Fss/Tbx6 is required for central dermomyotome cell fate in zebrafish

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Summary

The dermomyotome is a pool of progenitor cells on the surface of the myotome. In zebrafish, dermomyotome precursors (anterior border cells, ABCs) can be first identified in the anterior portion of recently formed somites. They must be prevented from undergoing terminal differentiation during segmentation, even while mesodermal cells around them respond to signaling cues and differentiate.

T-box containing transcription factors regulate many aspects of mesoderm fate including segmentation and somite patterning. The *fused somites* (*fss*) gene is the zebrafish ortholog of *tbx6*. We demonstrate that in addition to its requirement for segmentation, *fss/tbx6* is also required for the specification of ABCs and subsequently the central dermomyotome. The absence of Tbx6-dependent central dermomyotome cells in *fss/tbx6* mutants is spatially coincident with a patterning defect in the myotome.

Using transgenic fish with a heat-shock inducible *tbx6* gene in the *fss/tbx6* mutant background, we further demonstrate that ubiquitous fss/tbx6 expression has spatially distinct effects on recovery of the dermomyotome and segment boundaries, suggesting that the mechanism of Fss/Tbx6 action is distinct with respect to dermomyotome development and segmentation.

We propose that *Fss/Tbx6* is required for preventing myogenic differentiation of central dermomyotome precursors before and after segmentation and that central dermomyotome cells represent a genetically and functionally distinct subpopulation within the zebrafish dermomyotome.

Key words: Myogenesis, Slow muscle, Fast muscle, Muscle pioneer, Somite, Anterior Border Cells, Segmentation

Introduction

In vertebrates, the paraxial mesoderm is the source of all the skeletal muscle of the trunk and limbs as well as the dermis and axial skeleton of the trunk. The specification of paraxial mesoderm into the precursors of these cell types occurs during the segmentation period, with the appearance of the myotome, the dermomyotome, and the sclerotome within the somites.

The dermomyotome contains precursors to the embryonic myotome, and to satellite cells that underlie post-embryonic muscle growth and repair (Buckingham and Vincent, 2009). In avians and mammals, the earliest myotome cells differentiate after their incorporation into somites, at about the same time as the appearance of the dermomyotome (Denetclaw et al., 1997; Venters et al., 1999; Kahane et al., 2007). In contrast, in most teleosts, primary myotome cells start to differentiate prior to somite formation and prior to the appearance of the dermomyotome (Stellabotte and Devoto, 2007). In both amniotes and teleosts, signals from tissues surrounding the somite, including the notochord, spinal cord, and surface ectoderm trigger myogenic differentiation.

Much less is known about the initial development of the dermomyotome, or about the mechanisms that maintain a dermomyotome while some of its cells differentiate into muscle fibres during early embryogenesis. In zebrafish, dermomyotome precursors can first be identified in the anterior portion of recently formed somites (Hollway et al., 2007; Stellabotte et al., 2007). These anterior border cells (ABCs) migrate to the lateral surface of the somite, forming a layer of cells on the external surface of the myotome, as in other vertebrates (Devoto et al., 2006). These cells express *pax7*, *pax3*, *meox*, and *dacD* (Groves et al., 2005; Devoto et al., 2006; Feng et al., 2006; Hammond et al., 2007; Hollway et al., 2007).

The dermomyotome can be subdivided along the dorsal-ventral axis into separate domains based on gene expression. In amniotes, the central portion of the dermomyotome has been called the intercalated region, because it is located between the dorsal and the ventral regions (Spörle, 2001). It can be distinguished from the dorsal and the ventral dermomyotome by its expression of *engrailed* (Davis et al., 1991; Gardner and Barald, 1992) and *sim* (Spörle, 2001). In zebrafish, the central-most dermomyotome cells are distinguished by their expression of the chemokine SDF1a (David et al., 2002; Svetic et al., 2007).

Five genes are known to be required for segmentation of the paraxial mesoderm: *beamter*, *deadly seven*, *after eight*, *mind bomb*, and *fused somites* (van Eeden et al., 1996). The first four of these genes are *notch* pathway genes; they are not required for

the formation of the anterior three to nine somites but are required for the proper segregation of anterior and posterior half somite-markers, which in mutants are expressed in a 'salt and pepper' pattern. The *fused somites (fss)* gene is not part of the *notch* pathway, it encodes a transcription factor of the *tbx* gene family which is expressed in the anterior presomitic mesoderm (PSM) and in the anterior half of recently formed somites. *fss* is required for the formation of somites throughout the trunk and tail and for the development of anterior half-somite identity (Nikaido et al., 2002; van Eeden et al., 1996). *tbx* family members encode proteins with a conserved DNA binding motif known as the T-box and play important roles in embryonic mesoderm development. In mice, *eomesodermin* is required for the initial formation of the mesoderm (Showell et al., 2004), *brachyury* is required for notochord and posterior mesoderm development (Showell et al., 2004), *tbx6* is required for paraxial mesoderm development as well as somite patterning (Chapman and Papaioannou, 1998; White et al., 2003), and *tbx18* is required for the maintenance of anterior identity in newly formed somites (Bussen et al., 2004). Tbx proteins are sequence-specific DNA binding proteins which can serve as transcriptional repressors or activators (Wardle and Papaioannou, 2008).

We have investigated the role of the segmentation gene *fss* in the specification and differentation of paraxial mesoderm cell types. Sequence analysis demonstrates that the *fss* gene is the zebrafish ortholog of *tbx6*; we show that it is required for the specification of ABCs and for the development of the central dermomyotome and the underlying myotome in the posterior trunk and tail.

Using transgenic fish expressing heat-shock inducible *tbx6* in the *fss/tbx6* mutant background, we show that the ubiquitous expression of *fss/tbx6* has spatially distinct effects on restoration of central dermomyotome and on segmentation in mutant embryos. We propose that the role of Fss/Tbx6 is to prevent the differentiation of central dermomyotome precursors in response to myogenic signals before and after segmentation and that in zebrafish the central dermomyotome is a genetically distinct dermomyotome subpopulation.

Results

We have examined the expression of the dermomyotome marker Pax7 in segmentation mutants to investigate a possible connection between segmentation and the specification of dermomyotome precursors (ABCs) in zebrafish. We found that formation of the dermomyotome is unaffected in segmentation mutants of the *notch* gene pathway (not shown), suggesting that ABC specification is not dependent on segment boundary formation. However, Pax7 expression seemed to be altered in the *fused somites* (*fss*) mutant.

Orthology between the *fss* gene and *tbx6*

We have re-examined the homology of the *fss* gene to other members of the *tbx* gene family. *fss* was initially named *tbx24* (Nikaido et al., 2002), when few other teleost *tbx* gene sequences were available for comparison and the diversity within the *tbx6* subfamily was not apparent, and because there was a previously cloned zebrafish gene which had been named *tbx6* (Hug et al., 1997). With further sequences available, it is clear that the Fss

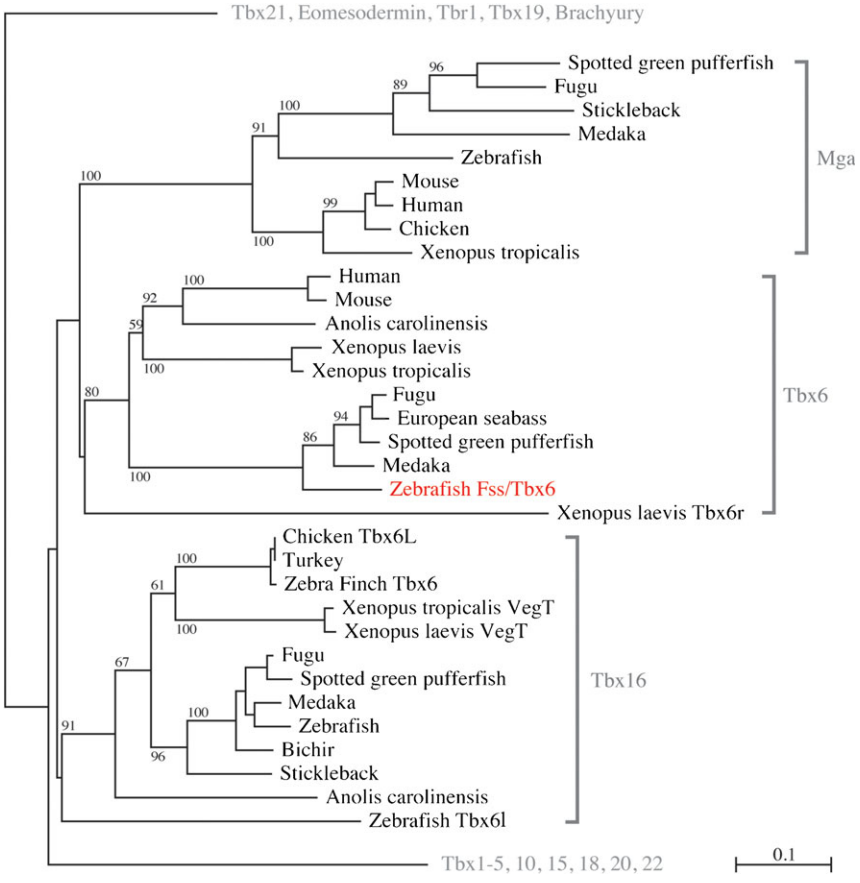

Fig. 1. Cladogram of Tbx6, Tbx16 and MGA members of the Tbx protein family. Tree is an internal branch taken from a neighbor-joining tree of the Tbx family of proteins based solely on their T-Box domain. Numbers represent bootstrap values (100 replications, values >50 shown). Sequence alignment using ClustalX 2.0 (Larkin et al., 2007), and tree building using SeaView 4.2.12 (Gouy et al., 2010). Note that zebrafish Fss/Tbx6 (red) nests within the tbx6 clade. For outgroup branching patterns and support values, see supplementary material Fig. S1. Protein accession numbers are listed in supplementary material Table S1.

amino acid sequence is homologous in its T-domain to the Tbx6 subfamily, nesting alongside Tbx6 proteins of other teleosts, amphibians, lizard, and mammals (Fig. 1; supplementary material Fig. S1, cladogram of the entire tree; supplementary material Table S1, list of gene accession numbers). The two nearest subgroups, Mga and Tbx16, also contain zebrafish genes nested with those of other species. The originally named zebrafish Tbx6 falls within the Tbx16 subgroup. In support of this, the chromosomal region containing the *fss* gene in zebrafish is syntenic with the chromosomal region containing tbx6 in mouse (34 paralogous genes), human (69 genes), and medaka (188 genes) (Catchen et al., 2009) (supplementary material Fig. S2). Using the same parameters, the zebrafish gene previously named *tbx6* has no neighboring genes in common with *tbx6* in mouse or human. According to the nomenclature guidelines of the zebrafish community, *fss/tbx24* has been renamed to *fss/tbx6*, to facilitate communication between those studying this gene in mammals and those studying it in fish. To avoid confusion, the gene previously named *tbx6* has been renamed to *tbx6l*.

Tbx6 is required for development of the central dermomyotome

Tbx6 is required for patterning of the paraxial mesoderm in mouse (Chapman et al., 1996). We examined the expression of dermomyotome markers (Pax7, *pax3* and *meox*) together with the expression of myogenic regulatory factors (MRFs) in *fss/tbx6* mutant embryos at different stages to characterize the role of Tbx6 in the development of the major paraxial mesoderm cell types in zebrafish.

We find that *fss/tbx6* is required for the development of the dermomyotome, but with regional differences: in the anterior trunk dermomyotome cells develop independently of *tbx6* function (Fig. 2A,C), whereas in the posterior trunk and tail the dermomyotome is subdivided into a *tbx6* independent dorsal/ventral domain and a *tbx6* dependent central domain at the level of the notochord (Fig. 2B,D–F).

During the early segmentation period, at the 13S stage, dermomyotome development has reached the level of somites 7–8 in wildtype embryos. Similarly, Pax7-positive dermomyotome cells are found on the lateral surface of the myotome of *fss/tbx6* mutant siblings (Fig. 2A,C). As the dermomyotome matures in the posterior trunk in wild type embryos, the dorsal/ventral domain is labeled by Pax7 antibody earlier than the central domain. During the same period, dorsal/ventral dermomyotome cells appears at the same axis levels. In *fss/tbx6* mutants, however, Pax7-positive cells are completely missing in the central domain of the posterior trunk in *fss/tbx6* mutants (Fig. 2E,F).

At the end of the segmentation period (24 h stage), the deficit in the central dermomyotome in *fss/tbx6* mutant embryos is highly specific and quite pronounced. Beginning in a region ranging from the equivalent of somite 6 to somite 8 of wild type siblings ($n=25$), Pax7-positive dermomyotome cells are absent in the central domain of all of the posterior trunk and tail (Fig. 2G, Fig. 3A,B). We found similar results with other markers of the dermomyotome, including *meox*, and *pax3* (data not shown).

To facilitate quantitative comparisons between embryos with and without segments we used the anal vent as a landmark (Fig. 2G). In *fss/tbx6* mutants the total number of Pax7-positive dermomyotome cells per cross section is reduced by more than 50 percent (Fig. 4A).

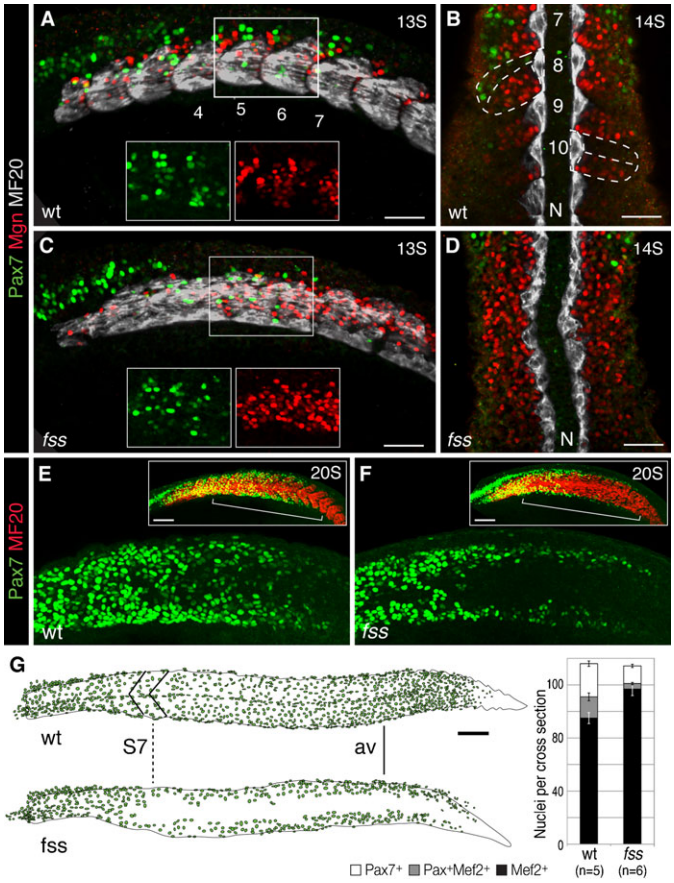

Fig. 2. *fss/tbx6* is required for the central dermomyotome development in the posterior trunk and tail. (**A–D**) Pax7 (green), Myogenin (Mgn, red), and MF20 (MyHC, white) expression in the anterior trunk (A,C; confocal z-stack, insets show higher magnification) and the posterior trunk (B,D) of wild type sibling (A,B) and *fss/tbx6* mutant (C,D) at 13–14S stage. N, Notochordd, numbers indicate somite number. Wild type siblings express all three markers within distinct compartments of the somites (dashed lines in B). In *fss/tbx6* mutants, Pax7 expressing central dermomyotome cells are restricted to the anterior trunk; the central posterior trunk shows more cells expressing Mgn. (**E**,**F**) Pax7 (green) and MF20 (MyHC, red) expression in wild type sibling (E) and *fss/tbx6* mutant (F) embryo at 20S stage. Boxes show flattened confocal z-stacks of the entire trunk. In the posterior trunk Pax7 is initially expressed in cells of the dorsal and ventral dermomyotome; central Pax7 expression is delayed in wild type sibling (E), but absent in *fss/tbx6* mutant (F). (**G**) Tracing of $Pax7^+$ dermomyotome nuclei in representative wild type sibling and *fss/tbx6* embryos at the 24 h stage. In this mutant embryo, the central dermomyotome deficit/defect in *fss/tbx6* mutants starts at trunk levels correlating to somite 7 in the wild type sibling (dashed line, S7). Graph showing number of $Pax7^+$, $Mef2^+$ and differentiating ($Pax7^+/Mef2^+$) cells (mean±s.e.m.) on sections at anal vent (av) level in wild type siblings and *fss/tbx6* mutants. Scale bars: 50 µm (A–D), 100 µm (E–G).

Loss of Tbx6 leads to myogenic differentiation of central dermomyotome progenitors

Specification of the precursors of slow muscle fibres, adaxial cells, is indistinguishable between *fss/tbx6* mutant and wildtype embryos (van Eeden et al., 1996) (Fig. 2B,D). However, specification of ABCs and fast muscle precursors, which develop from cells initially lateral to adaxial cells, is altered in mutants. In wild type embryos, MRF genes are expressed segmentally, in the posterior of recently formed somites, and not in the prospective dermomyotome precursors in the anterior (Weinberg et al., 1996). In *fss/tbx6* mutants in contrast, MRF

expression is more widespread in the paraxial mesoderm (Fig. 2B,D); we could not detect any myogenin-negative cells in the posterior trunk of *fss/tbx6* mutant embryos, suggesting that ABCs differentiate prematurely into primary myotome cells. To test this, we determined the number of dermomyotome and myogenic nuclei (Mef2-positive) (Ticho et al., 1996) in the paraxial mesoderm of embryos at the end of segmentation (24 h stage). The loss of more than half of the Pax7-positive dermomyotome cells correlates with a significantly higher number of myogenic nuclei ($P \leq 0.0002$), while the total number of labeled nuclei is not affected in *fss/tbx6* mutants (Fig. 2G). The simplest explanation of these results is that the precursors to the central dermomyotome have prematurely differentiated into primary myotome.

In wild type embryos at the end of segmentation, the central dermomyotome is distinguished from the dorsal and ventral dermomyotome by its higher proportion of differentiating cells, as indicated by the co-expression of Pax7 and MRFs (Fig. 3A). Approximately 50% of Pax7-positive cells in the central dermomyotome of wild type embryos co-express Mef2 at the 24 h stage, compared to about half that number in the dorsal/ventral domain. While Pax7-positive and differentiating dermomyotome cells are absent in the central domain in *fss/tbx6* mutants at the end of segmentation, the most dorsal and most ventral dermomyotome cells are present in approximately normal numbers and differentiate at percentages similar to wild type (Fig. 3B).

The deficit in central dermomyotome in *fss/tbx6* mutants spatially correlates with a myotome patterning defect

At the end of segmentation, the wild type zebrafish myotome consists of deep fast muscle fibres, covered laterally by a continuous monolayer of slow muscle fibres (Waterman, 1969). This slow muscle monolayer extends from the lateral surface of the myotome to the notochord, at the position of the future horizontal myoseptum, and separates the fast muscle fibres into epaxial and hypaxial regions (Fig. 3C–E). In *fss/tbx6* mutant embryos, the arrangement and morphology of slow and fast fibres is normal in the anterior trunk, where the central dermomyotome is present (not shown). However, it is profoundly disrupted in the posterior trunk and tail, where dermomyotome precursors have prematurely differentiated into muscle fibres. The continuity of the slow muscle monolayer is interrupted, with numerous fast muscle fibres superficial to the slow fibres in the central region (Fig. 3H–J). These superficial fast fibres can span several segment lengths, have very large cross sectional diameters, and have an unusually large number of nuclei, with several present in transverse sections (Fig. 3F,K). The morphology and position of these fast fibres suggest that the prematurely differentiated dermomyotome precursors joined the fast primary myotome. To examine muscle cell identities, we examined expression of Engrailed, which is a marker for both slow muscle pioneers and for medial fast muscle fibres in the central myotome (Hatta et al., 1991; Wolff et al., 2003). Despite the altered position of slow and fast fibres, Engrailed expression is similar to that in wild type embryos (Fig. 3G,L).

Dorsal/ventral dermomyotome cells are sufficient to support larval muscle growth in fss/tbx6 mutants

At the 24 h stage, *fss/tbx6* mutant embryos have fewer than half the number of dermomyotome cells per section through the posterior trunk, compared to wild type embryos (Fig. 2G, Fig. 4A). However, by the 48 h stage, wild type and mutant embryos have similar numbers of Pax7-positive dermomyotome cells. This is in part due to a decrease of central dermomyotome cells in wild type embryos as they differentiate into muscle fibres

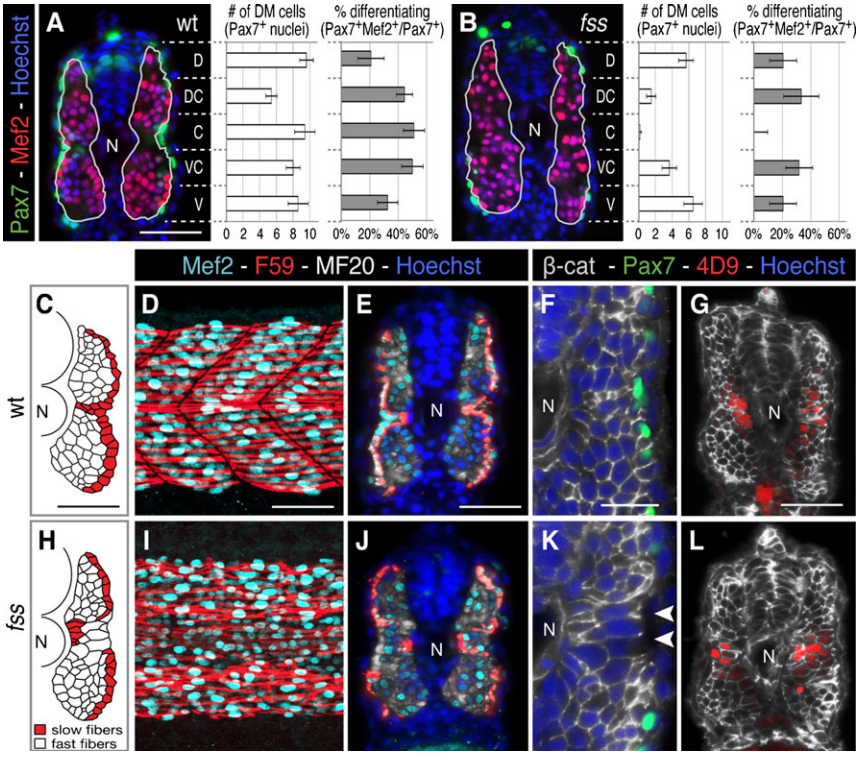

Fig. 3. Premature dermomyotome progenitor differentiation disrupts myotome patterning. (**A**,**B**) Cross-sections and graphs showing dorsal-ventral distribution of dermomyotome (average of $Pax7^+$ cells ± s.e.m.) and % differentiating dermomyotome ($Pax7^+/Mef2^+$) in wild type sibling and *fss/tbx6* mutant (green, Pax7; red, Mef2; blue, Hoechst 33258). (**C**–**L**) Myotome morphology in wild type siblings (D–H) and *fss/tbx6* (I–M) at the 24 h stage. (C,H) Tracing of fast (white) and slow (red) muscle fibres on representative cross-sections. In *fss/tbx6* mutants, the layer of slow fibres is disrupted and large fast muscle fibres are found lateral to the slow fibres in the central myotome. (D,E,I,J) Whole mounts (posterior trunk) and cross-sections labeled with F59 (red, labels slow myosin heavy chain at the 24 h stage), Mef2 (aqua) and MF20 (white). (F,G,K,L) β-catenin labeled cross-sections double-labeled for either Pax7 (green, F,G) or 4D9 (red, engrailed, G,L); nuclei labeled with Hoechst. Arrowheads indicate large diameter, lateral fast fibres. N, notochord. Scale bars: 50 μm (A,C–E,G), 25 μm (F). Corresponding images of fss are to the same scale.

(Fig. 3A), and in part due to an increase in the number of Pax7-positive dermomyotome cells in mutant embryos. Furthermore, the distribution of cells along the dorsal-ventral axis of the myotome is similar (Fig. 4B). This suggests the possibility that in *fss/tbx6* mutants the dorsal and ventral most dermomyotome provides a pool of precursor cells that populates the central domain. Accordingly we found Pax7-positive dermomyotome cells bridging the central domain at multiple locations throughout the posterior trunk and tail at the 36 h stage (not shown).

The premature differentiation of dermomyotome progenitors does not lead to an increased number of muscle fibres in mutant embryos at the end of segmentation, but rather to an increased number of nuclei per fibre in the ectopic fast fibres (Fig. 3K, Fig. 4A,C). The addition of muscle fibres in the fast myotome in *fss/tbx6* mutants is delayed at 48 h but recovers to wild type levels by 72 h (Fig. 4C). We presume this reflects the reduced number of myogenic precursor cells, with a 24 hour delay. Unusually large fast muscle fibres remain in the central domain of the myotome of *fss/tbx6* mutants at the 72 h stage, but new fibres, developing on the outside of the embryonic fast myotome during the period of stratified hyperplasia (Rowlerson and Veggetti, 2001) have diameters similar to those in wild type (Fig. 4D). These morphological differences between the wildtype and the *fss/tbx6* myotome persist into later larval stages (not shown).

Induced *fss/tbx6* expression rescues segmental gene expression and dermomyotome formation

To confirm that the segmentation defect and the loss of central dermomyotome in *fss/tbx6* mutants both result from the loss of the *fss/tbx6* gene, we created transgenic fish with *tbx6* under the regulation of the inducible promoter of the hsp70 gene (Halloran et al., 2000). We assayed the early response to Tbx6 induction by examining the expression of *ripply1*, *fgf8* and *myoD* shortly after heat shock. *ripply1* and *fgf8* are expressed in the anterior of newly formed somites in a Tbx6-dependent manner (Sawada et al., 2000; Kawamura et al., 2005), while the expression of the MRF *myoD* is restricted from the anterior of newly formed somites in a Tbx6-dependent manner (van Eeden et al., 1996). We found that a pulse of Tbx6 during the segmentation period restored partly segmented expression of *ripply1* and *fgf8*, and restored gaps in the *myoD* expression domain (Fig. 5), indicating the restoration of anterior half-somite identity in presumptive segments.

Heat shock induced expression of Tbx6 also restored central dermomyotome cells in mutant embryos. In wild type embryos during the segmentation period, most proliferative cells are dermomyotome cells on the surface of the somite, with the highest number at the dorsal and ventral extremes (Fig. 6A) (Barresi et al., 2001; Stellabotte et al., 2007). The loss of *fss/tbx6* leads to the complete absence of proliferative cells in the central region (Fig. 6A). Pulsed expression of Tbx6 restored proliferative cells in the central region of the somite, consistent with a rescue of the central dermomyotome. In the region of the trunk with maximal numbers of induced Pax7-positive cells, *fss/tbx6* mutants showed a similar number of dermomyotome cells as wild type siblings (Fig. 6B). These results suggest that segmentation and dermomyotome development are both restored after heat shock induced *fss/tbx6* expression.

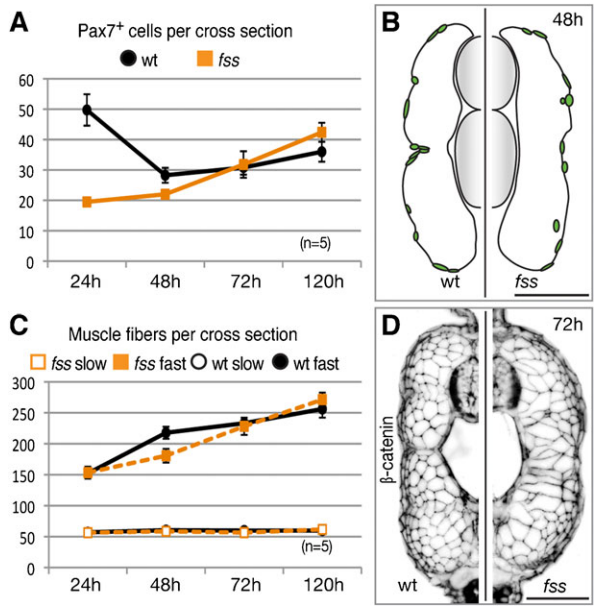

Fig. 4. Dermomyotome recovery and muscle growth. (**A**) Graph showing mean number of Pax7$^+$ cells (±s.e.m.) over time. (**B**) Tracing of Pax7$^+$ dermomyotome nuclei in representative transverse section of wild type sibling (left) and *fss/tbx6* mutant (right) at the 48 h stage. (**C**) Graph showing mean number of slow and fast muscle fibres over time (± s.e.m.). (**D**) Cross-sections showing myotome morphology (β-catenin) at the 72 h stage. Scale bars: 50 µm.

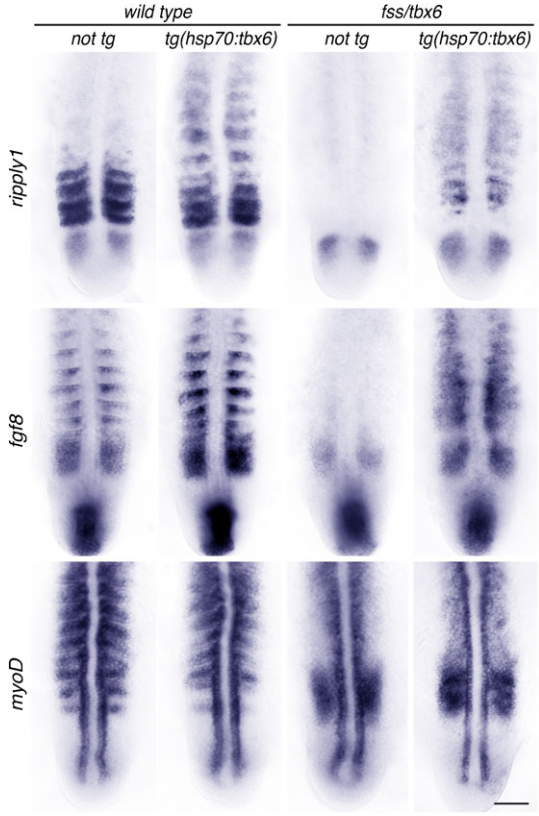

Fig. 5. A pulse of Tbx6 expression in fss/tbx6 mutants induces segmental expression of *ripply1* and *fgf8*, and locally represses *myoD*. Expression of genes expressed specifically in the anterior (*ripply1*, *fgf8*) and posterior (*myoD*) somite compartments in non-tg and *tg(hsp70:tbx6)* wild type sibling and *fss/tbx6* mutant embryos. Embryos were heat shocked at the 9–10S stage, fixed at the 13–14S (*myoD*) and 15–16S stage (*ripply1*, *fgf8*), and flat-mounted for documentation. Scale bar: 50 µm.

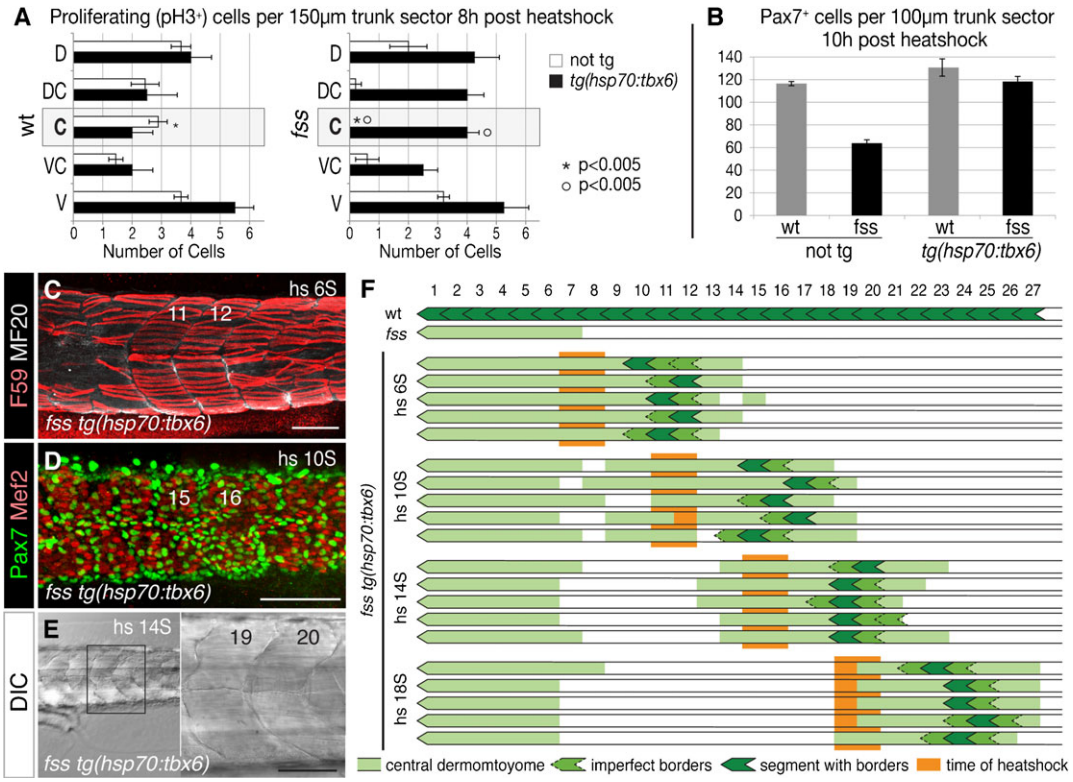

Fig. 6. Induced Tbx6 expression differentially rescues central dermomyotome and segmentation. (**A**) Dorsal/ventral distribution of proliferative (phosphorylated Histone H3, pH3^{+}) cells in non-tg and *tg(hsp70:tbx6)* wild type sibling and *fss/tbx6* mutant embryos 8 hours after heat-shocking at the 8S stage. Data are presented as mean ± s.e.m. (**B**) Graph showing number of Pax7^{+} cells in non-tg and *tg(hsp70:tbx6)* wild type sibling and *fss/tbx6* mutant embryos at 10 hours after heat-shocking at the 10S stage. (**C–E**) Rescue of dermomyotome and segmentation in *fss/tbx6* mutant *tg(hsp70:tbx6)* visualized using F59 (red) and MF20 (white) (C) and Pax7 (green) and Mef2 (white) (D) labeling, and DIC imaging (E). Specimen stages are 5 d (C,E) and 24 h (D), rescued segments are numbered according to the corresponding region in wild-type siblings. (**F**) Schematic showing the location of rescued dermomyotome (light green), segments (dark green) in individual embryos after heat shocks at various times (orange). Note that a Tbx6 pulse rescues the formation of 1–3 somite boundaries and central dermomyotome over 8–10 somite lengths. Scale bars: 100 µm (C,D), 50 µm (E).

Ubiquitous *fss/tbx6* expression differentially rescues dermomyotome and segmentation

To further characterize the requirement of *fss/tbx6* in the formation of central dermomyotome cells and segment boundaries, we induced a pulse of Tbx6 expression at varying times during the segmentation period, and monitored segmentation and the development of the dermomyotome. We found that a Tbx6 pulse rescued the formation of 1 to 3 remarkably normal somites with continuous somite boundaries, as visualized by DIC imaging and perfectly aligned slow muscle fibres, along with an 8–10 somite length region of rescued Pax7-positive central dermomyotome (Fig. 6C–E).

The best somite was consistently formed about 2 to 4 somite-lengths posterior to the somites that would have been forming at the time of heat shock. For example, if we heat shocked a clutch of embryos at the 14S stage, somites 15 and 16 were formed during heat shock and we consistently observed that *fss*$^{-/-}$*;hsp70:tbx6* embryos formed at least one complete segment in the trunk region equivalent to somite 19 to 20 in *fss* heterozygous siblings (Fig. 6D,F).

The heat shock pulse leads to ubiquitous expression of Tbx6, with the highest protein levels between one and two hours (corresponding to the addition of 2 to 4 somites) after the end of heat shock and rapidly decreasing levels thereafter (not shown). Thus, the cells becoming incorporated into a segment in response to heat shock induced *fss/tbx6* expression were located in the posterior presomitic mesoderm (PSM) at the beginning of heat shock and in the anterior PSM, about 2 somite-lengths posterior to the most recently formed segment in wild type siblings, when protein levels were highest. This indicates that the potential to form segment boundaries is spatially restricted to cells in the anterior PSM and that it requires high levels of fss/tbx6 expression.

Pax7-positive central dermomyotome cells were restored in a much broader region of the trunk and tail, extending anterior and posterior to the induced segment boundaries. This shows that the dermomyotome rescue is not dependent on the formation of segment boundaries, which is consistent with the observation that dermomyotome development is unaffected in segmentation mutants of the *notch* signaling pathway (data not shown) and suggests that lower levels of fss/tbx6 expression are sufficient for specification of central dermomyotome cells.

The restoration of central dermomyotome even anterior to the segments formed during heat shock indicates that Fss/Tbx6 can specify ABCs even after segment boundary formation. The difference in extent of dermomyotome restoration anterior to the segmentation rescue in different heat shock stages may be explained by different rates of cell maturation along anterior-posterior axis. Precursor cells remain uncommitted longer in the anterior segments than in the posterior segments of the trunk,

relative to their incorporation into a somite. Thus, the dermomyotome extends more anteriorly in embryos heat shocked during maturation of the trunk (10S stage) than it does in embryos heat shocked during maturation of the tail (18S stage).

Discussion

We have investigated the role of the segmentation gene *fss/tbx6* in the specification of paraxial mesoderm cells in zebrafish. We show that the loss of *fss/tbx6* leads to premature differentiation of central dermomyotome precursors into primary fast fibres, causing a patterning defect within the central myotome. The requirement for *fss/tbx6* subdivides the dermomyotome into distinct populations of cells along both the anterior-posterior axis and the dorsal-ventral axis.

Model for tbx6 action

Signals that promote primary myogenesis are uniformly distributed along the anterior-posterior axis, as they are derived from notochord, surface ectoderm, neural tube, and the pronephros (Cossu et al., 1996). This raises the question of how the segmentally arranged dermomyotome precursors, the ABCs, are protected from the influence of these myogenesis promoting signals. We propose that Fss/Tbx6 is a necessary component of a segmentally restricted myogenic inhibitor, and that this inhibition is required for the development of the dermomyotome.

Fss/Tbx6 is unlikely to be sufficient for the inhibition of myogenesis. It is expressed in all of the cells of the anterior PSM before it becomes restricted to the anterior of recently formed somites (Nikaido et al., 2002). Moreover, uniform expression of Tbx6 using a heat shock promoter does not prevent myogenesis in the posterior of newly formed somites. In the context of segmentation, *fss/tbx6* is part of a network of genes that regulate each other and other genes, including notch pathway genes, mesp-b, and ripply (Brend and Holley, 2009; reviewed by Oates et al., 2012). The spatial restriction of the activity of one or more of these gene products may restrict Fss/Tbx6 function to the ABCs.

The temporary, uniform expression of *fss/tbx6* function restores dermomyotome in a wider domain of the paraxial mesoderm than it restores segmentation. The spatial pattern of segmentation rescue is consistent with a role for Tbx6 in the wavefront region of the PSM. This region of the PSM is characterized by a gradual decrease in *fgf8* and *her13.2* in wildtype embryos and has been proposed to determine the position of future segment boundaries (reviewed by Holley, 2007). The spatial pattern of dermomyotome rescue after heat shock induced *fss/tbx6* expression might indicate that the competence of paraxial mesoderm cells to develop into dermomyotome is more widespread in the paraxial mesoderm than the competence to form a segment boundary and/or that lower levels of gene expression are sufficient to prevent dermomyotome precursors from prematurely differentiating into primary myotome.

The dermomyotome deficit in *fss/tbx6* mutant embryos correlates with altered muscle fibre morphologies and a disruption of cellular arrangements in the central trunk and tail. The presence of large muscle fibres with multiple nuclei per cross section—without any decrease in the number of muscle fibres—suggests that additional myonuclei join the fast fibre population in the central myotome. Variations in the shape of these fast fibres might result from the lack of myotome boundaries restricting their length (Henry et al., 2005) and/or the lack of central slow fibre migration promoting their elongation (Henry and Amacher, 2004).

Slow muscle fibres are specified normally in *fss/tbx6* mutant embryos. We suggest that the disrupted position of the central slow muscle fibres is an indirect effect of the *fss/tbx6* mutation on dermomyotome precursors. Ectopic differentiation of ABCs may cause a change in cell migration, preventing the normal displacement of the slow fibres by the fast fibres. Also, central dermomyotome cells may play a crucial role in displacing muscle fibres during cellular rearrangements in the maturing somite. These effects are likely independent of cell fate determination in the central domain of the myotome, as the muscle pioneers and the medial fast fibres express Engrailed, as in wild type (Wolff et al., 2003).

Whereas zebrafish *tbx6* mutants show a loss of segmentation and of the central dermomyotome, mouse *tbx6* mutants have an enlarged tailbud, transformation of paraxial mesoderm into neural tube, and loss of laterality in the node (Chapman and Papaioannou, 1998; Hadjantonakis et al., 2008). However, partial loss of *tbx6* in mouse has similar consequences to the complete loss of *tbx6* in zebrafish—a disruption of segmentation. In particular, partial loss of *tbx6* leads to the loss of anterior somite compartment identity, as indicated by the expression of tbx18 (White et al., 2003), and the appearance of fused ribs and vertebrae. These observations are consistent with the acquisition of additional functions by the *tbx6* gene in mouse. Those functions may be carried out in zebrafish by the *spt/tbx16* gene, which is expressed earlier in the maturation of the PSM, and which when mutant leads to the development of an enlarged tailbud and the loss of laterality in the zebrafish equivalent of the node (Ho and Kane, 1990; Gourronc et al., 2007). Although the *tbx16* gene is present in non-mammalian tetrapods, it has apparently been lost in mammals (Fig. 1). If *tbx6* in mammals has acquired the combined functions of the *tbx6* and *tbx16* genes, then the loss of *tbx6* would lead to a more severe phenotype in mouse than in zebrafish.

Regionalization of the dermomyotome along the anterior-posterior axis

fss/tbx6 is required for segmentation throughout the entire anterior-posterior extent of the trunk and tail (van Eeden et al., 1996), thus we were surprised to find that the requirement of *fss/tbx6* for dermomyotome development is regionally restricted. The regional specificity for the *fss/tbx6* requirement along the anterior-posterior axis is reminiscent of other developmental differences between anterior and posterior paraxial mesoderm. In the anterior-most somites, cell differentiation is delayed relative to segmentation: expression of *myoD*, *snail1a*, and *engrailed* arises simultaneously in roughly the first six somites, but sequentially with the formation of each segment in the posterior trunk and tail (Hatta et al., 1991; Ekker et al., 1992; Weinberg et al., 1996; Jülich et al., 2005). Similarly, slow muscle fibres are displaced to the lateral surface simultaneously in the anterior six somites, but sequentially with segmentation in the posterior somites (S.H.D., unpublished; Felsenfeld et al., 1991). The Notch pathway mutants also reveal a regional specificity, as they disrupt segmentation in the posterior trunk and tail but not in the anterior three to nine somites (reviewed by Holley, 2007).

Differences between the anterior-most and the more posterior segments are also observed in amniotes. Mouse embryos mutant for either of the transcription factors *mesogenin* and *tbx6* form only the anterior seven somites: the posterior trunk and tail lacks segmented paraxial mesoderm (Chapman and Papaioannou, 1998; Yoon and Wold, 2000).

We suggest that the dermomyotome phenotype in *fss/tbx6* mutants indicates that the dermomyotome, like many other aspects of somite patterning, is distinctly regulated in the anterior somites compared to the rest of the trunk and tail.

Regionalization of the dermomyotome along the dorsal-ventral axis

In amniotes, *engrailed* and *sim* are expressed in the central portion of the dermomyotome, but not in the dorsal and the ventral dermomyotome (Davis et al., 1991; Gardner and Barald, 1992; Spörle, 2001). Also, the central portion of the dermomyotome de-epithelializes earlier than the dorsomedial and ventrolateral lips of the dermomyotome (Ben-Yair and Kalcheim, 2005; Gros et al., 2005).

In zebrafish, the central dermomyotome is adjacent to the engrailed-expressing muscle pioneer slow fibres, and specifically expresses the *sdf1a* gene product, which serves as a guidance cue for the migrating lateral line primordium (David et al., 2002; Svetic et al., 2007). We show that the cells of the central dermomyotome begin to express Pax7 later than the cells of the dorsal/ventral dermomyotome and are more likely to differentiate (co-express Pax7 and MRFs) before the end of the segmentation period. Dorsal/ventral dermomyotome cells, in contrast, have higher rates of proliferation after the end of segmentation and show increased MRF co-expression beginning in early larval development (data not shown). These observations suggest that in zebrafish at 24 h, the central dermomyotome is responsible for the initial growth of the primary myotome, while the dorsal/ventral dermomyotome is responsible for a later phase of myogenesis, such as stratified hyperplasia (Steinbacher et al., 2006; Steinbacher et al., 2007).

The requirement for *fss/tbx6* provides the first genetic distinction between these two subdivisions of the dermomyotome, and also suggests that the dorsal/ventral portion of the dermomyotome can compensate for the central region. Thus, embryos lacking the tbx6-dependent central dermomyotome domain show only a very early and temporally restricted deficit in muscle growth. We propose that the dorsal and/or ventral dermomyotome is the source of the cells responsible for the recovery of myogenesis in the early larval period. Whether these dorsal/ventral dermomyotome cells and central dermomyotome cells have distinct embryonic origins remains to be determined.

Materials and Methods

Zebrafish, transgenesis, and heat shock

All experiments were done on zebrafish (*Danio rerio*) from the Wesleyan University strain of wild type fish, and the ti1 or te314a allele of *fss/tbx6*, which behaves as a null (Nikaido et al., 2002). We detected no differences between wild type and heterozygotes, all embryos labeled "wt" in figures are heterozygous siblings of the homozygous mutants in the same figure. Embryos were cared for using standard procedures (Westerfield, 1995).

The full open reading frame of zebrafish *tbx6* was amplified by PCR from a cDNA kindly provided by Scott Holley (New Haven, Connecticut, USA), with Gateway recombination sites added at each end. The PCR product was recombined into the donor vector pDONR221 to make the plasmid pME-TBX6. pME-TBX6 was recombined with the 5′ entry clone containing the zebrafish hsp70promoter (p5E-hsp70), and with the 3′ entry clone containing 6× myc tag plus the SV40 late poly adenylation signal (p3E-MTpA), and a destination vector with tol2 sites and egfp under the control of the cardiac myosin light chain 2 promoter (pDestTol2CG2) (Villefranc et al., 2007). The resulting plasmid (RAD510) is hsp70-tbx6-myc. We also recombined pME-TBX6 with p5E-hsp70, a 3′ entry clone containing cherry (p3Emcherry), and pDestTol2CG2. This resulting plasmid (RAD521) is hsp70-tbx6-cherry.

Single cell *fss/tbx6* mutant embryos were injected with hsp70-tbx6-myc or hsp70-tbx6-cherry and tol2 mRNA, and grown to adulthood. A founder was identified on the basis of cardiac eGFP expression in its offspring. These offspring were raised to adulthood and outcrossed to *fss/tbx6* homozygous mutants. All crosses generated 50% transgenic embryos, suggesting the presence of only one transgene insertion. All of the shown experiments were done on the v8 allele of tg(hsp70:tbx6-myc), but we obtained qualitatively similar results using tg(hsp70:tbx6-cherry), the allele we used is v7.

Embryos were heat shocked at the indicated time by transferring them in small mesh-bottom wells to embryo medium pre-warmed to 37℃ for one hour, and then returned to the standard temperature (28.5℃) for development after heat shock.

In situ hybridization and immunocytochemistry

RNA *in situ* hybridization and immunohistochemistry was carried out as previously described (Barresi et al., 2000). Embryos in Fig. 2B,D are shown anterior to the top. All other embryos are shown dorsal to the top, and for whole mount labeling, anterior to the left. Imaging of whole mount labeling was with a Zeiss LSM510 confocal microscope, individual optical sections were flattened for each image, except for Fig. 2B,D, which show single optical sections. Black and white were inverted in Fig. 4D for clarity of presentation. Tracings of individual labeled nuclei (Fig. 2G, Fig. 4B) and muscle fibres (Fig. 3C,H) were done on computer projections.

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Competing Interests

The authors have no competing interests to declare.

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