T-box Protein Tbx18 Interacts with the Paired Box Protein Pax3 in the Development of the Paraxial Mesoderm*

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The compartmentalization of somites along their anterior-posterior axis is crucial to the segmental organization of the vertebral column. Anterior-posterior somite polarity is generated in the anterior presomitic mesoderm by Mesp2 and Delta/Notch signaling and is further maintained by two transcriptional regulators, Uncx4.1 and Tbx18, acting in the posterior and anterior somite compartment, respectively. Here, we report that the paired box transcription factor Pax3 cooperates with the T-box protein Tbx18 in maintaining anterior somite half identity. Our findings that both genes are co-expressed in the anterior presomitic mesoderm and in early somites, that Pax3 and Tbx18 proteins physically interact, and that the loss of Pax3 gene function enhances the vertebral defects (i.e. the gain of vertebral elements derived from posterior somite halves in Tbx18 mutant mice) suggests that the two proteins cooperatively regulate the gene expression program necessary for maintaining anterior-posterior somite polarity. Genetic interaction of Pax3 with Tbx18 and the closely related T-box gene Tbx15 was also observed in the development of the scapula blade, indicating an additional cooperative function for these genes in the paraxial mesoderm.

The metameric organization of the vertebral column derives from the somites, segmentally repeated units in the paraxial mesoderm. Somites form in a highly periodic and synchronized fashion by condensation and subsequent epithelialization of groups of mesenchymal cells at the anterior end of the presomitic mesoderm (PSM) on both sides of the neural tube. Under the influence of signals from surrounding tissues, somites start to differentiate along their dorso-ventral axis. The ventral part undergoes an epithelial-mesenchymal transition to form the sclerotome, which contains precursors of the vertebral column and parts of the ribs. The dorsal part remains epithelial and generates the dermomyotome, from which skeletal muscles and the dermis of the skin will develop. In addition to differentiation along the dorso-ventral axis, somites become subdivided into distinct anterior and posterior compartments. Anterior-posterior (AP) polarization of somites underlies the segmental arrangement of the peripheral nervous system, since trajectories of neural crest and spinal nerves are confined to anterior somite halves. On the level of the sclerotome, the differential contribution of either compartment to the forming vertebra affects the structure of the axial skeleton. Vertebral bodies, laminae with the spinal processes, the rib heads, and the distal ribs derive from both somite halves, whereas pedicles with their transverse processes and proximal ribs derive from posterior somite halves only (1–3).

Establishment of somitic AP polarity is closely coupled to the segmentation process. Work from a variety of vertebrate model systems has shown that somite formation is governed by an oscillator known as the segmentation clock that operates in the PSM (4, 5). It is now believed that synchronized oscillations of a number of signaling pathways, including Wnt, fibroblast growth factor, and Notch signaling, are involved in the mechanism of the segmentation clock. Gradients of secreted signaling molecules cooperatively define the segmentation border within the anterior PSM. In this region, Notch oscillation is stabilized to a narrow domain, in which cells with a high Notch pathway activity will constitute the posterior half of a newly forming somite. In an adjacent stripe of cells, Notch signaling is suppressed by the action of the basic helix-loop-helix transcription factor Mesp2. The expression domain of Mesp2 thereby defines the anterior somite half, and its anterior limit demarcates the next segmental border to be formed (6). Correspondingly, loss of Mesp2 activity leads to posteriorization of somites, whereas loss of Delta-like1 (Dll1) gene function and Notch signaling results in somites that bear only features of anterior halves (7).

Molecular players required for the further maintenance of somitic AP polarity have recently surfaced. Genetic evidence from both loss- and gain-of-function studies in the mouse suggest that this process is controlled by the combined action of a pair of transcription factors, the T-box (Tbx) protein Tbx18 and the paired type homeobox protein Uncx4.1, which are expressed in anterior and posterior somite halves, respectively (8, 9). Uncx4.1 is specifically required for the development of pedicles and proximal ribs (10, 11), elements exclusively derived from the posterior lateral sclerotome. In contrast, loss of Tbx18 function results in expansion of pedicles and proximal ribs in the cervical and thoracic region of the axial skeleton (12). Notably, the forced misexpression of Tbx18 in posterior somite...
halves results in reduction of pedicles and proximal ribs (12), suggesting that Tbx18 is sufficient to specify anterior versus posterior somite fates. Opposing phenotypic consequences of loss of either factor are based on molecular cross-regulation. In Uncx4.1 mutants, Tbx18 expression is derepressed in posterior somite halves, whereas in Tbx18 mutants, expression of Uncx4.1 progressively expands in anterior somite halves (12). On the molecular level, Uncx4.1 may therefore act as transcriptional repressor of Tbx18, whereas Tbx18 may regulate Uncx4.1 indirectly by controlling expression of the Notch ligand Dll1 (13). To get further insight into the molecular function of Tbx18, thus into the control of AP-somite compartmentalization, we sought to identify and characterize protein binding partners of Tbx18. This may also help to define transcriptional targets of Tbx18 and their molecular regulation.

Here, we report on the identification of the paired box (Pax) transcription factor Pax3 as a protein binding partner of Tbx18. We characterize this interaction on the biochemical level and define genetically that both transcription factors synergize in the development of the paraxial mesoderm, including anterior-posterior somite compartmentalization and scapula development.

EXPERIMENTAL PROCEDURES

Expression Constructs—Bacterial expression constructs were generated as N-terminal glutathione S-transferase (GST)-fusions in pGEX-4T3 (GE Healthcare). Generation of GST-Tbx18 fusion proteins has been described (13), and constructs covering the T-box region of mouse Tbx15 (aa 41–225) were PCR-amplified from the cDNAs (Clontech) following the manufacturer's instructions. Brachyury (aa 41–225) were PCR-amplified from the cDNAs covering the T-box region of mouse Tbx15 (aa 1–345), Pax3 (aa 1–361), Pax7 (aa 1–503), and Pax9 (aa 1–342) and Tbx18 (aa 1–345) was cloned into pGBKT7 (Clontech). All constructs were sequenced; details on cloning strategies and primer sequences are available upon request.

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 5’ NcoI and 3′ XhoI sites. Some of the eight murine family members, including anterior-posterior somite compartmentalization and scapula development.

RESULTS

T-box and Pax Proteins Interact in Vitro—In order to identify protein interaction partners of Tbx18, we performed a yeast two-hybrid screen. We initially tested a number of fusion constructs of the GAL4-DNA-binding domain with subregions of Tbx18 protein for their quality as bait. A construct encoding a fusion protein with the N terminus and the T-domain of Tbx18, which was expressed and lacked autoactivation in yeast, was transformed into yeast, and the resulting bait strain was mated to a strain pretransformed with a mouse cDNA library from E11.5 whole embryos. One of the clones identified by the yeast two-hybrid screen harbored a partial cDNA for Pax3, a member of the gene family encoding paired box transcription factors (data not shown).

To validate and further investigate the interaction between Tbx18 and Pax3, we performed a series of in vitro binding assays using bacterially expressed subregions of Tbx18 fused to GST and in vitro expressed HA-tagged Pax3 protein (Fig. 1, A and B). In GST pull-down assays, Pax3 was specifically bound to GST-Tbx18 fusion proteins harboring the N-terminal domain and the T-box region (GST-Tbx18(N+T)) and the T-box region alone (GST-Tbx18(T)), respectively. Binding was observed neither with GST nor with GST-Tbx18(N) or GST-Tbx18(C), indicating that the T-domain of Tbx18 mediated the binding to Pax3 (Fig. 1C).

We next generated a series of deletion mutants of the Pax3 cDNA for expression in vitro as HA-tagged peptides to determine which region of Pax3 confers interaction with Tbx18 (Fig. 1, D and E). Pax proteins are characterized by the presence of a conserved N-terminal DNA-binding region, the paired domain. Some of the eight murine family members, including
Pax3, additionally contain a homeodomain as a second DNA-binding region. Pax3 peptides containing the paired domain were efficiently bound to GST-Tbx18(N + T), whereas peptides containing the homeodomain only and/or the C terminus of Pax3 were not efficiently retained (Fig. 1E). In summary, our in vitro binding assays showed that binding of Tbx18 and Pax3 is mediated by the two conserved DNA binding regions, the T-domain and the paired domain.

The interaction between Tbx18 and Pax3 was additionally validated in a mammalian cell system using a nuclear recruitment assay. Transfection of an expression construct for HA-tagged Pax3 revealed constitutive nuclear localization of Pax3. In contrast, Myc-tagged Tbx18 protein lacking the nuclear localization signal (Tbx18ΔNLS) (13) was excluded from the nucleus and localized to the cytoplasm. Upon co-transfection of constructs encoding HA-tagged Pax3 protein and Myc-tagged Tbx18ΔNLS protein, nuclear localization of Tbx18 was regained (Fig. 1F), suggesting that Tbx18ΔNLS in complex with Pax3 is shuttled to the nuclear environment.

Furthermore, co-immunoprecipitation assays were performed in HEK293 cells transfected with full-length constructs for Myc-tagged Tbx18 alone or in the presence of HA-tagged Pax3. In immunoprecipitates obtained with the HA antibody, an enrichment of Myc-tagged Tbx18 protein was detected only upon co-transfection of the Pax3 expression construct (Fig. 1G, left). Conversely, Pax3.HA protein was specifically coimmunoprecipitated with the anti-Myc antibody when Tbx18.Myc protein was present (Fig. 1G, right), providing further proof for complex formation of Tbx18 and Pax3 in a cellular system.

T-box and Paired Box Interaction Is Promiscuous—We next investigated whether binding of Tbx18 to Pax3 is unique among T-box and Pax proteins or whether Tbx18 and Pax3 interact with additional members of the other family as well. In a GST pull-down assay, we found that, similar to Pax3, the closely related Pax7 and the more divergent proteins Pax1 and Pax9 exhibited binding to the T-domain of Tbx18 (Fig. 2A). This interaction was confirmed in the nuclear recruitment assay, where Tbx18ΔNLS was shuttled to the nucleus upon co-expression of HA-tagged Pax1 and Pax9 but not with unrelated nuclear proteins (data not shown).

Conversely, we analyzed if Pax3 is able to bind to other members of the T-box protein family. Therefore, GST fusions of the T-box region of the closely related Tbx15, Tbx18, and Tbx22 proteins and the distant family member Brachyury (Fig. 2B) were expressed in bacteria, purified (Fig. 2C), and incubated with in vitro expressed Pax3 protein. Binding of Pax3 protein was detected to all T-box proteins analyzed; however, binding of Pax3 to Tbx18 was the strongest (Fig. 2D).

Together, these findings suggest promiscuity of binding between T-box and paired box regions, but binding affinities between individual family members might differ substantially.

Comparative Expression Analysis of Tbx15, Tbx18, Tbx22, and Pax3—The facts that we only detected Pax3 but not other Pax family member in our yeast two-hybrid screen and the high affinity binding of Tbx18 with Pax3 in the in vitro assays prompted us to analyze whether this interaction is functionally relevant in vivo. To determine in which tissues such a molecular interaction may occur, we compared the expression patterns of Pax3 and Tbx18 and the two closely related Tbx15 and Tbx22 genes using in situ hybridization analysis of E9.5 wild-type mouse embryos (Fig. 3).
Pax3 was strongly expressed in the dorsal neural tube (Fig. 3D, arrow). Furthermore, Pax3 expression was found in the anterior PSM and in epithelial somites (Fig. 3, D and G). Expression was maintained in the dermomyotomal compartment (Fig. 3G, arrow) and in migrating precursors of the limb musculature (Fig. 3D, arrowhead) (17).

Hence, Tbx18, Tbx22, and Pax3 are co-expressed in the PSM and undifferentiated somites, but expression domains segregate during the differentiation of the sclerotome, myotome, and dermomyotome.

Tbx18 and Pax3 Cooperate in the Development of the Axial Skeleton—The observed physical interaction and the co-expression of Tbx18 (and Tbx22) with Pax3 during somitogenesis suggested that these factors also interact genetically during this process. We analyzed this possibility by generating embryos compound mutant for null alleles of Tbx18 (Tbx18GFP) and Pax3 (Pax3lacZ). On the outbred background on which we maintained these alleles, Pax3−/− embryos were viable at E14.5. This is in contrast to studies where lethality of Pax3−/− embryos was observed between E13.5 and E14.5 when the mutant allele was kept on an inbred background, such as a mix of C3H/101 and BA/Ca or C57Bl6 (17, 18). Tbx18−/− embryos died shortly after birth as reported before (12).

Mice double heterozygous for Tbx18GFP and Pax3lacZ mutant alleles were viable and fertile and were intercrossed to obtain all possible allelic combinations. We harvested embryos at E14.5 and analyzed the skeletons as a read-out of defects of somite patterning and differentiation. We noted that embryos double homozygous for Pax3 and Tbx18 null alleles were severely underrepresented at this stage. Of a total of 123 embryos harvested, we only obtained two double mutants (1.6%) instead of the expected eight (1 of 16; 6.3%). Similarly, the observed number of nine Tbx18−/−,Pax3+−/− embryos (7.3%) displayed a reduction from the expected value (15 embryos; 1 of 8; 12.5%), suggesting that the removal of one or two copies of one wild-type allele in the mutant background of the other gene dramatically enhanced the severity of the embryonic defects. All other genotypes were found in the expected Mendelian frequencies (data not shown).

In wild-type embryos of E14.5, the cartilagenous preskeleton was invested with a segmental array of orderly spaced ribs and vertebra (Fig. 4A). At the thoracic level, ribs were connected to vertebral pedicles (Fig. 4G, black arrowhead).

Strikingly, in 12 of 30 embryos (40%) double heterozygous for both Tbx18 and Pax3 null alleles, we detected isolated expansions of proximal ribs (Fig. 4, A and H, white arrowhead), whereas these malformations were never observed in single heterozygous embryos.

In Tbx18−/− embryos, pedicles and proximal ribs were expanded and formed contiguous cartilagenous bands in the vertebral column at the cervical and thoracic levels and the rib cage, respectively (Fig. 4, C [brackets] and I [arrowheads]) (12).

In all Tbx18−/−,Pax3+−/− embryos analyzed (n = 9), expansions of proximal ribs were increased in frequency and extended more caudally (Fig. 4D, brackets) and mediially (Fig. 4J, white arrowheads) compared with Tbx18−/− embryos. A further expansion of pedicles was not observed (Fig. 4J, black arrowhead).
In mutants double homozygous for Tbx18 and Pax3 null alleles, the body axis was dramatically shortened ($n = 2$; Fig. 4E). The severity of the skeletal defects in cervical vertebrae was unchanged compared with Tbx18 mutant embryos. In contrast, the lateral parts of the vertebrae, neural arches and pedicles, were largely expanded at the thoracic and lumbar level (Fig. 4E, black arrowhead) and frequently misconnected to the vertebral bodies that were often split (Fig. 4E, asterisks and white arrow, respectively). Proximal parts of ribs constituted large contiguous plates of cartilage on both sides of the vertebral column (Fig. 4K, white arrowheads).

In Pax3$^{-/-}$ embryos, defects of the axial skeleton, including fusions of neural arches of adjacent vertebrae, occurred mainly in the lumbar-sacral region (Fig. 4F, arrowhead), and rib fusions and bifurcations were apparent (Fig. 4F, arrow). However, skeletal defects were generally less severe than the ones described for Pax3 alleles maintained on inbred genetic backgrounds (17, 18). Notably, and in contrast to Tbx18 mutants, the proximal ribs were unaffected, and the pedicles were spaced regularly (Fig. 4L).

Together, our results demonstrate genetic interaction of Pax3 and Tbx18 in the formation of the axial skeleton. Removal of Pax3 function enhances the phenotypic changes associated with the loss of Tbx18, namely the expansion of derivatives of the posterior lateral sclerotome, pedicles, and proximal ribs.

**Tbx18 and Pax3 Cooperate in the Maintenance of Anterior Somite Halves—Co-expression of Tbx18 and Pax3 in undifferentiated somites suggests that not only the lateral sclerotome but also other somitic compartments could be affected by the combined loss of Pax3 and Tbx18 functions. To determine patterning and differentiation of the somitic mesoderm into myotome and sclerotome more carefully, we analyzed expression of molecular markers at E10.5. Within the collected embryos at this stage ($n = 221$), all genotypes were found in the expected frequencies, indicating that lethality of Tbx18/Pax3 double mutant embryos occurred between E10.5 and E14.5.

**FIGURE 4. Dose-dependent requirement of Tbx18 and Pax3 in the formation of the axial skeleton.** Alcian blue-stained preparations of cartilaginous preskeletons of E14.5 Tbx18/Pax3 compound mutant embryos with genotypes indicated on the top, A–F, dorsal views; G–L, magnifications of the boxed regions. The arrangement of pedicles (black arrowheads) and proximal ribs (white arrowheads) is highlighted. In contrast to wild-type embryos (A and G), a fraction of Tbx18$^{+/+}$/Pax3$^{+/+}$-embryos (12 of 30; 40%) displays expansions of proximal ribs ($B$ and $H$). Tbx18$^{+/−}$/Pax3$^{+/−}$-embryos show contiguous bands of proximal ribs (C, bracket) and expanded pedicles (I, black arrowhead). Rib defects are further increased in severity in Tbx18$^{-/-}$/Pax3$^{+/−}$ compound (D, bracket; J, white arrowheads) and Tbx18$^{-/-}$/Pax3$^{-/-}$ embryos (K, white arrowheads). In Tbx18$^{-/-}$/Pax3$^{+/−}$ embryos, pedicles and neural arches are contiguous in thoracic and lumbar regions (E and K, black arrowheads) and frequently unconnected to the vertebral bodies (E, asterisks). The white arrow indicates split vertebra. In contrast, fusions of neural arches in Pax3 single mutants are present mainly in lumbar regions (F, arrowhead), and rib fusions and bifurcations occur more distally (F, arrow).
Tbx18 and Pax3 in Somitogenesis

FIGURE 5. Tbx18 and Pax3 cooperate in AP-somite compartmentalization. A–M, expression analysis of somite differentiation markers in E10.5 Tbx18/Pax3 compound mutant embryos. Lateral views of differentiated somites at the interlimb level, with anterior to the left. Genotypes are indicated on top. A–D, Myogenin expression reveals that combined loss of Tbx18 and Pax3 does not affect formation of the myotome. The hypaxial domain of the dermomyotome is truncated equally in Pax3−/− (C) and Tbx18−/−, Pax3−/− embryos (D, arrows). E–H, polarized expression of the lateral sclerotome marker Pax9 is cooperatively regulated by Tbx18 and Pax3. Segment boundaries are highlighted with black lines, demonstrating expansion of Pax9 expression in Tbx18−/− embryos into anterior (a) somite halves (F). In Tbx18−/−, Pax3−/− embryos (n = 3), Pax9 expression appears almost homogenous between anterior and posterior (p) somite halves (H). Note that in somites of Pax3−/− embryos, polarization of Pax9 expression is normal (G). I–M, restriction of Uncx4.1 expression to the sclerotome of posterior somite halves depends on both Tbx18 and Pax3. Anterior expansion of Uncx4.1 somite expression in Tbx18−/− embryos (J) is further increased by additional loss of Pax3 (n = 2) (L). Note that in Pax3−/− embryos, Uncx4.1 is restricted to the sclerotome of posterior somite halves (n = 2) (K). Isolated expansions of adjacent Uncx4.1-positive segments were observed in 3 of 10 Tbx18−/−, Pax3−/− compound mutant embryos (M, arrowheads). N–Q, comparative in situ hybridization analysis of Tbx18 expression in E9.5 wild-type (N) and Pax3−/− embryos (O) and of Pax9 expression in E9.5 wild-type (P) and Tbx18−/− embryos (Q) shows that somitic expression of Tbx18 and Pax3 is independent of each other.

enhanced compared with Tbx18−/− embryos, demonstrating a further expansion of posterior somitic identity (Fig. 5I). In embryos heterozygous mutant for Pax3 or Tbx18, Uncx4.1 expression was normal. In contrast, in 3 of 10 double heterozygous embryos, expansions of Uncx4.1 expression into anterior halves of differentiated somites were detected (Fig. 5M, arrowheads).

Cooperativity of Pax3 and Tbx18 in AP polarization of somites may also derive from mutual regulation of the two genes in early somitogenesis. However, unchanged expression of Tbx18 in Pax3−/− somites (Fig. 5O) and of Pax9 in Tbx18−/− embryos (Fig. 5Q) indicates that genetic cooperativity more likely stems from co-regulation of transcriptional target genes.

Genetic Interactions in the Development of the Scapula Blade—Upon inspection of the skeletal preparations of E14.5 Tbx18/Pax3 compound mutant embryos (for numbers see above), we detected additional defects in the appendicular skeleton indicative of a genetic interaction of the two genes in scapula development. In 4 of 19 embryos of the Tbx18−/−, Pax3−/− genotype (21%), a central hole was present in the scapular blades on both sides (Fig. 6E). This phenotype was not observed in Tbx18−/− and Pax3−/− single or in Tbx18−/−, Pax3−/− compound mutant embryos (Fig. 6A–D), demonstrating a stronger contribution of Pax3 in the genesis of this phenotypic trait. Interestingly, the appendicular skeleton of the pelvic girdle was unaffected (data not shown).

Since a similar scapula phenotype has been reported for mice homozygous mutant for Tbx15 (15, 25), the gene most closely related to Tbx18, we decided to test whether Pax3 shows genetic interaction with Tbx15 in scapula development as well. All combinations of compound mutants were found in the expected ratio in E14.5 embryos (n = 63).

Similar to Tbx18, we observed the scapula defect in Tbx15−/−, Pax3−/− embryos (Fig. 5F), although with a much higher penetrance (5 of 7 embryos; 71%). In addition, we noted that loss of Pax3 in the Tbx15 mutant background caused a dose-dependent increase in the phenotypic severity of the scapula defects (Fig. 5, G–I). In fact, in Tbx15, Pax3 double mutant embryos, the proximal region of the scapula was almost completely absent (Fig. 5I).

Comparative expression analysis of Pax3 and Tbx15, Tbx18, and Tbx22 during limb development revealed co-expression of Tbx15 and Tbx18 in mesenchymal precursor cells of the scap-
Members of the T-box and the paired box families of transcriptional regulators control a diverse array of processes during vertebrate embryonic development. Here, we provide evidence that two individual members of these gene families, Tbx18 and Pax3, cooperatively regulate subprograms in the development of the axial and appendicular skeletons. Direct interaction of Tbx18 and Pax3 proteins in vitro and in mammalian cells, co-expression of Tbx18 and Pax3 in the anterior PSM and in newly formed somites, and enhancement of phenotypic defects of Tbx18 mutant embryos upon loss of Pax3 gene function in the derivatives of the lateral sclerotome and the scapula blade suggest that these proteins cooperatively regulate gene expression programs necessary for the maintenance of the AP-somite compartmentalization and the formation of the scapula blade.

**DISCUSSION**

Members of the T-box and the paired box families of transcriptional regulators control a diverse array of processes during vertebrate embryonic development. Here, we provide evidence that two individual members of these gene families, Tbx18 and Pax3, cooperatively regulate subprograms in the development of the axial and appendicular skeletons. Direct interaction of Tbx18 and Pax3 proteins in vitro and in mammalian cells, co-expression of Tbx18 and Pax3 in the anterior PSM and in newly formed somites, and enhancement of phenotypic defects of Tbx18 mutant embryos upon loss of Pax3 gene function in the derivatives of the lateral sclerotome and the scapula blade suggest that these proteins cooperatively regulate gene expression programs necessary for the maintenance of the AP-somite compartmentalization and the formation of the scapula blade.

**FIGURE 6. Genetic interaction between Pax3 and Tbx18/Tbx15 in the development of the scapula blade.** A–I, Alcian blue-stained preparations of cartilaginous preskeletons of E14.5 forelimbs of compound mutant embryos. Limbs of Tbx18+/− (B) and Pax3+/− (D) single mutants were unaffected. However, in 4 of 19 Tbx18+/−,Pax3−/− embryos (21%), a central hole in the scapula blade is present (E, arrow). In an allelic series of Pax3 and Tbx15 mutant alleles, we noted a similar defect in Tbx15+/−,Pax3−/− embryos (P) although with an increased frequency (5 of 7 embryos; 71%). The scapula defect of Tbx15+/− (G) embryos increases in severity with the additional loss of one (H) or both alleles (I) of Pax3 (arrows). J–Q, comparative expression analysis of Tbx15 (J and N), Tbx18 (K and O), Tbx22 (L and P), and Pax3 (M and Q) in the forelimb region. Shown is an overview of expression patterns in E11.5 wild-type embryos (A–I) and a higher magnification of forelimb buds (N–Q). Tbx15 and Tbx18 are co-expressed in the proximal region of the limb bud, whereas Tbx22 and Pax3 are not expressed. In N–Q, the scapula-forming region (26) is surrounded with a red line.

 ula in the proximal region of the E11.5 embryo limb bud (Fig. 6, J, K, N, and O) (26). Expression of Tbx22 was not detected in this region (Fig. 6, L and P). Surprisingly, Pax3 was not expressed in these cells (Fig. 6, M and Q), suggesting that scapular defects might arise from an earlier requirement of Tbx15, Tbx18, and/or Pax3 in precursor cells of the somitic mesoderm from which the scapula is derived.
somite halves. Most notably, in double heterozygous embryos, we observed expansions of proximal ribs, which we never detected in single heterozygotes. On the molecular level, these phenotypic changes were paralleled by a further expansion of the expression domain of Uncx4.1, demonstrating a requirement of both Pax3 and Tbx18 in maintenance of the anterior somite fate.

Tbx18 and Pax3 are coexpressed in the anterior PSM and epithelial somites, but expression domains subsequently segregate to the anterior lateral sclerotome in the case of Tbx18 and the dermomyotome for Pax3, respectively. This and the finding that Pax3 and Tbx18 interact on the protein level (see below) suggest that Pax3 and Tbx18 cooperativity results from molecular interaction occurring in early somite development.

It is noteworthy that a functional requirement for Pax3 in early somite development has previously also been suggested by Schubert et al. (17), who studied defects of the axial skeleton in the Pax3 mutant mice maintained on the C57/B16 inbred genetic background. There, defects in AP-somite patterning and disturbed somite boundaries were noted and also correlated with Pax3 expression during early somite formation. The skeletal phenotype of Tbx18/Pax3 double heterozygous embryos exhibited a partial penetrance only, further indicating that the function of Pax3 is subject to genetic modification. Genetic modifiers might be represented by other members of the T-box and paired box gene families that are co-expressed with Pax3 and Tbx18 in early somitogenesis. We have shown that Tbx22, a gene closely related to Tbx18, is co-expressed with Tbx18 in anterior somite halves and exhibits similar biochemical properties (13, 29). Similarly, Pax3 gene function might be partially compensated by Pax1 and Pax9, which are co-expressed with Pax3 in early somites (24). Since we have shown that Pax3 can bind to other T-box family members, including Tbx22, and that Tbx18 can also interact with Pax1 and Pax9 in vitro, a complex network of Pax and T-box proteins may cooperate in early somite development.

The Tbx18/Pax3 double mutants displayed contiguous cartilaginous elements, similar to Mesp2 mutant embryos, which have completely caudalized somites (30). Moreover, isolated expansions of proximal ribs, as seen in the Tbx18/Pax3 double heterozygotes, have also been reported for a hypomorphic allele of Mesp2 (31). Although the expression of Mesp2 is unchanged in Tbx18/Pax3 double mutants (data not shown), the somitic expression of Tbx18 is absent, and the expression of Pax3 is strongly reduced in Mesp2 null embryos (32, 33), arguing that after the establishment of somite AP polarity, Tbx18 and Pax3 act downstream of Mesp2 to maintain AP-somite compartmentalization.

The cellular and genetic programs that are co-regulated by Tbx18 and Pax3 are currently unclear. We have previously hypothesized that Tbx18 controls maintenance of anterior somite fates by direct repression of Delta-like 1 (Dll1) transcription (13) and, thus, suppression of Notch signaling that confers posterior somite fates (7). When testing the same Dll1 promoter fragment in transactivation assays using HeLa cells, we failed to detect a cooperative effect of Pax3 and Tbx18 on Dll1 repression (data not shown). However, elements mediating Pax3 binding may reside in promoter regions outside the fragment tested. A role for Pax3 as a transcriptional repressor that synergizes with Tbx18 is compatible with the described interaction of Pax3 with the transcriptional co-repressors HIRA and Daxx (34).

Pax3 and Tbx18 Interaction Is Mediated by Conserved DNA-binding Regions—In this study, we identified Pax3 as a binding partner of Tbx18 in a yeast two-hybrid screen and validated this interaction in vitro and in mammalian cells. We mapped the interaction domain to the T-box region of Tbx18 and the paired domain of Pax3, demonstrating that these DNA-binding domains have an additional role as protein–protein interaction motifs. Although we and others have shown that the T-box region mediates binding to the homeobox region in other homeodomain transcription factors, including Nkx2-5 (13, 35), the homeodomain of Pax3 was not efficiently bound by Tbx18, suggesting that interaction of DNA-binding regions is selective.

However, we found that the interaction between paired box and T-box regions is promiscuous among divergent members of both families, suggesting functional co-operativity of other T-box and Pax proteins in tissues of co-expression. One example has previously been presented by the pair of Tbx5 and Pax6 that may cooperate to regulate dorso-ventral patterning of the optic cup (36).

Interaction among different classes of DNA-binding transcription factors is likely to represent a common mechanism to increase specificity of target gene recognition. For T-box proteins, the combinatorial function with other classes of DNA-binding proteins in target promoter regulation is well established. One example is the interaction of Tbx2, Tbx5, Tbx18, and Tbx20 with the transcription factors Gata4 and Nkx2–Nkx5 to regulate cardiac expression of Nppa (natriuretic peptide precursor type a) (13, 35, 37–41). Likewise, Pax proteins have been shown to interact with a number of different DNA-binding proteins, including Pax3 interacting with Sox10 and Mox2 and Pax6 binding to pRB (34). Future approaches for the identification of target genes of Tbx18 could benefit from a search of the combined presence of conserved T-sites and paired binding sites (42).

Pax3 Cooperates with Tbx15 and Tbx18 in the Development of the Scapula Blade—Our analysis of the phenotypes of Tbx18/Pax3 compound mutants uncovered an additional requirement for both genes in the formation of the scapula blade. In compound mutant embryos, the scapula blade exhibited a central hole. In addition, loss of Pax3 dose-dependently increased the severity of the scapula defect in the mutants for Tbx15 (15, 25), the T-box gene most highly related to Tbx18.

The formation of the scapula blade, the thin posterior extension of the shoulder girdle is unusual in the respect that it neither derives from the sclerotomal cells of the somite nor from the lateral plate mesoderm like the limb skeleton but from a set of eight dermomyotomes from somites 17 to 24 as revealed by chick-quail chimeric analysis (43). Hence, a common Pax3 positive pool of precursor cells contributes to both limb muscles and the scapula. Under the influence of signaling pathways from the surroundings, including the surface ectoderm, the subpool of scapula precursor cells switches expression from Pax3 to Pax1 and enters chondrogenic differentiation (44). The scapula blade is an extremely thin bone that is exquisitely sen-
Tbx18 and Pax3 in Somitogenesis

sitive to slight alterations in proliferation and thus reduction of the pool of precursor cells. Since we did not detect co-expression of Pax3 with Tbx15 and Tbx18 in the scapula-forming region (26), Pax3 and Tbx15/Tbx18 may act subsequently rather than simultaneously. Loss of Pax3 function may lead to a reduction of the dermatomyotomal precursor pool that becomes available for Tbx15/Tbx18 to act on. Further loss of Tbx15 and Tbx18 function may then reduce the number of mesenchymal precursor cells of the scapula under a critical threshold required for the condensation and/or chondrification process. Alternatively, Pax3 protein may persist in the precursor cells of the scapula for some time, given an opportunity for molecular interaction with these T-box transcription factors.

Interestingly, Tbx15 has been shown to synergize with a number of other transcription factor genes, including Gli3 and aristaless-type homeobox genes (Alx4 and Cart1) in scapula development (25), arguing for a complex network of developmental regulators involved in patterning/differentiation of this bone.

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