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Tbx4 and *Tbx5* Acting in Connective Tissue Are Required for Limb Muscle and Tendon Patterning

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SUMMARY

Proper functioning of the musculoskeletal system requires the precise integration of bones, muscles, and tendons. Complex morphogenetic events ensure that these elements are linked together in the appropriate three-dimensional configuration. It has been difficult, however, to tease apart the mechanisms that regulate tissue morphogenesis. We find that deletion of *Tbx5* in forelimbs (or *Tbx4* in hindlimbs) specifically affects muscle and tendon patterning without disrupting skeletal development, thus suggesting that distinct cues regulate these processes. We identify muscle connective tissue as the site of action of these transcription factors and show that N-Cadherin and β-Catenin are key downstream effectors acting in muscle connective tissue and regulating soft-tissue morphogenesis. In humans, *TBX5* mutations lead to Holt-Oram syndrome, which is characterized by forelimb musculoskeletal defects. Our results suggest that a focus on connective tissue is required to understand the etiology of diseases affecting soft tissue formation.

INTRODUCTION

Dissecting the cues involved in patterning specific tissues in the developing embryo has proven to be a challenge. The vertebrate limb has been a useful model by which to study these processes, and much effort has been aimed at identifying the cues that pattern the limb skeleton (Mariani and Martin, 2003). For the limb skeleton to function properly, it is critical that the appropriate associated muscles become anchored to the skeletal scaffold via the correct tendons. These three tissues (bone, muscle, and tendon) must interact with each other in three-dimensional (3D) space with high fidelity to form a functional musculoskeletal system. Although much is known about the molecular pathways that determine muscle cell fate and subsequent differentiation (Biressi et al., 2007), very little is known about the mechanisms that regulate the morphogenesis of individual muscles and their associated tendons.

The T-box transcription factors *Tbx5* and *Tbx4* are expressed in the forelimb- and hindlimb-forming regions, respectively, of the lateral plate mesoderm prior to and during limb bud initiation. Once limb buds have formed, both genes are expressed broadly in the limb mesenchyme (Logan et al., 1998; Rallis et al., 2003) until later stages of development (Hasson et al., 2007; Naiche and Papaioannou, 2007b). Their temporal and spatial expression pattern suggests that *Tbx5* and *Tbx4* may have roles in limb patterning processes. Mutations in human, *TBX5* and *TBX4*, are associated with Holt-Oram syndrome (HOS) (Basson et al., 1997; Li et al., 1997) and Small Patella syndrome (SPS) (Bongers et al., 2004), respectively, and both syndromes are characterized by various limb defects in addition to other abnormalities.

Using a conditional deletion strategy in mice and 3D imaging techniques (Sharpe et al., 2002; Weninger and Mohun, 2002), we show that in the absence of *Tbx5* in the forelimb and *Tbx4* in the hindlimb, limb muscle and tendon patterning is disrupted. Although limb muscles undergo terminal differentiation and myotubes fuse to form muscle bundles, the muscles that form in these mutant limbs are the incorrect size and shape, undergo abnormal splitting, and insert at the inappropriate locations on bone. The associated tendons also show abnormal patterning. Significantly, the limb skeleton is not affected by this *Tbx4*/*Tbx5*-deletion regime (Hasson et al., 2007; Naiche and Papaioannou, 2007b), indicating that the patterning of these tissues can be separated from one another and suggesting that cues required for muscle and tendon patterning are independent from those of the skeleton. This is consistent with the observation that some muscle phenotypes in HOS patients are not associated with corresponding skeletal abnormalities (Newbury-Ecob et al., 1996; R.A. Newbury-Ecob, personal communication). We identify the temporal developmental window in which this activity is carried out and demonstrate that reduction of N-Cadherin and β-Catenin causes muscle connective tissue (MCT) deformities that account for this phenotype. This study reveals a molecular mechanism affecting soft tissue patterning in the limb and sheds light on the previously unappreciated role of connective tissues in the development of and diseases affecting the limb musculoskeletal system.

RESULTS

Tbx5 and *Tbx4* Are Required for Limb Muscle Patterning

Tbx5 is expressed in the cells of the lateral plate mesoderm that ultimately form the forelimb bud and is known to be an essential component of the genetic cascade that triggers limb initiation since in its absence no forelimb bud forms (Agarwal et al., 2003; Ahn et al., 2002; Rallis et al., 2003). *Tbx4* appears to have an equivalent role in the hindlimb (Minguillon et al., 2005), and, although a nascent bud does form in the *Tbx4* homozygous null mutant, it fails to develop further (Naiche and Papaioannou, 2003). Both *Tbx5* and *Tbx4* continue to be expressed broadly in the limb bud mesenchyme beyond limb initiation stages; however, neither gene is required to maintain outgrowth and patterning of the limb skeleton. To test whether *Tbx5* and *Tbx4* are required for patterning other tissues of the musculoskeletal system, namely, the muscles and tendons, we carried out conditional deletion of these genes at early stages of limb outgrowth (embryonic day [E] 8.5–E12.5) and subsequently monitored muscle and tendon development.

By E14.5, tissues of the musculoskeletal system have largely assumed their mature arrangements, and individual muscle bundles and associated tendons are identifiable (DeLaurier et al., 2006). To follow muscle pattern, we used an antibody that enables us to identify terminally differentiated muscles by using whole-mount staining (Figure 1). Deletion of *Tbx5* and *Tbx4* between E9.5 and E10.5 leads to equivalent disruptions of normal muscle splitting patterns and muscle sizes and alters the sites of individual muscle origins and insertions. For example, in the wild-type forelimb, the spinodeltoidus (Spd), the M. Triceps brachii longus (Tbl), and the M. Triceps brachii lateralis (Tblt) have characteristic origin and insertion sites (Figure 1A). In the *Tbx5* mutant, the muscle bundles in the equivalent region have a common origin at one focus (Figure 1B, white arrow) and have split to form smaller muscle bundles that insert at aberrant positions. Significantly, if deletion regimes are carried out at later stages (E11.5 for *Tbx5*, E12.5 for *Tbx4*; see Table S1 available online), limb development is apparently unaffected. To analyze the muscle splitting and insertion phenotypes in more detail, we carried out a 3D analysis of muscle morphology with optical projection tomography (OPT) (DeLaurier et al., 2006; Sharpe et al., 2002) and high-resolution episcopic microscopy (HREM) (Weninger and Mohun, 2002). As an example of abnormal muscle splitting and insertion in the mutant, we focused on the three triceps muscles of the forelimb, Tbl, M. triceps brachii medialis (Tbm), and Tblt, that insert on the olecranon process of the ulna (Figure 1C). In the *Tbx5* mutant, the muscles in the equivalent region have split into additional bundles (short arrow), some of which now insert in the more distal shaft of the ulna (Figure 1D, long arrow; Movies S1 and S2). Zeugopodal muscles, such as those occupying the region of the M. extensor digitorum communis (Edc), are similarly ectopically split in the *Tbx5*-deleted limb (Figures 1E and 1F). This perturbation of normal muscle pattern can also be observed in the muscles of the autopods (data not shown). Similar muscle mispatterning is also observed in *Tbx4* mutant hindlimbs. For example, in the analogous region to the M. Lumbricales (Lum) and M. flexor digitorum brevis (Fdb) of the wild-type, hindlimb (Figure 1G) muscles in the *Tbx4* mutant misinsert and assume the wrong shape (Figure 1H).

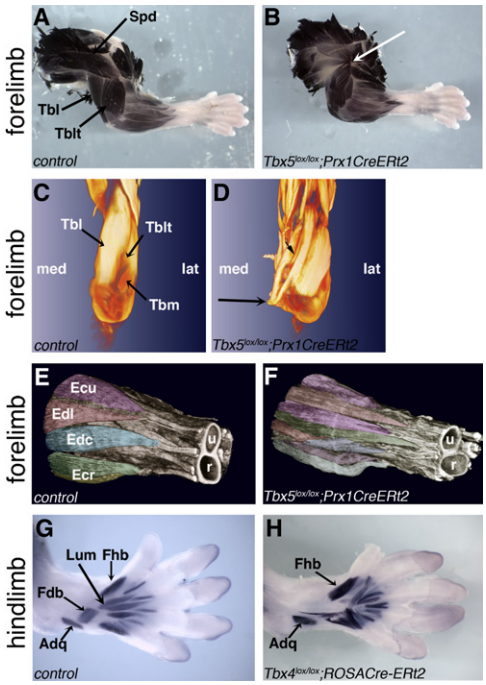

Figure 1. Limb Muscle Pattern Is Disrupted after Deletion of Tbx5 or Tbx4

(A–H) Whole-mount immunohistochemistry of (A–D) E16.5 forelimbs or (G and H) E15.5 hindlimbs with the anti-muscle myosin antibody. The (A) normal pattern of forelimb muscles is disrupted in the (B) *Tbx5* mutant (*Tbx5*$^{lox/lox}$; *Prx1CreERt2*) (dorsal views). Still images of an OPT, 3D analysis, viewed from the back of the upper arm, comparing (C) control and (D) *Tbx5* mutant forelimbs, showing ectopic splitting (small arrow) and insertions of muscles (long arrow) at the region of the Tbl, Tblt, and Tbm. Dorsal view with HREM of (E) control and (F) *Tbx5* mutant forelimb, showing ectopic splitting of muscles in the region of the zeugopod (individual muscle bundles have been shaded for clarity; however, colors do not indicate muscle type). (G) Muscle pattern in the ventral footplate is disrupted in the (H) *Tbx4*-deleted hindlimb. Control littermates shown are (A and C) *Tbx5*$^{lox/+}$; *Prx1CreERt2* or (E and G) wild-type. CreERt2 was activated by TM administration at (A, B, G, and H) E10.5 or at (C–F) E9.5. Spd, M. spinodeltoideus; tbl, M. triceps brachii (long); Tblt, M. triceps brachii (lateral); Tbm, M. triceps brachii (medial); Amg, M. Adductor magnus; Sm, M. semimembranosus; Gra, M. gracilis anticus; Ecu, M. extensor carpi ulnaris; Edl, M. extensor digitorum lateralis; Edc, M. extensor digitorum communis; Ecr, M. extensor carpi radialis (longus and brevis); r, radius; u, ulna; Lum, M. lumbricales; Adq, M. abductor quinti; Fhb, M. flexor hallucis brevis; Fdb, M. Flexor digitorum brevis. See also Table S1, Movies S1 and S2, and Figure S1.

After conditional deletion of *Tbx5* and *Tbx4*, all of the limb musculature is affected, and it is not possible to detect any limb muscles that retain all aspects of their normal pattern. Terminal differentiation of the limb muscle, however, is not apparently affected in either *Tbx5* or *Tbx4* mutants. The sarcomeric marker muscle myosin is expressed normally, as judged by immunohistochemical staining (Figure 1); myoblasts undergo fusion to form muscle fibers and bundles; and the cytoarchitecture of the sarcomere in the *Tbx5* mutant limbs, analyzed by transmission electron microscopy, is not affected (data not shown). Significantly, although deletion of *Tbx5* from E10.5 leads to dramatic muscle and tendon patterning defects (see below), the formation and patterning of the limb skeleton are unaffected

(Hasson et al., 2007) (data not shown; Table S1). Likewise, conditional deletion of *Tbx4* at E10.5 produces some minor skeletal abnormalities, but deletion at E11.5 leads to muscle mispatterning without affecting the skeleton (Naiche and Papaioannou, 2007b) (Table S1), indicating that at least some aspects of the patterning of two elements of the musculoskeletal system, the muscles and tendons (see below), can be uncoupled from that of the skeleton. Comparable muscle phenotypes are obtained when a dominant-negative form of *Tbx5* (*Tbx5*-EN) (Rallis et al., 2003) is misexpressed in chick wings (Figure S1), suggesting that the underlying molecular mechanisms regulated by *Tbx5* (and by inference *Tbx4*) in limb muscle patterning are conserved across vertebrates.

Tbx5 Regulates Tendon Patterning

The limb muscles are connected to the limb skeleton via tendons. For the correct musculoskeletal pattern to be elaborated, specific groups of tendon progenitors must associate with the appropriate muscle bundles before they make their attachment to the skeleton. Experiments in the chick to generate either muscleless or limbs lacking specific tendons demonstrated that patterning of each tissue is initially independent, but at later stages the two tissues become interdependent (Kardon, 1998). To follow muscle origins and insertions and hence the interactions of muscles with tendons, we deleted *Tbx5* by tamoxifen (TM) administration at E9.5 in mice that also carry a *Scx-GFP* transgene that marks tendons and their progenitors (*Tbx5$^{lox/lox}$; Prx1CreERt2;Scx-GFP*) and analyzed both muscle and tendon pattern in 3D by using OPT (Sharpe et al., 2002) (Figure 2). Deleting *Tbx5* at this stage gives rise to limbs with minor skeletal deformities similar to those commonly seen in HOS individuals, such as triphalangeal thumb. Muscle pattern is altered similarly to that shown in Figure 1, and disruption of tendon pattern is visible (Figure 2). The normal pattern of tendon fibers that connect forearm (zeugopodal) muscles to the skeletal elements of the handplate (Figures 2A and 2C) is unrecognizable in the *Tbx5* mutant (Figures 2B and 2D). Fewer tendon fibers are present; some are thinner than normal, whereas some have fused. Significantly, mispatterned muscles make myotendinous attachments to tendons, and the tendons develop entheses on the forming skeleton, indicating that the signals required for the crosstalk between muscle and tendon and tendon and bone, enabling these fundamental interactions, remain functional in the mutant.

Early Alterations of Muscle and Tendon Pattern

The majority of the myoblasts have migrated into the forelimb by E10.5 (33 somites) (Houzelstein et al., 1999). Previously, we have shown that after TM administration, 18–24 hr are required for full Cre activity from the *Prx1CreERt2* transgene (Hasson et al., 2007). Deletion of *Tbx5* during or after myoblasts have migrated into the limb (i.e., TM administration at E9.5 and E10.5, respectively) results in similar muscle patterning defects, suggesting that the muscle phenotypes are not the result of the myoblasts failing to migrate properly. Consistent with this interpretation, we find that even in limbs in which TM is administered at E8.5 to delete *Tbx5* in the limb at stages at which myoblast progenitors first migrate into the limb, *Pax3* expression in the limb is unaffected at E10.5 (data not shown). Furthermore, the *Tbx5*- and *Tbx4*-deleted limbs do not show a reduction in muscle

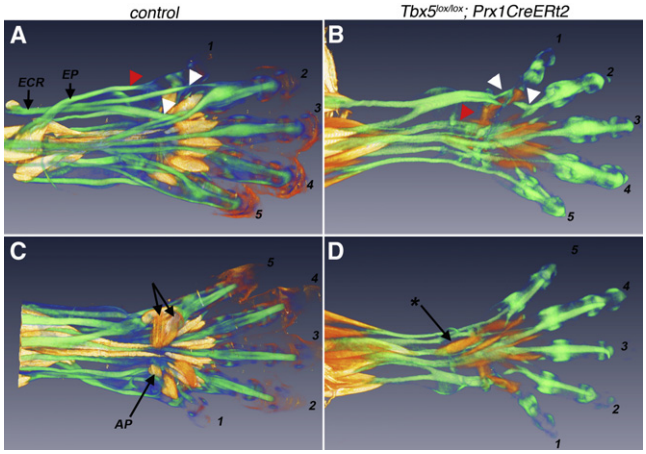

Figure 2. Mispatterning of Tendons Does Not Prevent Their Interactions with Muscles and Skeleton

(A–D) OPT analysis of whole-mount immunohistochemistry of (A and C) E15.5 control and (B and D) *Tbx5*-deleted autopods, of which TM was administered at E9.5, showing mispatterning of muscles (red) and tendons (green). (A and C) Control littermates shown are *Scx-GFP*. (A and B) Dorsal views; (C and D) ventral views. Some tendons and muscles are designated. Note the lack of analogous muscles and tendons in the mutant limbs. ECR, M. extensor carpi radialis; EP, M. extensor pollicis; AP, M. Abductor pollicis; *, ectopic muscle. Triangles mark tendons that in control limbs insert onto digit 1 (red) and digits 2 and 3 (white) but misinsert in the mutant.

mass compared with control littermates (e.g., Figure 1) or in *MyoD* staining at E12.5 (see below); therefore, the total number of myoblasts migrating into the limb in both mutants and wild-type appear to be the same. Taken together, these results suggest that *Tbx5* and *Tbx4* do not regulate the initial migration of myoblast progenitors into the limb.

To identify the underlying mechanism by which *Tbx5* and *Tbx4* exert their muscle- and tendon-patterning activity, we first wished to identify the temporal window of their activity and the earliest observable defects after their deletion. After TM administration into pregnant females at E10.5, we harvested litters at E12.5 and stained for muscle (*MyoD*) and tendon markers (*Scx*). As early as E12.5, when muscle splitting and subdivision into distinct muscle bundles can first be observed, the expression patterns of both *MyoD* and *Scx* are abnormal in *Tbx5* mutants (Figures 3A, 3B, 3E, and 3F, arrows) and *Tbx4* mutants (Figure S2). These phenotypes are further enhanced by E13.5, when the pattern of the emerging muscles is altered from wild-type and ectopic splitting of nascent muscle bundles is observed (Figures 3C and 3D, note arrow; Figure S2). These results indicate that the muscle and tendon phenotypes observed at E15.5–E16.5 are caused by a disruption of earlier *Tbx5*/*Tbx4*-dependent processes that occur at ~E11.5–E12.5, but not earlier. Significantly, these results demonstrate that *Tbx5* and *Tbx4* regulate muscle and tendon patterning before E12.5, when the progenitor pools of these two tissues are developing independently of each other (Kardon, 1998).

Tbx5 Regulates Muscle Patterning in a Nonautonomous Manner

Our results demonstrate that *Tbx5* and *Tbx4* have roles in coordinating forelimb and hindlimb muscle pattern, respectively. To

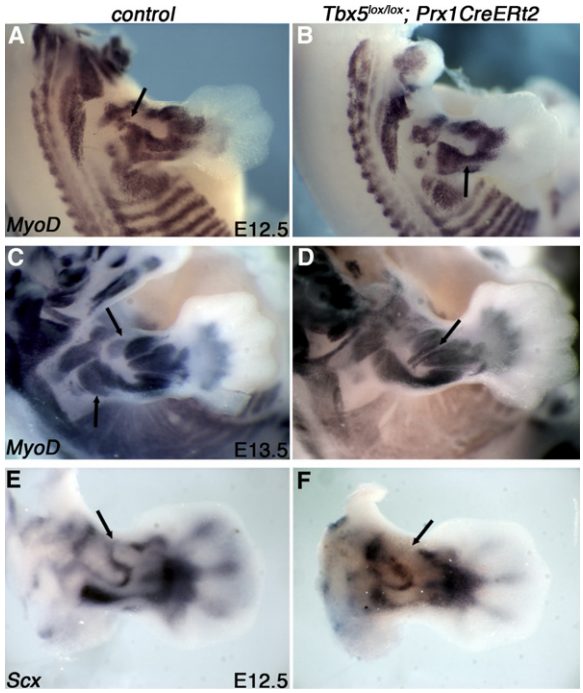

Figure 3. Tbx5 Exerts Its Muscle and Tendon Patterning Activity at ~E11.5–E12.5

(A–F) (A and B) Whole-mount in situ hybridization for *MyoD* at E12.5 reveals subtle differences between (A) control and (B) *Tbx5*-deleted limbs. (C and D) Aberrant splitting of nascent muscle bundles (arrowed in [D]) is clear at E13.5. (E and F) Similarly, tendon progenitors, monitored by *Scx* expression, are mispatterned in the *Tbx5* mutant limbs by E12.5. (A, C, and E) Control littermates shown are *Tbx5$^{lox/+}$; Prx1CreERt2*. TM was administered to pregnant females at E10.5, and embryos were harvested at E12.5–E13.5. See also Figure S2.

further understand the activity of these genes, we wished to identify the cells in which they are acting. Classical embryology and recent molecular data suggest that extrinsic cues are required for patterning limb muscles (e.g., Christ et al., 1977; Kardon et al., 2003), although there are some data suggesting that some nonlimb myoblasts are patterned by intrinsic cues (Alvares et al., 2003). The *Prx1Cre* deleter line is expressed in all limb mesenchymal cells, including the myoblast progenitors once they migrate into the limb (Durland et al., 2008; Logan et al., 2002), and therefore cannot distinguish between autonomous and nonautonomous Tbx5 activity. To overcome this problem, we took advantage of the *Pax3CreKI* deleter line in which *Cre* is inserted into the *Pax3* locus (Engleka et al., 2005) that enables Cre activity, and hence *Tbx5* deletion, in the myoblasts prior to their migration into the limb field, rendering all limb myoblasts *Tbx5* null. Deletion of *Tbx5* in the myoblasts does not affect their patterning (Figure S3), demonstrating that *Tbx5* controls limb muscle patterning nonautonomously, consistent with the model that extrinsic cues are critical for muscle morphogenesis.

Tbx5 Regulates Muscle Connective Tissue Organization

The results reported above demonstrate that *Tbx5* does not function autonomously to pattern the limb muscles. A possible alternative explanation is that *Tbx5* acts in muscle connective tissue (MCT), found adjacent to forming muscles and which has been shown to influence muscle formation (Grim and Wachtler, 1991). *Tbx5* is strongly expressed in MCT cells that are embedded within and ensheath the *MyoD*-positive muscle progenitors (Figures 4A and 4B). To test the function of *Tbx5* in MCT, we deleted this gene and analyzed the MCT at E16.5, a stage at which it can be identified histologically. Deletion of *Tbx5* leads to a disruption of normal MCT organization (Figures 4C and 4D, arrows).

We also analyzed the expression patterns of a battery of molecular markers that have been implicated in limb MCT development or muscle patterning after deletion of *Tbx5*. Recently, Tcf4, a nuclear component of the canonical Wnt signaling pathway, has been shown to be expressed in MCT and tendon progenitors and to be involved in determining the basic pattern of limb muscles (Kardon et al., 2003). *Tcf4* expression is still detectable in the *Tbx5* mutant limbs, demonstrating that MCT is still present; however, the distribution of *Tcf4*-positive cells is altered (Figures 4E and 4F). We also analyzed other genes expressed in limb mesenchyme or MCT that have been implicated in limb muscle formation, such as SD*F1α* and SD*F1β* (Vasyutina et al., 2005), *Mox2* (Mankoo et al., 1999), *SF/HGF* (Dietrich et al., 1999), *Lbx1* (Schafer and Braun, 1999), *Osr1* and *Osr2* (Stricker et al., 2006), and *BMP2/4* (Bonafede et al., 2006), and they all showed the same features in that they continued to be expressed in the mutant although their pattern of expression was altered (Figures 4G–4L; data not shown).

In *Tbx5* conditional mutants, MCT is disorganized throughout the limb (e.g., Figure 4D), consistent with our observations that all limb muscles and tendons appear to be affected. This suggests that *Tbx4/Tbx5* regulate a fundamental process within the limb mesenchyme, such as cell:cell adhesion, a process known to play key roles in development and tissue morphogenesis (Gumbiner, 2005). Several classes of proteins and signaling cascades have been shown to participate in cell adhesion. Among these, β-Catenin is a focal player that has major roles in both cell:cell adhesion as well as signaling (Ben-Ze'ev and Geiger, 1998; Brembeck et al., 2006). Furthemore, Wnt signaling has been implicated in limb muscle patterning in the MCT via the activity of Tcf4 (Kardon et al., 2003) as well as in limb muscle development (Anakwe et al., 2002). In wild-type E12.5 limbs, β-Catenin is clearly detectable at the cell membrane in the Tcf4-expressing MCT cells, whereas in *Tbx5*- and *Tbx4*-deficient limbs there is a marked decrease in its levels at the cell membrane (Figures 5A and 5B; Figure S4). No difference in *β-Catenin* transcription is observed between control and *Tbx5*-deleted limbs with in situ hybridization (data not shown). To verify and quantify these observations, we performed quantitative PCR (qPCR). No difference is observed in *β-Catenin* transcript levels in *Tbx5*-deleted limbs (see Experimental Procedures) and their heterozygous littermate controls (Figure 5H), suggesting that the disruption of β-Catenin expression in *Tbx4*- and *Tbx5*-deleted limbs is not at the transcriptional level.

After the loss of membrane-tethered β-Catenin, a concomitant reduction of its membranal anchors, the Cadherins, are observed (Cali et al., 2007). Like β-Catenin, Cadherins participate in multiple processes and play cardinal roles in cell adhesion (Halbleib and Nelson, 2006). Consequently, we tested

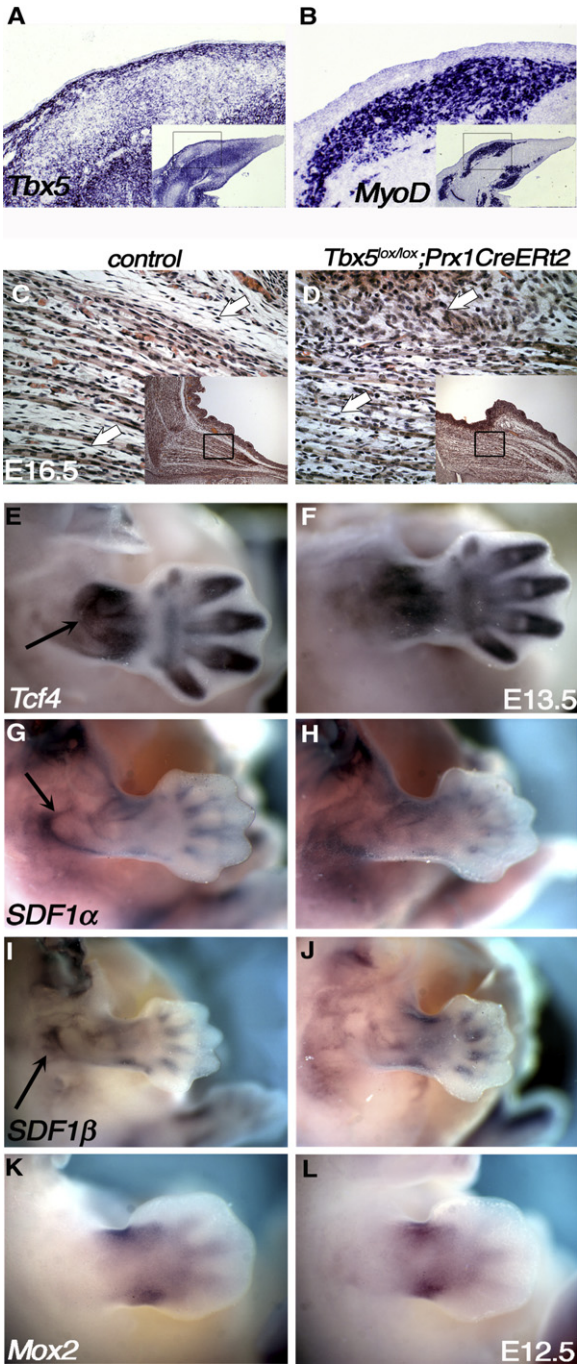

Figure 4. Candidate Muscle Patterning Genes Are Still Expressed after Deletion of Tbx5

(A–L) (A and B) In situ hybridization of serial sections at E12.5 of (A) *Tbx5* and (B) *MyoD Tbx5* is expressed in the cells ensheathing and embedded within the muscles. H&E staining comparing the histology of (C) control and (D) *Tbx5* mutant limbs. The connective tissue (white arrows) is disorganized in the mutant. The black box within the smaller inset indicates the region covered by the magnified view. No gross changes in expression levels of (E and F) *Tcf4*, (G and H) SD*F1*α, or (I and J) SD*F1*β by E13.5 or (K and L) *Mox2* (by E12.5) are detected by whole-mount in situ hybridization between control and mutant limbs. Black arrows point to normal domains of expression in the (E, G, and I) control that are altered in the (F, H, and J) mutant examples. TM administration at (C, F, H, and J) E10.5 or (L) E9.5. See also Figure S3.

whether certain Cadherins are also affected in the *Tbx4*- and *Tbx5*-deleted limbs. In E12.5 limbs in which *Tbx5* has been deleted at E8.5, there is a marked reduction in pan-Cadherin antibody staining (data not shown), suggesting that one or more Cadherins are affected. N-Cadherin is a classical, mesenchymally expressed Cadherin that has been suggested to participate in limb myoblast pathfinding (Brand-Saberi et al., 1996). After deletion of *Tbx5* or *Tbx4*, N-Cadherin is downregulated in Tcf4-positive MCT cells (Figures 5C and 5D). RNA in situ staining and qPCR analysis confirmed that as with β-Catenin, *Tbx4*/*Tbx5* regulation of N-Cadherin is not at the transcriptional level (Figure 5H; data not shown). Finally, *Tcf4* transcription was previously shown to be downstream of Wnt signaling (Kardon et al., 2003); however, neither RNA in situ hybridization (Figures 4E and 4F) nor qPCR showed any difference in its levels after the deletion of *Tbx5* (Figure 5H), reinforcing a model in which reduction of β-Catenin in the MCT does not affect Wnt signaling.

To further characterize this reduction, we marked the forming muscles with MyoD and costained with N-Cadherin. As expected, a strong reduction is observed in the cells ensheathing the muscles (Figures 5E and 5F). Western blots from wild-type and *Tbx5* mutant limbs with N-Cadherin and β-Catenin antibodies confirmed a decrease in the levels of both proteins in the mutant (Figure 5G). Expression levels of another mesenchymally expressed Cadherin, Cadherin 11, are unaffected, however (data not shown), suggesting that there is not a general Cadherin downregulation, and that the response to the loss of β-Catenin in MCT may be limited to N-Cadherin.

Recently, it has been reported that β-Catenin is not required cell autonomously within limb muscles for their embryonic development and patterning (Hutcheson et al., 2009). Our results suggest that N-Cadherin/β-Catenin expressed in MCT have a role in muscle patterning. To directly test this model, we used a *β-Catenin* conditional allele in combination with a Cre transgene (*Prx1Cre(98)*) to delete β-Catenin activity in the limb bud mesenchyme. Embryos were harvested at E13.5, and the limb buds were analyzed *MyoD* expression to assess whether the forming muscles were mispatterned. Deletion of *β-Catenin* in the limb mesenchyme leads to ectopic muscle splitting and muscle mispatterning in both forelimbs and hindlimbs (Figures 6A–6D), presumably due to the disruption of MCT organization, similar to that observed in the *Tbx5* mutant limbs (Figures 6E and 6F, cf. with Figure 4D) and consistent with a model in which the N-Cadherin/β-Catenin complex in the MCT is critical for muscle patterning.

DISCUSSION

Our results reveal a spatiotemporal window in which *Tbx4* and *Tbx5* are required for patterning the soft tissues (muscles and tendons) of the musculoskeletal system. *Tbx4* and *Tbx5* exclusively regulate muscle and tendon patterning while having no apparent effect on the generation, proliferation, or migration of the progenitors of these tissues, strongly suggesting that they regulate a distinct patterning signal(s), which our results indicate is dependent on the proper organization of MCT. Regulation of the Tcf4-expressing connective tissue can account for the independent patterning activity that Tbx5 and Tbx4 have on both muscles and tendons since Tcf4 is also expressed in

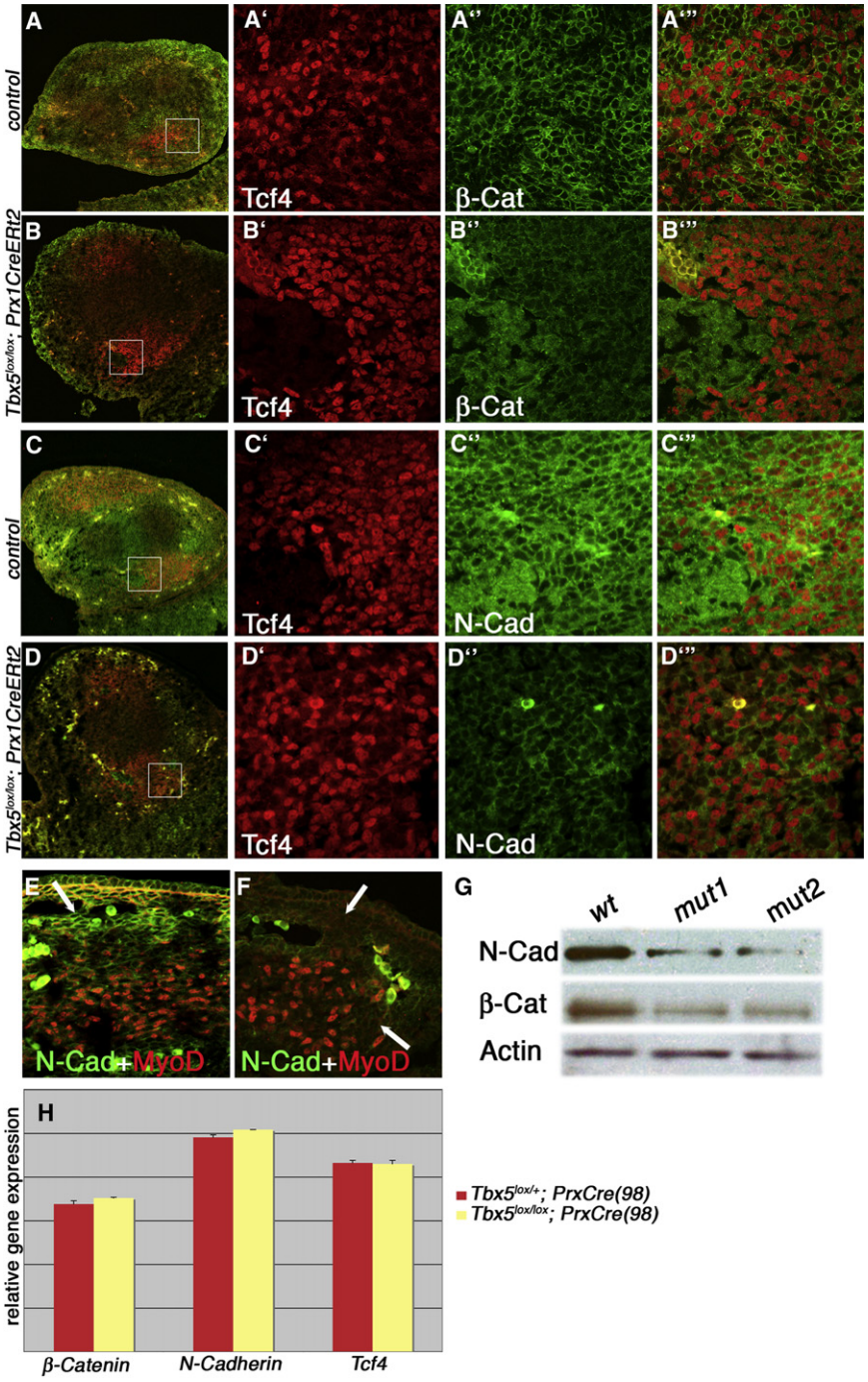

Figure 5. Tbx5 Regulates β-Catenin and N-Cadherin in Muscle Connective Tissue

(A–H) Immunohistochemistry of transverse sections of limbs at E12.5. (A) β-Catenin (green) is localized at the cell membrane of Tcf4 (red)-positive MCT cells. (B) Membrane-localized β-Catenin (green) is lost in Tbx5 mutant Tcf4 (red)-positive MCT cells. (C) N-Cadherin (green) expression is localized at the cell membrane of Tcf4-expressing MCT cells (red). (D) Membrane-localized N-Cadherin (green) is lost in Tbx5 mutant Tcf4 (red)-positive MCT cells. (A–D) Tagged panels show high magnifications of boxed areas. (E and F) Sagittal sections of (E) control and (F) *Tbx5*-deleted limbs stained with MyoD (red) to mark the muscles. N-Cadherin (green) expression in the cells ensheathing the muscles in (E) control (white arrow) is not present in the (F) mutant (white arrows) (bright green cells are autofluorescent red blood cells). (G) Western blot analysis from wild-type or two different*Tbx5* mutant limbs confirmed the reduction of N-Cadherin and β-Catenin.(H) qPCR analysis showing *β-Catenin*, *N-Cadherin*, and *Tcf4* transcripts levels found in control (red bar) do not change in the *Tbx5* mutant (yellow bar). Error bars mark standard deviation. See also Figure S4.

domains where tendon progenitors arise (Kardon et al., 2003). We propose a model in which *Tbx4*/*Tbx5* expressed in the MCT positively regulate expression of N-Cadherin and β-Catenin, which are required for the proper integrity and organization of this tissue that, in turn, is critical for the correct patterning of the adjacent muscles (Figure 6F) and tendons. The loss of *Tbx4*/*Tbx5* leads to downregulation of N-Cadherin and β-Catenin and disorganization of the MCT, resulting in mispatterning of muscles (Figure 6G). Consistent with this model, we show that deletion of β-Catenin results in similar phenotypes.

N-Cadherin has been previously implicated in limb myoblast pathfinding (Brand-Saberi et al., 1996). In addition, Cadherins and β-Catenin expressed in craniofacial connective tissue have been suggested to play a role in patterning adjacent head muscles (Rinon et al., 2007). Interestingly, *β-Catenin*$^{lox/lox}$*; Prx1Cre(98)* mice also exhibit some cranial muscle mispatterning (not shown) since this Cre deleter is also expressed in the ventral part of the first branchial arch (Logan et al., 2002). Together these data suggest that Cadherin/β-Catenin activity in connective tissue could be a general mechanism regulating vertebrate muscle patterning. Recent data suggest that, like β-Catenin, N-Cadherin does not function solely in cell adhesion, but has many other roles, such as in cell signaling and transcriptional regulation (Halbleib and Nelson, 2006). A major challenge now will be to precisely determine how the N-Cadherin/β-Catenin complex functions in connective tissue to regulate muscle and tendon morphogenesis.

Tcf4 is expressed in MCT, but not myoblasts themselves, and has also been implicated in muscle patterning and formation. *Tcf4* continues to be expressed in *Tbx5* mutants, suggesting that it either acts in a parallel pathway or potentially upstream. Although the *Tbx5*-dependent β-Catenin reduction we observe could, in principle, effect Tcf4/Wnt signaling in the MCT, all our data suggest that this is not the case. First, after the deletion of β-Catenin, protein levels of its associated membranal cofactor,

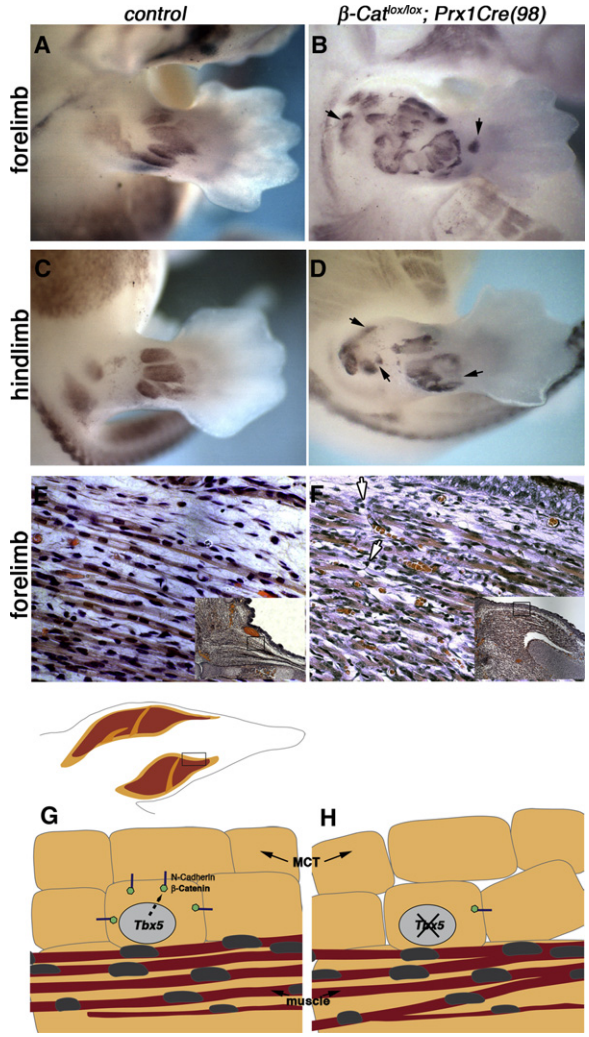

Figure 6. Disrupting the β-Catenin/N-Cadherin Complex Leads to MCT Disorganization and to Ectopic Muscle Splitting and Mispatterning

(A–D) Whole-mount in situ hybridization for *MyoD* on E13.5 limbs to detect the forming muscles in (A and C) control (*β-Catenin*$^{lox/+}$*;Prx1Cre(98)* compared to (B and D) mutant (*β-Catenin*$^{lox/lox}$*;Prx1Cre(98)*) in forelimbs and hindlimbs. Histological (H&E) staining of an E16.5.

(E and F) (E) Control (wild-type) and (F) *β-Catenin*$^{lox/lox}$*;Prx1Cre(98)* forelimb shows that the MCT (arrow) is disorganized (note the white arrows).

(G) Model depicting activity of *Tbx5*/*Tbx4* in normal MCT (orange) and underlying muscles (red) in the developing limb. In wild-type limbs, *Tbx5* (and *Tbx4*) expressed in the MCT facilitates the expression of N-Cadherin and β-Catenin required for MCT organization and integrity.

(H) In the *Tbx5* (and *Tbx4*) mutant limbs, a strong reduction in N-Cadherin and β-Catenin is observed, leading to the disorganization of the MCT and mispatterning of the adjacent muscles.

N-Cadherin, are reduced in a Wnt-independent manner (Cali et al., 2007). Second, blocking the Wnt pathway by using dominant-negative Tcf4 (Tcf4-EN) affects myotube differentiation, leading to some muscles failing to form while the patterning of other muscles is affected variably (Kardon et al., 2003). In contrast, in the *Tbx4*/*Tbx5*-deleted limbs, all muscles are uniformly affected, and there is no effect on muscle differentiation. Finally, *Tcf4* mRNA was shown to be responsive to Wnt signaling (Kardon et al., 2003); however, we do not detect any changes in *Tcf4* transcript levels after the deletion of *Tbx5* (Figure 5H). Collectively, these differences suggest that the *Tbx4*/*Tbx5*-dependent β-Catenin loss in the MCT affects muscle patterning via a mechanism that is distinct from the Wnt-dependent Tcf4 pathway.

Tbx4 and *Tbx5* have equivalent roles in initiating limb outgrowth during a narrow, early time window at ~E9.0 (Hasson et al., 2007; Naiche and Papaioannou, 2007b) Our current results demonstrate that at later stages of limb development, when the genes are no longer required to initiate limb outgrowth, both genes have a role patterning the limb muscles and tendons. This second pulse of activity lasts for 24–48 hr. Limb muscles are formed from a subpopulation of the hypaxial myoblasts that migrate into the limb buds, and it is when they have entered this environment that these cells receive instructive cues that dictate ultimate muscle morphology (Buckingham et al., 2003). Our data suggest that *Tbx4* and *Tbx5* have been co-opted to pattern limb muscles by regulating a general Cadherin/β-Catenin-dependent muscle patterning "cassette" after myoblast migration has terminated and coincident with the onset of terminal differentiation. Little is known about the tissue interactions that occur during tendon patterning, and the deletion approaches we have taken do not distinguish whether these *T-box* genes are acting autonomously or nonautonomously on tendon progenitors.

Together, our results point to MCT organization and integrity being critical for normal patterning of soft tissues. Accordingly, we suggest that disruption of MCT development, and specifically the Cadherin/β-Catenin complex, play a role in human soft tissue pathologies. In humans, HOS patients can present with soft tissue abnormalities that are not associated with skeletal defects (Newbury-Ecob et al., 1996; Spranger et al., 1997; R.A. Newbury-Ecob, personal communication), consistent with the observations that, despite the widespread soft tissue defects produced in our mouse models after deletion of *Tbx4*/*Tbx5*, the skeleton could be unaffected. We propose that defects in MCT integrity should be explored as an explanation for soft tissue abnormalities and the influence of connective tissue considered in developing strategies for musculoskeletal tissue regeneration therapies.

EXPERIMENTAL PROCEDURES

Transgenic Mice and Embryos

Mouse embryos were staged according to Kaufman (2001). Noon on the day a vaginal plug was observed was taken to be embryonic day (E) 0.5. The mouse lines carrying a conditional allele of *Tbx5* (Bruneau et al., 2001), *Tbx4* (Naiche and Papaioannou, 2007b), *β-Catenin* (Huelsken et al., 2001), *Scx-GFP* (DeLaurier et al., 2006), *RosaCreERt2* (de Luca et al., 2005), and a *Prx1CreERt2* (Hasson et al., 2007) transgene have been described previously. *Prx1Cre(98)* is an independent transgenic line generated with the same construct used to produce the *Prx1Cre* (Logan et al., 2002) transgene. Cre activity in the limbs is detected at slightly later stages (E8.5–E9.0) than that reported for the orginal *Prx1Cre* line.

Tamoxifen Induction

Tamoxifen (TM) preparation and induction was done as described in the Joyner lab webpage (http://www.mskcc.org/mskcc/shared/graphics/ski/Tamoxifen_Preparation.pdf). Adult dam females were gavaged with 6.5 mg TM in corn oil or by intraperitoneal injection of 6 mg 4-hydroxy-TM in 1:10

(v/v) ethanol:sunflower oil (from a stock of 20 mg/ml) at the indicated time points. Cre activity from deleter strains, including *RosaCreERt2* (used here), has been reported to cause apoptosis in embryos (Naiche and Papaioannou, 2007a). Muscle and tendon patterning phenotypes were only observed in animals homozygous for *Tbx5*, *Tbx4*, or β-*Catenin* conditional alleles and carrying a Cre deleter transgene. Animals heterozygous for conditional allele and carrying a Cre deleter transgene had entirely normal limb muscle and tendon patterns, and, in most cases, these are the control examples shown.

Quantitative PCR

RNA was extracted from E12.5 *Tbx5*-deleted or heterozygous limbs by using the RNeasy Mini Kit (QIAGEN), and cDNA was subsequently prepared by using SuperScript III Reverse Transcriptase (Invitrogen). A total of 50 ng cDNA was loaded per qPCR well. The following TaqMan probes (Applied Biosystems) were used: *GAPDH* (4352932), *Cdh2* (682189), *β-Catenin* (705555), *Tcf4* (676819).

In Situ Hybridization

Whole-mount and section in situ hybridization was carried out essentially as previously described (Riddle et al., 1993; Schaeren-Wiemers and Gerfin-Moser, 1993). A minimum of three mutant embryos were analyzed at each stage described with each probe. Most probes have been described previously: *MyoD* (Davis et al., 1987), *SDF1α/β* (Vasyutina et al., 2005), *Osr1/2* (Stricker et al., 2006), *TCF4* (Kardon et al., 2003), *Mox2* (Mankoo et al., 1999), *SF/HGF* (kindly provided by F. Maina), *β-Catenin* (Hill et al., 2005), and *N-Cadherin* (kindly provided by M. Takeichi).

Immunohistochemistry

Whole-mount immunohistochemistry and OPT analysis were done as previously described (DeLaurier et al., 2006). The following antibodies were used: mouse anti-skeletal myosin (My32; 1:800; Sigma), mouse anti-Tcf4 (6H5-3; 1:100; Upstate), rabbit anti-N-Cadherin (abcam; 1:500), mouse anti-N-Cadherin (GC4; 1:200; Sigma), mouse anti-MyoD1 (1:50; Dako), mouse anti-β-Catenin (1:200; Sigma), rabbit anti-β-Catenin (1:500; Sigma); mouse anti-sarcomeric myosin (MF20; 1:20; DSHB); rabbit anti-Cadherin 11 (1:800; kindly provided by R. Mege [Marthiens et al., 2002]).

Histology

For histology, limbs were fixed in 4% PFA, dehydrated in a graded series of ethanol, cleared in xylene, and embedded in fibrowax (VWR International, UK). Sections of 6 μm thickness were stained with hematoxylin and eosin (H&E).

SUPPLEMENTAL INFORMATION

Supplemental Information include four figures, one table, and two movies and can be found with this article online at doi:10.1016/j.devcel.2009.11.013.

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