5, 80 mM NaCl, 50 mM NaF, 1 mM MgCl2, 0.1% Nonidet P-40) with 1× Complete protease inhibitor mixture (Roche Applied Science) and 1 μg of double-stranded poly(dI-dC). Reactions were preincubated for 20 min on ice before 10,000 counts of probe were added. For supershift experiments, 1 μl of anti-myc antibody (9E10, Sigma) was added to the lysate. Complexes were allowed to form at room temperature for 20 min, before the reactions were loaded on a native 4% polyacrylamide gel (0.5× Tris-borate-EDTA). Gels were run at 10 V/cm at 4 °C for 5 h before they were dried and exposed to autoradiography film.

Cell Culture, Transient Transfections, and Reporter Assays—HEK293 and HeLa cells were seeded at 20–30% confluency in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, grown overnight, and transfected using the calcium phosphate method. For reporter assays, HeLa cells were seeded in six-well dishes and transfected with constant amounts of reporter plasmids and 25 ng of pCMVβ (Clontech) for normalization. The total amount of expression plasmid was kept constant by adding empty pcDNA3. Per transfection, 250 ng of 5xGAL4UAS-tk-luciferase reporter plasmid (a kind gift of J. Milbrandt), 75 ng of pGL3.Nppa-luciferase, containing a 0.7-kb fragment of the mouse Nppa promoter (a kind gift of V. Christoffels), or 75 ng of pKS.msd-luciferase (kindly provided by B. Herrmann) were used (18, 19); luciferase and β-galactosidase activities were measured 48 h after transfection. All transfections were performed in duplicates, and experiments were repeated at least three times. After normalization, the mean luciferase activities and standard deviations were plotted as “fold activation” when compared with the empty expression plasmid. p values were determined with the Student’s t test.

Immunofluorescence—Experiments in HEK293 cells were performed according to standard protocols. Primary antibodies used were rat anti-HA (3F10, Roche Applied Science) and mouse anti-myc (9E10, Sigma), both at 1:200 dilutions, and secondary antibodies were donkey anti-rat IgG(H + L) fluorescein isothiocyanate and donkey anti-mouse IgG(H + L) rhodamine (both Diano, at 1:200 dilutions). Immunofluorescent detection of proteins was repeated at least three times, and representative examples were photographed on a Leica DM5000 microscope with DFC300FX camera (Leica).
Transcriptional Repression by Tbx18 and Tbx15

GST Pulldown and Co-immunoprecipitation Assays—GST and GST-Tbx18 fusion proteins were produced in the Escherichia coli strain BL21 and bound to glutathione-Sepharose 4T beads (Amersham Biosciences). HA- or myc-tagged prey proteins were produced in HEK293 cells that were lysed in pull-down buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 10 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 5% glycerol, 0.05% Triton X-100, and 1 mM diethiothreitol). The binding reaction was performed as described (21). For co-immunoprecipitation assays, HEK293 cells were seeded in 10-cm dishes and either transfected with myc-tagged expression construct alone or transfected together with a HA-tagged bait construct. After 48 h, cells were lysed in 500 µl of Nonidet P-40 buffer, cellular debris was precipitated by centrifugation for 30 min at 4 °C, and the supernatant was precleared from nonspecific binding by incubation with 25 µl of protein A-agarose (Roche Applied Science). 5 µl of anti-HA antibody and 25 µl of protein A-agarose were added to the supernatant for 2 h at 4 °C, before the beads were washed three times with 500 µl of Nonidet P-40 buffer. Proteins eluted from the beads were analyzed by Western blot with anti-myc and anti-HA antibodies, and 5% of the input was loaded as a control.

Quantification of Endogenous Nppa Expression—The mouse atrial cardiomyocyte tumor cell line HL-1 was cultured as described (22). For transfections, 1.3 × 10⁶ cells were seeded in 60-mm dishes. Transfections were performed on the following day using FuGENE HD (Roche Applied Science, 20 µl of each) according to the supplier’s instructions. Each transfection was performed in a well of a 60-mm dish. Transfections were carried out using anti-HA antibody and 25 µl of protein A-agarose added to the supernatant for 2 h at 4 °C, before the beads were washed three times with 500 µl of Nonidet P-40 buffer. Proteins eluted from the beads were analyzed by Western blot with anti-myc and anti-HA antibodies, and 5% of the input was loaded as a control.

Signal Transduction

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Signal Transduction
HEK293 cells and detected the myc tag by immunofluorescence (Fig. 1B). Full-length Tbx18 protein showed exclusive nuclear localization in HEK293 (Fig. 1B) and in NIH3T3 cells (data not shown). Deletion of the C-terminal region did not alter this distribution, whereas removal of the N-terminal region resulted in relocalization to the cytoplasm. The N-terminal region alone was found in the nucleus, demonstrating that it is not only necessary but also sufficient for nuclear import. Similar results were obtained for Tbx15. However, a truncation mutant of Tbx15 lacking the N-terminal region retained partial nuclear localization (Fig. 1B, white arrowhead). Inspection of the primary structure of Tbx18 and Tbx15 revealed a cluster of 5 basic amino acid residues resembling a classical NLS at a conserved position at the N terminus of the two proteins (Fig. 1C, marked in red). Deletion of this cluster resulted in exclusive localization, whereas removal of the N-terminal region resulted in relocalization to the cytoplasm. The N-terminal region alone was found in the nucleus, demonstrating that it is not only necessary but also sufficient for nuclear import.

The DNA-binding properties of Tbx18, Tbx15, and the distant family member Brachyury by EMSA. All three proteins failed to bind to DNA fragments harboring single T half-sites under our experimental conditions (data not shown). In contrast, DNA fragments with repeats of T half-sites supported protein-DNA binding of full-length, in vitro translated Tbx18, Tbx15, and Brachyury (Fig. 3). All three proteins formed specific complexes with the consensus sites BS.inv, BS.dir, and the palindromic binding site identified for Brachyury, BS.p (see Ref. 1) and was therefore designated BS.inv. For Tbx15, nine of fifteen selected oligonucleotide sequences matched the BS.inv consensus sequence (Fig. 2, middle panel). A smaller fraction (6 of 15 sequences) featured a direct repeat of the T half-site, indicating differences in binding specificity between the two proteins (Fig. 2, lower panel). The consensus of this directly repeated binding site was named BS.dir.

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that the N- and C-terminal regions participate in protein-DNA complex formation. T-boxes of Tbx18 and Tbx15, but not of Brachyury, exclusively bound to the BS.inv site (Fig. 3D, open arrowheads), suggesting that the T-boxes of Tbx18 and Tbx15 possess an inherent binding specificity for this sequence. A strong increase in protein-DNA complex formation was observed for all truncated T-box peptides and full-length proteins after the addition of anti-myc antibody (Fig. 3, C and D, black arrowheads). Since GST-Tbx18 full-length protein could not be expressed in bacteria, we used a series of bacterially expressed fusion proteins of GST with the N- and C-terminal region and the T-box of Tbx18 instead (Fig. 4A, left). Tbx18 specifically bound to GST-Tbx18(N + H11001 T) and -Tbx18(T) but not to GST-Tbx18(N) or -Tbx18(C) (Fig. 4A, right), showing that dimerization via the T-box region occurred in the absence of DNA binding. A weaker interaction was observed with Tbx15 but not with Brachyury, indicating that related T-box proteins are able to form heterodimers.

To address whether dimerization occurs in cells, we analyzed the subcellular distribution of the myc-tagged Tbx18ΔNLS or Tbx15ΔNLS proteins (described above) by immunofluorescence in HEK293 cells upon coexpression of wild-type Tbx18 or Tbx15 (HA-tagged) proteins. Unlike for NLS mutants alone, we now detected nuclear localization of both Tbx18ΔNLS and Tbx15ΔNLS proteins (Fig. 4B), strongly suggesting that dimerization has occurred in these cells. The absence of nuclear recruitment upon coexpression of unrelated nuclear proteins confirmed the specificity of the interaction (data not shown).

**Tbx18 and Tbx15 Are Potent Transcriptional Repressors**—Members of the T-box gene family encode specific DNA-binding proteins that can activate or repress RNA polymerase II-mediated transcription (11). We performed luciferase reporter assays in HeLa cells to analyze how Tbx18 and Tbx15 modulate transcription. We used full-length Tbx18 and Tbx15 proteins fused to the GAL4-DNA binding domain cotransfected with a reporter plasmid containing five copies of the GAL4 binding site in front of the thymidine kinase minimal promoter (5xGal4UAS-tk-luciferase, Fig. 5A). We observed a strong and
dose-dependent repression of reporter gene activity by GAL4-Tbx18 with a maximal reduction of the basal activity to 5.5 ± 0.2% (Fig. 5B). The GAL4-DNA binding domain alone did not affect the transcription of the reporter gene. GAL4-Tbx15-mediated repression of the reporter to 8.4 ± 0.1% demonstrates that Tbx18 and Tbx15 do not only share similar DNA binding properties but also strong transcriptional repressor activities.

We then asked whether Tbx18 contains any identifiable domain that mediates this repression. GAL4-Tbx18(N–T), GAL4-Tbx18(N), and GAL4-Tbx18(C) proteins (data not shown) all repress 5xGal4UAS-TK-luciferase reporter activity in HeLa cells, although more weakly than the full-length protein (Fig. 5C), suggesting that Tbx18 contains multiple regions capable of transcriptional repression.

**Tbx18 and Tbx15 Interact with Groucho Corepressors** — Transcriptional repression by tissue-specific transcription factors is mediated by binding to corepressor complexes that in turn modify chromatin structure. We wondered whether we could identify corepressors of Tbx18 by analyzing the primary structure of Tbx18 for motifs known to recruit such molecules. Near the N terminus of the protein, we identified an eh1 motif (engrailed homology 1) (Fig. 6A) that had also recently been noted in a genome-wide screen (29). The eh1 motif comprises a stretch of 7 amino acid residues that is present in a large number of transcription factors of various classes and is necessary for binding of Groucho proteins (30). Groucho proteins constitute a family of highly conserved corepressors, with the members Grg1–5 (Groucho-related genes) in mouse and TLE1–4 (Transducin-like enhancers of split) in humans (31). Interestingly, the eh1 motif is also found at a conserved N-terminal position in murine Tbx15, Tbx22, and Tbx20 proteins (Fig. 6A), suggesting that these members of the Tbx1 subfamily may also act as Groucho-dependent repressors.

To experimentally explore this possibility, we tested Tbx18 binding to Groucho proteins in vitro. Pulldown assays were performed using GST-Tbx18 fusions (compare Fig. 4A) that were incubated with lysates of HEK293 cells transfected with myc-tagged Tbx18, Tbx15, and Brachyury full-length expression constructs. GST-Tbx18 cells were transfected with expression constructs for myc-tagged Tbx18(NLS) or Tbx15(NLS) (red) in the presence or absence of HA-tagged wild-type Tbx18 or Tbx15 (green). Both NLS-deficient proteins are efficiently relocalized to the nucleus upon coexpression of wild-type Tbx18 or Tbx15 (compare 4′,6-diamidino-2-phenylindole (DAPI) nuclear counter staining).

**FIGURE 4.** Tbx18 and Tbx15 form homo- and heterodimers. A, dimerization in vitro. Left, GST and fusion proteins of GST and N + T, N−T, and C-domains of Tbx18 were purified from E. coli extracts and analyzed for integrity and quantity by Coomassie Brilliant Blue staining of SDS-polyacrylamide gels. Right, GST pulldown assays with protein extracts from HEK293 cells transfected with myc-tagged Tbx18, Tbx15, and Brachyury full-length expression constructs. B, dimerization in cells. HEK293 cells were transfected with expression constructs for myc-tagged Tbx18/NLS or Tbx15/NLS (red) in the presence or absence of HA-tagged wild-type Tbx18 or Tbx15 (green). Both NLS-deficient proteins are efficiently relocalized to the nucleus upon coexpression of wild-type Tbx18 or Tbx15 (compare 4′,6-diamidino-2-phenylindole (DAPI) nuclear counter staining).

**FIGURE 5.** Tbx18 and Tbx15 act as transcriptional repressors. A, schematic representation of the 5xGal4UAS-tk-luciferase-reporter plasmid containing a pentamer of the GAL4 binding site, the minimal promoter of the thymidine kinase gene (tk), and the luciferase reporter gene. B, HeLa cells were cotransfected with the reporter plasmid and increasing amounts of GAL4, GAL4-Tbx18, or GAL4-Tbx15 expression constructs. Tbx18 and Tbx15 dose-dependently repress luciferase reporter gene activity. C, full repression activity of Tbx18 requires N- and C-terminal regions as well as the T-box. HeLa cells were cotransfected with GAL4-Tbx18 deletion constructs (each 25 ng) and the 5xGal4UAS-tk-luciferase reporter plasmid.
Transcriptional Repression by Tbx18 and Tbx15

A

| Protein          | Consensus Sequence |
|------------------|--------------------|
| Tbx18            | HAF5VEALIGA-28     |
| Tbx15            | HAF5VEALIGS-26     |
| Tbx22            | HAF5VEALIMOR-18    |
| Tbx20            | HAF5ISA1AMLSM-26   |
| eh1 consensus    | a. lp. lb.         |

B

- TLE3
- 10% input GST
- GST-Tbx18

C

- IP: α-HA
- α-myc
- TLE3.HA
- α-HA
- α-myc
- TLE3

D

- Tbx18.WD40.HA
- Tbx15.WD40.HA
- α-HA
- α-myc
- DAPI

E

- 5xGal4UAS-tk-luciferase
- Fold activation

**FIGURE 6.** Tbx18 and Tbx15 interact with the corepressor Groucho. A, sequence alignment of mouse Tbx18 (AF30666), Tbx15 (NM_009323), Tbx22 (NM_145224), and Tbx20 (NM_194263) demonstrates the presence of an N-terminal eh1 motif; the numbers refer to the position within the protein. The eh1 consensus sequence (a, aromatic; l, aliphatic; p, polar; b, bulky amino acids) is shown. B, interaction of Tbx18 with Groucho protein (TLE3) in vitro. Pulldown assays with GST-Tbx18 deletion mutants were carried out as outlined in the legend for Fig. 4 with extracts from HEK293 cells transfected with a TLE3.HA expression construct. C, interaction of Tbx18 and Tbx15 with Groucho in vivo requires the eh1 motif. HEK293 cells were transfected with expression constructs for wild-type (wt) or eh1-deleted (Δeh1) myc-tagged Tbx18 (left) and Tbx15 (right), respectively, either alone or in combination with a construct encoding HA-tagged TLE3. Lysates were immunoprecipitated with anti-HA antibody followed by Western blot (WB) analysis of input (in) and immunoprecipitated fractions (IP) with anti-myc and anti-HA antibodies. Signals for Tbx18 and Tbx15 (open arrowheads), TLE3 (black arrowheads), and IgH-bands (asterisk) are highlighted. D, nuclear recruitment assay. Coexpression of myc-tagged Tbx18 or Tbx15 (middle row) translocates the cytoplasmic WD40 domain of TLE3 (HA-tagged, upper row) to the nucleus as shown by immunofluorescence in HEK293 cells after cotransfection. DAPI, 4’6-diamidino-2-phenylindole. E, repression activity of Tbx18 and Tbx15 partially depends on the presence of the eh1 motif. Expression constructs for fusion proteins of GAL4 with wild-type Tbx18 or Tbx15 and eh1 mutants (25 ng), respectively, were transfected in HeLa cells together with the 5xGal4UAS-thymidine kinase-luciferase reporter plasmid. *, p < 0.05.

(Fig. 6C, left). Site-directed mutagenesis of the eh1 motif by replacing the amino acids phenylalanine and serine by leucine and isoleucine (Tbx18Δeh1) abolished complex formation. Not unexpectedly, Tbx15 also showed eh1-dependent binding to TLE3 (Fig. 6C, right). Groucho proteins are known to bind the eh1 motif with their C-terminal WD40 domain (32). To delineate the region in TLE3 that mediates interaction with the eh1 motif in Tbx18 and Tbx15, we performed immunofluorescence experiments in HEK293 cells expressing the HA-tagged WD40 domain of TLE3 in the presence or absence of myc-tagged Tbx18 or Tbx15. When expressed alone, the WD40 domain of TLE3 is distributed in the cytoplasm as it lacks a functional NLS. Coexpression of Tbx18 or Tbx15 led to nuclear translocation of the WD40 domain (Fig. 6D), an effect that was not observed following coexpression of Tbx18 or Tbx15 protein with a mutant eh1 motif, confirming the specificity of the interaction. Next, we determined whether the interaction of Tbx18 or Tbx15 with Groucho is required for transcriptional repression. Therefore, we compared the repression activities of wild-type and eh1 mutant versions of GAL4-Tbx18 and GAL4-Tbx15 on the 5xGal4UAS-tk-luciferase-reporter in HeLa cells. Wild-type and eh1 mutant proteins were expressed at equal levels (data not shown). Both eh1 mutant proteins exhibit an approximate 50% reduction of repressor activity (Fig. 6E) with a release of repression from 17.8 ± 3.4 to 31.4 ± 2.1% for GAL4-Tbx18 and 35.0 ± 0.0 to 64.3 ± 4.7% for GAL4-Tbx15. We conclude that a major part of the repression activity of Tbx18 and Tbx15 is mediated through the eh1 motif. Overexpression of Groucho protein failed to increase repression by Tbx18 and Tbx15 wild-type proteins (data not shown), indicating that abundant endogenous expression in mammalian cell lines was sufficient to saturate the repression (33).

Tbx18 Interacts with Gata4 and Nkx2–5 and Represses the Nppa Promoter by Competition with Tbx5—Cooperativity between transcription factors in DNA binding plays a crucial role in target promoter specificity. For T-box proteins, this may be achieved by dimerization on multiple T half-sites and/or through interaction with other proteins (10, 28, 34–36). A well established paradigm for the latter is the interaction of Tbx2, Tbx5, and Tbx20 with the transcription factors Gata4 and Nkx2–5 to regulate cardiac expression of Nppa (natriuretic peptide precursor type a, also known as atrial natriuretic factor, ANF) (4–7, 18, 37, 38). The 700-bp upstream region of Nppa contains all the necessary control elements to confer correct spatial expression in the developing heart (18). Binding of Tbx5, Tbx20, NK-type homeodomain proteins, and GATA proteins to their respective recognition sequences synergistically activates this promoter, whereas binding of Tbx2 releases activa-
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Binding of Tbx18 to Nkx2–5 and Gata4 as well as coexpression of Gata4, Tbx5, and Tbx18 in the sinus horn mesenchyme (15, 39) suggest that failure of Nppa activation in this region of the developing heart might at least partly be caused by the repression activity of Tbx18. We tested this hypothesis by analyzing the effect of Tbx18 in an Nppa-luciferase reporter assay (pGL3.Nppa-luciferase, Fig. 7C). Low concentrations of Tbx18 alone had little effect on the activity of the reporter. Coexpression of Gata4 with Nkx2–5 caused a moderate activation, in agreement with previous observations (40). Low concentrations of Tbx18 caused further activation when constant amounts of Gata4 and Nkx2–5 were present, suggesting that Tbx18 efficiently recruited the activators Gata4 and Nkx2–5 to the Nppa promoter. However, at higher doses of Tbx18, this activation was reversed to base line levels, indicating the dominance of Tbx18-mediated repression. Coexpression of Gata4 and Nkx2–5 with Tbx5 led to strong activation, demonstrating synergism of the three transcription factors in activating the Nppa promoter (4–6, 18). This activation was efficiently repressed in a dose-dependent manner upon the addition of Tbx18, suggesting that Tbx18 repressed the Nppa promoter by competition with the activator Tbx5 (Fig. 7D). An activator form of Tbx18 (Tbx18-VP16) caused transcriptional activation, which was synergistically increased when Gata4 and Nkx2–5 were coexpressed (Fig. 7E), demonstrating cooperative binding of Tbx18, Gata4, and Nkx2–5 proteins to DNA binding sites in the Nppa promoter.

To address whether Tbx18 is also sufficient to repress endogenous Nppa expression, we performed overexpression experiments in HL-1 cardiomyocytes (22). Since we achieved only low transfection efficiency of these cells, we enriched transfected cells by coexpression of a cell surface marker followed by magnetic cell separation. Endogenous Nppa expression was measured by quantitative RT-PCR. Mean relative mRNA expression levels were derived from two independent transfections and triplicate RT-PCR measurements. *, **, p < 0.02; ***, p < 10^-4.

Although Tbx2, Tbx5, and Tbx20 belong to different T-box subfamilies, they share several interacting partners. We therefore decided to investigate whether Tbx18 was also capable of interacting with GATA zinc finger and NK-type homeodomain proteins. Indeed, we found in GST pulldown assays that HEK293-expressed Gata4 and Nkx2–5 in vitro. Pulldown assays with GST-Tbx18, as described in the legend for Fig. 4, with lysates of HEK293 cells transfected with expression constructs for HA-tagged Gata4 or Nkx2–5, were performed. Bound protein was detected by anti-HA Western blot. B, nuclear recruitment of Tbx2 and Tbx5 by Gata4 or Nkx2–5. Immunofluorescence in HEK293 cells transfected with an expression construct for gyc-tagged Tbx2 or NLS either alone or in the presence of HA-tagged Gata4 or Nkx2–5. DAPI, 4',6-diamidino-2-phenylindole. C, schematic diagram of the Nppa luciferase reporter (pGL3.Nppa-luciferase). The numbers indicate the genomic positions relative to the transcription start site. D, Tbx18 counteracts the transcriptional activation of the Nppa promoter mediated by Tbx5. HeLa cells were transfected with increasing amounts of an expression construct for Tbx18 (0–200 ng), alone (white bars), together with constant amounts of Gata4 and Nkx2–5 expression constructs (100 ng of each, gray bars), or with expression constructs for Gata4, Nkx2–5, and Tbx5 (100 ng each of black bars). E, the expression of Tbx18-VP16 (white bars) activates the Nppa promoter and causes a synergistic activation in combination with Gata4 and Nkx2–5 (gray bars). F, forced expression of Tbx18 or Tbx2 decreases Nppa mRNA levels in HL-1 cardiomyocytes. Transfected cells were enriched, and endogenous Nppa expression was measured by quantitative RT-PCR. Mean relative mRNA expression levels were derived from two independent transfections and triplicate RT-PCR measurements. *, **, p < 0.02; ***, p < 10^-4.

FIGURE 7. Tbx18 binds Gata4 and Nkx2–5 and represses the Nppa promoter by competition with Tbx5. A, interaction of Tbx18 with Gata4 and Nkx2–5 in vitro. Pulldown assays with GST-Tbx18, as described in the legend for Fig. 4, with lysates of HEK293 cells transfected with expression constructs for HA-tagged Gata4 or Nkx2–5, were performed. Bound protein was detected by anti-HA Western blot. B, nuclear recruitment of Tbx18 and NLS by Gata4 or Nkx2–5. Immunofluorescence in HEK293 cells transfected with an expression construct for myc-tagged Tbx18 and NLS either alone or in the presence of HA-tagged Gata4 or Nkx2–5. DAPI, 4',6-diamidino-2-phenylindole. C, schematic diagram of the Nppa luciferase reporter (pGL3.Nppa-luciferase). The numbers indicate the genomic positions relative to the transcription start site. D, Tbx18 counteracts the transcriptional activation of the Nppa promoter mediated by Tbx5. HeLa cells were transfected with increasing amounts of an expression construct for Tbx18 (0–200 ng), alone (white bars), together with constant amounts of Gata4 and Nkx2–5 expression constructs (100 ng of each, gray bars), or with expression constructs for Gata4, Nkx2–5, and Tbx5 (100 ng each, black bars). E, the expression of Tbx18-VP16 (white bars) activates the Nppa promoter and causes a synergistic activation in combination with Gata4 and Nkx2–5 (gray bars). F, forced expression of Tbx18 or Tbx2 decreases Nppa mRNA levels in HL-1 cardiomyocytes. Transfected cells were enriched, and endogenous Nppa expression was measured by quantitative RT-PCR. Mean relative mRNA expression levels were derived from two independent transfections and triplicate RT-PCR measurements. *, **, p < 0.02; ***, p < 10^-4.
restricted to anterior somite halves. We therefore hypothesized that Tbx18 antagonizes Tbx6-mediated activation to restrict the expression of Dll1 to the posterior somite compartment. We failed to detect ectopic expression of Dll1 in anterior somite halves in Tbx18−/− embryos; however, coexpression of the closely related Tbx22 gene in newly formed somites may compensate for the loss of Tbx18 function in this context (43). Restriction of Tbx18 expression to anterior somite halves is achieved by the transcription factor Uncx4.1 in posterior somite halves (13). In Uncx4.1 mutant embryos, Tbx18 expression is found throughout the somites, providing a natural situation to study the effect of ectopic Tbx18 on Dll1 somitic expression. Indeed, we found a down-regulation of Dll1 expression and of the Notch target gene Hey1 in Uncx4.1−/− somites (Fig. 8A), suggesting that Tbx18 represses Dll1, and thus, Notch signaling in this tissue. To confirm this possibility at the molecular level, we performed reporter assays in HeLa cells using a luciferase reporter under control of the Dll1 msd upstream enhancer fused to the Dll1 minimal promoter (pKS.msd-luciferase) (Ref. 19). The 1.4-kbp msd fragment is sufficient to direct Dll1 transcription to the presomitic mesoderm, the somites, and the dermomyotome (45) and contains six T half-sites as well as four Tcf/Lef binding sites that are required to mediate this activity in vivo (19). Neither expression of Tbx6 at various concentrations (0–250 ng) nor of Tcf1e together with β-catenin (both 100 ng) stimulated the activity of the msd-luciferase reporter in our experiments. Cotransfection of Tbx6 and Tcf1e/β-catenin dose-dependently activated the reporter (Fig. 8B) in accordance with previous data (19). In contrast, Tbx18 alone repressed the basal reporter activity moderately and was unable to synergistically activate the msd reporter when coexpressed with Tcf1e/β-catenin (Fig. 8C). An activator form of Tbx18 (Tbx18-VP16) caused transcriptional activation of the reporter gene, arguing that the effects of Tbx18 on the msd promoter are caused by competitive binding to T half-sites and not merely by protein interactions (data not shown).
DISCUSSION

Genetic analyses have shown that the closely related T-box genes Tbx18 and Tbx15 are crucial players in the formation of the heart, the ureter, the vertebral column, and the skin during mouse development. As a first step toward understanding the molecular pathways regulated by these two proteins, it is critical to define their molecular properties and the mode of regulation of direct transcriptional targets. Here, we have shown that Tbx15 and Tbx18 act as transcriptional repressors that may exert their function by antagonizing transcriptional activators of the same family. During cardiac development, Tbx18 may counteract Tbx5-mediated activation of the Nppa promoter and Tbx6-mediated activation ofDll1 during somitogenesis. Interaction with Groucho corepressors is at least partially responsible for transcriptional repression. DNA binding specificity and protein interaction partners are likely to dictate target specificity of Tbx15 and Tbx18. Finally, we show that Tbx15 and Tbx18 are biochemically equivalent, suggesting redundant function in embryogenesis.

Repression by Tbx18 through Antagonizing T-box Activators in Somite and Heart Development—Mice carrying a null allele of Tbx18 die shortly after birth due to severe malformations of the axial skeleton, a phenotype that was traced to the function of Tbx18 in maintaining anterior-posterior somite polarity (13). Anterior-posterior somite polarity is established in the anterior presomitic mesoderm by the combined action of Mesp2 and Notch-Delta signaling (46). The latter induces expression of Uncx4.1 in posterior somite halves (47) that is required to restrict the expression of Tbx18 to anterior somite halves (13). In Tbx18/−/− embryos, Uncx4.1 expression gradually expands into anterior somite halves, suggesting inhibition of Uncx4.1 activation by Tbx18 (13). To date, the lack of clarity on the mode of target gene regulation by Tbx18 has made it difficult to hypothesize on the molecular pathways controlled by Tbx18 in the somitic mesoderm. Our studies have now shown that Tbx18 is likely to act as a transcriptional repressor in vivo. Expansion of Uncx4.1 expression in Tbx18−/− somites is therefore compatible with a direct transcriptional repression of Uncx4.1, and alternatively, with an indirect inhibition of Uncx4.1 activation by repression of components of the Delta/Notch signaling pathway. The first possibility seems unlikely given the relatively slow expansion of Uncx4.1 expression in Tbx18−/− somites. However, coexpression of Thx22, a closely related Thx gene, in anterior somite halves of newly formed somites (43) might prevent an immediate derepression of Uncx4.1 transcription. The second possibility gains support from our data on the transcriptional control of the Dll1 promoter in vitro and from analysis of molecular changes accompanying Tbx18 overexpression in Uncx4.1−/− embryos. Tbx18 abrogates the Tbx6-mediated activation of the Dll1 promoter, most likely by competition for T half-sites in vitro, and ectopic expression of Tbx18 in posterior somites coincides with the down-regulation of Dll1 and Notch-Delta signaling in vivo. Expression of Tbx6 mRNA and protein is restricted to the presomitic mesoderm (42), arguing for a role of Tbx6 in activating rather than maintaining somitic expression of Dll1. Tbx18 might therefore counteract Tbx6 to restrict Dll1 expression to posterior stripes in the anterior presomitic mesoderm and/or might antagonize an as yet unidentified somitic activator to restrict Dll1 to posterior somite halves. As a third possibility, expansion of Uncx4.1 expression in Tbx18−/− embryos may merely reflect immigration of cells from posterior into anterior somite halves due to loss of adhesion or repulsion mechanisms.

The Nppa promoter is subject to a spatially complex pattern of regulation in which both activating and repressing T-box proteins may bind and compete for the same set of binding sites. Nppa expression in the chamber myocardium of the heart is established by synergistic action of Nkx2–5, Gata4, and Tbx5 transcriptional activators (4–6). Exclusion of Nppa expression from regions of the primary myocardium in the atrioventricular canal and the outflow tract is achieved by binding of the Tbx2 repressor to T half-sites in the Nppa promoter, competing Tbx5 activation (18, 38). Our studies show that Tbx18 might play a similar role in the sinus horn mesenchyme that is also devoid of Nppa expression. However, repression of Nppa expression in the posterior pole of the heart does not exclusively depend on the presence of Tbx18 but also on the absence of Nkx2–5 (15), providing an additional level of safety to exclude Nppa expression from this area.

Our analysis of Nppa repression by Tbx18 sheds light onto the molecular control of target site specificity of T-box genes. Tbx5 and Tbx18 are members of distantly related Tbx subfamilies, arguing for diverse modes of DNA and protein interactions. However, regulation of the same promoter clearly indicates that both proteins bind to the same DNA binding sites, a situation that is similarly found for Tbx6 and Tbx18 in the Dll1 promoter. To date, all T-box proteins analyzed, including Tbx15 and Tbx18 tested here, recognize DNA binding sites containing a T half-site 5′-AGGTGT-GAA-3′. Earlier reports and this study suggest that in vitro selected (strong) binding sites are not necessarily present in the genome as such but that combinations of two or more (less conserved) T half-sites including their particular orientation and spacing influence DNA binding specificity in vivo (1–3, 28, 34). Along this line, we failed to detect the selected perfect binding sites of Tbx15 and Tbx18 in the mouse genome. Since binding affinities of Tbx18 and Tbx15 for paired T half-sites was considerably higher than for a single half-site, a cooperative mode of DNA binding seems mandatory. Indeed, we found that Tbx15 and Tbx18 are able to dimerize in the absence of DNA in vitro and in cells, providing a mechanism to stabilize weak monomer-DNA interactions. Moreover, the enhanced dimerization by the addition of antibody greatly increased the DNA binding affinity, an effect that was previously reported for other T-box proteins (1, 3). The available DNA-protein co-crystal structure of the Xenopus Brachyury homolog Xbra together with the palindromic binding site has implicated critical residues of the T-domain that mediate dimerization (27). Most of these residues are conserved in Tbx18 and Tbx15 (data not shown). However, the binding to paired T half-sites of inverted or
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directly repeated orientations implies an alternative quaternary structure of the protein dimer-DNA complex, arguing for additional dimerization interfaces in Tbx18 and Tbx15 proteins. Furthermore, our finding that flanking N- and C-terminal protein regions confer high affinity DNA binding suggests that regions outside the T-box participate in dimer formation.

An additional level of target specificity might be achieved by protein-protein interaction with other DNA binding transcription factors. Our study has shown that Tbx18 can directly bind to the homeodomain transcription factor Nkx2–5 and the zinc finger protein Gata4 in vitro as well as in mammalian cells, two proteins that were previously identified as binding partners of Tbx1, Tbx2, Tbx5, and Tbx20 (4–7, 10). During heart development, transcription factors of all three families cooperatively regulate cardiac gene expression programs.

We found that the binding to Nkx2–5 and Gata4 is mediated by the T-box and (more weakly) by the N-terminal region, indicating that both transcription factors either bind to two different sites in the T-box and N-terminal region or bind within the short stretch of overlapping amino acids present in both fusion proteins of Tbx18 (aa 148–157). This is in line with previous studies that have shown that interaction of Tbx5 and Nkx2–5 proteins is mediated both by the N-terminal region outside the T-box as well as by the N-terminal part of the T-box (4). The high conservation of the T-box domain of different family members might thus not only be a prerequisite for shared DNA binding specificity but also for conserved binding to protein interaction partners. Nonetheless, it is likely that specific protein interaction domains are present in the regions outside the T-box, providing an additional level of specificity in target gene recognition by T-box transcription factors.

Repression by Recruitment of Corepressors—Transcription factors of various classes including basic helix-loop-helix, Tcf/HMG (high mobility group), homeodomain, runt domain, and zinc finger domain proteins have previously been reported to function as Groucho-dependent repressors (31). Groucho proteins are known to recruit histone deacetylases (48, 49) that function as Groucho-dependent repressors. Widespread expression of vertebrate Groucho was not only identified at a conserved N-terminal tors. The eh1 motif that mediates binding to the WD40 domain of Groucho-dependent repressors (31). Groucho proteins are known to recruit histone deacetylases (48, 49) that remove acetyl groups from histone tails, thus rendering the chromatin inaccessible for transcriptional activation. Our analysis provides the first evidence that this mode of repression also extends to members of the T-box family of transcription factors. The eh1 motif that mediates binding to the WD40 domain of Groucho was not only identified at a conserved N-terminal position within the vertebrate homologues of Tbx18, Tbx15, Tbx22, and Tbx20 but also in the ancestral Amphioxus Tbx15/18/22 protein as well as in the Drosophila Tbx20 homologues Midline and H15 (data not shown). This evolutionary conservation strongly implies that all T-box proteins within the Tbx18/15/22/20 branch of the Tbx1 subfamily act as Groucho-dependent repressors. Widespread expression of vertebrate Groucho proteins is compatible with a corepressor function for these T-box proteins in diverse developmental contexts. However, it will be important to determine the functional significance of this interaction in vivo since Groucho recruitment might be tissue-specific as shown for the transcription factor Runx3 (50).

Mutation of the eh1 motif resulted in only partial loss of Tbx18 and Tbx15 repression activities, indicating the presence of additional repression domains, i.e., interfaces for recruitment of other corepressors. In the case of Tbx15, a recruitment motif for C-terminal-binding protein (CtBP) is present at amino acid positions 39–43. Two members of this corepressor family in the mouse, CtBP1 and CtBP2, also interact with histone deacetylase (HDAC) (51), suggesting an additional mode of gene silencing by Tbx15 that can be experimentally explored in the future. The mode of repression exerted by Tbx15 and Tbx18 is clearly distinct from that of the T-box factors Tbx2 and Tbx3 since the C-terminal motif that mediates repression by direct binding to HDAC1 is not found in Tbx15 and Tbx18 (52). Restriction of Tbx15 and Tbx18 function to transcriptional repression is clearly precocious at this point. The close relative Tbx20 was reported to contain both activation and repression domains, arguing for a more complicated context-dependent transcriptional modulation by T-box proteins (7).

Functional Redundancy of Tbx15 and Tbx18—Tbx15 and Tbx18 form a pair of structurally related T-box proteins. Sequence conservation between Tbx18 and Tbx15 amounts to 92% in the T-box region and extends to short stretches in the less well conserved N- and C-terminal domains, suggesting conservation of important functional interfaces for DNA and protein binding. Indeed, our biochemical analysis of Tbx15 and Tbx18 has demonstrated identical DNA binding properties, subcellular localization, and Groucho-dependent transcriptional repression activities. Together with our finding that Tbx18 and Tbx15 hetero- and homodimerize, this indicates that the two proteins are likely to regulate a similar set of targets when coexpressed in one tissue. Although Tbx15 and Tbx18 show highly specific and largely non-overlapping expression during mouse development (39, 53), they are coexpressed in the proximal region of the developing limb bud. Lack of defects in the appendicular skeleton of Tbx18−/− mice and mild phenotypic changes in Tbx15−/− limbs might thus indicate functional redundancy in the development of this structure.

This biochemical equivalence group is likely to include Tbx22, the third member of the Tbx15/18/22 subgroup in the Tbx1 subgroup of murine T-box genes. The T-box of Tbx22 is highly related to those of Tbx15 and Tbx18, exerting similar DNA binding preference to T half-sites, and also acts as a transcriptional repressor in reporter assays (54). Coexpression of Tbx18 and Tbx22 in anterior halves of epithelial somites suggests functional redundancy in anterior-posterior somite patterning and may explain the delayed expansion of posterior somite fates in Tbx18−/− embryos (13, 39, 43). Phenotypic characterization of compound mutants of Tbx15, Tbx18, and Tbx22 will clarify functional redundancy of these T-box family members in vivo.

As a single copy representative of the subgroup is present in urochordates (Tbx15/18/22 of Ciona intestinalis) and in cephalochordates (Branchiostoma floridae Tbx15/18/22) (55, 56), the three vertebrate genes probably arose from a chordate-specific precursor by two gene duplication events. Evidence suggests that basic transcriptional properties of the members of this subgroup have been preserved throughout vertebrate evolution. Functional specificity may have been acquired by unique
transcriptional repression by Tbx18 and Tbx15

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References

1. Kispert, A., and Herrmann, B. G. (1993) EMBO J. 12, 3211–3220
2. Conlon, F. L., Fairclough, L., Price, B. M., Casey, E. S., and Smith, J. C. (2001) Development (Camb.) 128, 3749–3758
3. Sinha, S., Abraham, S., Gronostajski, R. M., and Campbell, C. E. (2000) Gene (Amst.) 258, 15–29
4. Hiroi, Y., Kudoh, S., Monzen, K., Ikeda, Y., Yazaki, Y., Nagai, R., and Komuro, I. (2001) Nat. Genet. 28, 276–280
5. Bruneau, B. G., Nemer, G., Schmitt, P. J., Charonn, F., Rotbiteille, L., Caron, S., Conner, D. A., Gessler, M., Nemer, M., Seidman, C. E., and Seidman, J. G. (2001) Cell 106, 709–721
6. Garg, V., Kathiriya, L. S., Barnes, R., Schluterman, M. K., King, I. N., Butler, C. A., Rothrock, C. R., Eppen, R. S., Hirayama-Yamada, K., Joo, K., Matsumuka, R., Cohen, J. C., and Srivastava, D. (2003) Nature 424, 443–447
7. Stennard, F. A., Costa, M. W., Elliott, D. A., Rankin, S. A., Lai, D., McDonald, I. P., Niederreither, K., Kolle, R., Bruneau, B. G., Zorn, A. M., and Harvey, R. P. (2003) Dev. Biol. 262, 206–224
8. Leconte, L., Lecoin, L., Martin, P., and Saule, S. (2004) J. Biol. Chem. 279, 47272–47277
9. Takeuchi, J. K., Mileikovskia, M., Koshiba-Takeuchi, K., Heidt, A. B., Monzen, K., Aikawa, R., Akazawa, H., Yamazaki, T., Kudoh, S., and Yazaki, Y. (1999) J. Biol. Chem. 274, 8231–8239
10. Plageman, T. F., Jr., and Yutzey, K. E. (2004) J. Biol. Chem. 279, 19026–19034
11. Naiche, L. A., Harrelson, Z., Kelly, R. G., Goldin, S. N., Gibson-Brown, J. J., Bollag, R. J., Silver, L. M., and Papaioannou, V. E. (2004) Development (Camb.) 131, 5041–5052
12. Kraus, F., Haenig, B., and Kispert, A. (2001) Mech. Dev. 100, 83–86
13. Shiojima, I., Komuro, I., Oka, T., Hiroi, Y., Mizuno, T., Takimoto, E., Monzen, K., Aikawa, R., Kazakawa, H., Yamazaki, T., Kudoh, S., and Yazaki, Y. (1999) J. Biol. Chem. 274, 8231–8239
14. Becker, J. (2006) Mol. Cells 21, 331–336
15. Benisty, A., and Herrmann, B. G. (2001) Mech. Dev. 108, 141–149