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1 Fgf-driven Tbx protein activities directly induce *myf5* and *myod* to initiate zebrafish  
2 myogenesis

3

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29

**30 Abstract**

31 Skeletal muscle derives from dorsal mesoderm that is formed during vertebrate  
32 gastrulation. Fibroblast growth factor (Fgf) signalling is known to cooperate with  
33 transcription factors of the Tbx family to promote dorsal mesoderm formation, but the role  
34 of these proteins in skeletal myogenesis has been unclear. Using the zebrafish, we show  
35 that dorsally-derived Fgf signals act through Tbx16 and Tbx16 to induce two populations of  
36 slow and fast trunk muscle precursors at distinct dorsoventral positions. Tbx16 binds to  
37 and directly activates the *myf5* and *myod* genes that are required for commitment to  
38 skeletal myogenesis. Tbx16 activity depends on Fgf signalling from the organiser. In  
39 contrast, Tbx16 is not required for *myf5* expression. However, Tbx16 binds to a specific site  
40 upstream of *myod* not bound by Tbx16, driving *myod* expression in the adaxial slow  
41 precursors dependent upon Fgf signals, thereby initiating muscle differentiation in the  
42 trunk. After gastrulation, when similar muscle cell populations in the post-anal tail are  
43 generated from the tailbud, declining Fgf signalling is less effective at initiating adaxial  
44 myogenesis, which is instead initiated by Hedgehog signalling from the notochord. Our  
45 findings provide insight into the ancestral vertebrate trunk myogenic pattern and how it  
46 was co-opted during tail evolution to generate similar muscle by new mechanisms.

47

48

**49 Introduction**

50 Sarcomeric muscle arose early in animal evolution, creating a defining feature of the  
51 metazoa: efficient multicellular movement. Prior to protostome-deuterostome divergence,  
52 the bilaterian animal likely acquired sensorimotor specializations, including divisions  
53 between cardiac, skeletal/somatic and visceral muscles, which are today regulated by  
54 similar gene families in *Drosophila* and vertebrates e.g. *mef2* genes (Taylor and Hughes,  
55 2017). Skeletal myogenesis is initiated during gastrulation, shortly after mesendoderm  
56 formation, but conserved bilaterian pathways leading specifically to skeletal muscle (as  
57 opposed to cardiac or visceral) have been harder to discern. One likely reason for this is  
58 that new regulatory steps have evolved in each lineage since their divergence.

59 A key step in vertebrate evolution was the chordate transition through which animals  
60 acquired the notochord, post-anal tail, gill slits and a more complex dorsal nerve cord,

61 facilitating swimming (Brunet et al., 2015; Gee, 2018; Gerhart, 2001; Satoh et al., 2012).  
62 Throughout vertebrates, the notochord patterns the neural tube and paraxial mesodermal  
63 tissue by secreting Hedgehog (Hh) signals that promote motoneuron and early muscle  
64 formation (Beattie et al., 1997; Blagden et al., 1997; Du et al., 1997; Münsterberg et al.,  
65 1995; Roelink et al., 1994). Nevertheless, in the absence of either notochord or Hedgehog  
66 signalling, muscle is formed in vertebrate somites (Blagden et al., 1997; Dietrich et al.,  
67 1999; Du et al., 1997; Grimaldi et al., 2004; Zhang et al., 2001). How might deuterostome  
68 muscle have formed prior to evolution of the notochord?

69 A change in function of the *Tbxt* gene, a T-box (Tbx) family paralogue, occurred during  
70 chordate evolution such that *Tbxt* now directly controls formation of posterior mesoderm,  
71 notochord and post-anal tail in vertebrates (Chiba et al., 2009; Showell et al., 2004).  
72 Hitherto, *Tbxt* may have distinguished ectoderm from endoderm and regulated formation  
73 of the most posterior mesendoderm (Arenas-Mena, 2013; Kispert et al., 1994; Woollard  
74 and Hodgkin, 2000; Yasuoka et al., 2016). In vertebrates, *Tbxt* genes also promote slow  
75 myogenesis (Coutelle et al., 2001; Halpern et al., 1993; Martin and Kimelman, 2008;  
76 Weinberg et al., 1996). Other Tbx genes, such as *Tbx1*, *Tbx4*, *Tbx5*, *Tbx16* and *Tbx6*,  
77 also influence sarcomeric muscle development (Chapman and Papaioannou, 1998; Griffin  
78 et al., 1998; Hasson et al., 2010; Kimmel et al., 1989; Manning and Kimelman, 2015;  
79 Weinberg et al., 1996; Windner et al., 2015). For example, the Tbx6 family is implicated in  
80 early stages of paraxial mesoderm commitment, somite patterning and the generation and  
81 positioning of muscle precursor cells (Bouldin et al., 2015; Chapman and Papaioannou,  
82 1998; Kimmel et al., 1989; Manning and Kimelman, 2015; Nikaido et al., 2002; White et al.,  
83 2003; Windner et al., 2012). It is unclear, however, whether the Tbx genes promote  
84 myogenesis directly, and/or are required for earlier events in mesoderm development that  
85 are necessary for subsequent myogenesis.

86 In vertebrates, a key essential step in skeletal myogenesis is activation of myogenic  
87 regulatory factors (MRFs) encoded by the *myf5* and *myod* genes (Hinits et al., 2009; Hinits  
88 et al., 2011; Rudnicki et al., 1993). Distinct myogenic cell populations initiate *myf5* and  
89 *myod* expression in different ways, the genes being induced by distinct signals through  
90 distinct *cis*-regulatory elements in different skeletal muscle precursor cells (Buckingham  
91 and Rigby, 2014). As the anteroposterior axis forms and extends, *de novo* induction of  
92 *myf5* and *myod* mRNAs in slow and fast muscle precursors occurs in tissue destined to  
93 generate each successive somite. Once expressed, these MRF proteins have two

94 functions: to remodel chromatin and directly enhance transcription of muscle genes  
95 (reviewed in (Buckingham and Rigby, 2014). In zebrafish, myogenesis begins at about  
96 75% epiboly stage when adaxial cells that flank the shield/organizer/nascent notochord  
97 (hereafter called pre-adaxial cells; diagrammed in Fig. 1A), begin MRF expression (Hinits  
98 et al., 2009; Melby et al., 1996). Pre-adaxial cells express both *myf5* and *myod*, converge  
99 to form two rows of adaxial cells flanking the notochord, become incorporated into somites  
100 and differentiate into slow muscle fibres (Coutelle et al., 2001; Devoto et al., 1996;  
101 Weinberg et al., 1996). Loss of either *Myf5* or *Myod* alone is not sufficient to prevent slow  
102 myogenesis, but loss of both completely inhibits adaxial slow muscle formation (Hinits et  
103 al., 2009; Hinits et al., 2011). Dorsal tissue immediately lateral to the pre-adaxial cells, the  
104 paraxial mesoderm (Fig. 1A), expresses *myf5* but little *myod* and subsequently generates  
105 fast muscle once somites have formed, upregulating *myod* in the process. A key to  
106 understanding myogenesis in both paraxial and adaxial cells is thus the mechanism(s) by  
107 which *myf5* and *myod* expression is regulated.

108 Intrinsic factors such as Tbx proteins likely interact with extrinsic positional cues within the  
109 embryo to pattern myogenesis. Fgf and Tbx function have long been known to interact to  
110 drive early mesendoderm patterning, but how directly they control early embryonic  
111 myogenesis remains unclear (Kimelman and Kirschner, 1987; Showell et al., 2004; Slack  
112 et al., 1987). Various Fgf family members are expressed close to myogenic zones during  
113 vertebrate gastrulation (Isaacs et al., 2007; Itoh and Konishi, 2007; Wilkinson et al., 1988).  
114 In zebrafish, Fgf signalling is required for mesendoderm formation, tailbud outgrowth and  
115 normal fast myogenesis (Draper et al., 2003; Griffin et al., 1995; Groves et al., 2005;  
116 Reifers et al., 1998; Yin et al., 2018). Fgf signalling is also thought to be involved in early  
117 expression of *myf5* and *myod* in pre-adaxial cells, but the mechanism of initial induction of  
118 *myf5* and *myod* is unknown (Ochi et al., 2008). Expression of *fgf3*, *fgf4*, *fgf6a*, *fgf8a* and  
119 *fgf8b* has been detected in the chordoneural hinge (CNH, Fig. 1A) adjacent to pre-adaxial  
120 cells (Draper et al., 2003; Groves et al., 2005; Thisse and Thisse, 2005). Subsequently,  
121 Hedgehog (Hh) signalling from the ventral midline maintains MRF expression and  
122 progression of the pre-adaxial cells into terminal slow muscle differentiation (Coutelle et  
123 al., 2001; Hirsinger et al., 2004; Lewis et al., 1999). Here, we show how both Fgf and Hh  
124 extracellular signals cooperate with Tbx genes to control fast and slow myogenesis. In the  
125 trunk, Fgf signalling is required for the initiation of myogenesis and acts in cooperation with  
126 Tbx16/Tbx1 function directly on *myf5* and *myod*. In the tail, by contrast, direct MRF gene

127 induction by Fgf is not required and the evolutionary novelty of midline-derived Hh-  
128 signalling accounts for adaxial myogenesis.

129

## 130 **Results**

### 131 ***Fgf signalling is essential for induction of adaxial myf5 and myod expression***

132 Adaxial myogenesis is driven by successive Fgf and Hh signals. When Hh signalling was  
133 prevented with the Smo antagonist cyclopamine (cyA), *myf5* and *myod* mRNAs in pre-  
134 adaxial cells were unaffected at 90% epiboly (Fig. 1A). In contrast, when Fgf signalling  
135 was inhibited with SU5402 both *myf5* and *myod* mRNAs were lost (Fig. 1A)(Ochi et al.,  
136 2008). To show that lack of MRFs was not due to failure of gastrulation caused by  
137 SU5402-treatment, we analysed expression of *aplnrb* mRNA, an anterior mesodermal  
138 marker (Zeng et al., 2007). At 80% epiboly, *aplnrb* mRNA has a complex and informative  
139 expression pattern, marking the anterior invaginating mesoderm cells around the germ ring  
140 and the pre-adaxial cells, but appears down-regulated in more lateral regions expressing  
141 *myf5* but not *myod* (Figs 1A; S1, see Table S1 for quantification of this and subsequent  
142 experiments). In SU5402-treated embryos, *aplnrb* mRNA reveals the normal invagination  
143 of mesoderm and *aplnrb*-expressing cells flanking the organiser. Both the downregulation  
144 of *aplnrb* mRNA in paraxial trunk mesoderm that normally expresses *myf5* alone, and pre-  
145 adaxial *aplnrb* up-regulation were absent in SU5402-treated embryos (Fig. 1A). Thus,  
146 early Fgf signalling is required for the initiation of skeletal myogenesis in future trunk  
147 regions.

148 As trunk myogenesis proceeds, Hh signalling becomes essential for adaxial slow  
149 myogenesis. At 6ss, in *smo* mutants, cyA-treated embryos and even cyA-treated *smo*  
150 mutants, all of which lack Hh signalling, *myod* mRNA is lost from adaxial slow muscle but  
151 persists in paraxial fast muscle precursors (Fig. 1B). Nevertheless, *myod* mRNA  
152 transiently accumulates in pre-adaxial cells of presomitic mesoderm (PSM) destined to  
153 make trunk somites, but is then lost in anterior PSM (Fig. 1B)(Barresi et al., 2000; Lewis et  
154 al., 1999; Osborn et al., 2011; van Eeden et al., 1996; van Eeden et al., 1998). Thus, as  
155 suggested previously (Coutelle et al., 2001; Ochi et al., 2008), in the wild type situation  
156 trunk pre-adaxial *myod* expression is maintained and enhanced by Hh. In contrast, during  
157 tail myogenesis at 15ss and thereafter, no pre-adaxial *myod* expression was detected in  
158 *smo* mutants or cyA-treated embryos (Fig. 1B and data not shown). These data suggest

159 that whereas Hh is necessary for induction of adaxial myogenesis in the tail, Fgf-like  
160 signals initiate *myod* expression in trunk pre-adaxial cells.

161 Additional evidence emphasizes the greater importance of Hh in tail myogenesis. In *shha*  
162 mutants, slow muscle is lost from tail but remains present in trunk somites (Fig. 2A).  
163 Moreover, injection of *myod* or *myog* mRNA into embryos lacking Hh signalling can rescue  
164 slow myogenesis in trunk but not in tail (Fig. S2). Similarly, absence of notochord-derived  
165 signals in *noto* (*flh*) mutants, in which the nascent notochord loses notochord character  
166 and converts to muscle (Coutelle et al., 2001; Halpern et al., 1995), ablates tail but not  
167 trunk slow muscle (Fig. 2B). Treatment of *noto* mutants with cyA shows that *myod*  
168 expression is initiated in trunk pre-adaxial cells adjacent to the transient pre-notochordal  
169 tissue, but fails to be maintained owing to the blockade of floorplate-derived Hh signals  
170 (Fig. 2C). Taken together, these data show that Hh can initiate and then maintain MRF  
171 gene expression, but that other signals initiate slow myogenesis in the trunk.

172 We next tested whether Hh-independent *myod* expression and *myf5* up-regulation in trunk  
173 pre-adaxial cells requires Fgf signalling. Treatment with cyA left residual pre-adaxial *myod*  
174 and *myf5* mRNA flanking the base of the notochord at trunk levels, but ablated adaxial  
175 expression in slow muscle precursor cells (Fig.2D). The residual expression was ablated  
176 when, in addition to cyA, SU5402 was used to block *fgf* signalling from 30% epiboly (Fig.  
177 2D). Application of SU5402 alone diminished *myf5* and *myod* mRNA accumulation up to  
178 tailbud stage, but caused little if any reduction of adaxial *myf5* and *myod* mRNAs in the  
179 tailbud region at the 6 somite stage (6ss), after midline *shha* function had commenced  
180 (Fig. 1B,C)(Krauss et al., 1993). Nevertheless, SU5402 greatly diminished *myod*  
181 expression in somitic fast muscle precursors and reduced the extent of *myf5* expression in  
182 paraxial PSM (Fig. 1B,C)(Groves et al., 2005; Reifers et al., 1998).

183

#### 184 ***Fgf3, fgf4, fgf6a and fgf8a collaborate to promote MRF expression***

185 To identify candidate Fgf regulators of pre-adaxial myogenesis, the expression patterns of  
186 *fgf3, fgf4, fgf6a* and *fgf8a* were investigated on wild type embryos (Fig. S3A). As reported  
187 previously, *fgf4, fgf6a* and *fgf8a* mRNAs were all detected in the posterior dorsal midline at  
188 80% epiboly, followed by *fgf3* in the chordoneural hinge (CNH) and posterior notochord  
189 (Fig. S3A)(Kudoh et al., 2001; Thisse and Thisse, 2005; Yamauchi et al., 2009). The  
190 temporal and spatial expression of *fgf3, fgf4, fgf6a* and *fgf8a* during gastrulation and early

191 somitogenesis make them excellent candidates for Fgf regulators of *myf5* and *myod*.  
192 To evaluate the role of Fgfs in *myf5* and *myod* expression, each Fgf was knocked down  
193 with previously validated antisense morpholino oligonucleotides (MOs) in wild type  
194 embryos (Fig. S3B). At 80% epiboly, there was little or no decrease of *myf5* or *myod*  
195 mRNA in individual *fgf* morphants or *fgf8a* mutant embryos (Fig. S3B). Combinatorial  
196 knockdown of several *fgfs* led to progressively more severe loss of *myod* mRNA and  
197 reduction of the raised pre-adaxial and paraxial levels of *myf5* mRNA (Fig. 3A). Thus,  
198 specific Fgfs collaborate to drive the initial expression of *myod* and *myf5* in pre-adaxial and  
199 paraxial cells.

200 By tailbud stage, however, *fgf4+fgf8a* MO treatment alone had little effect on *myod* mRNA  
201 accumulation, presumably due to the presence of Hh in the midline (Fig. 3B).  
202 Congruently, *cyA*-treatment reduced anterior adaxial *myod* mRNA, but pre-adaxial  
203 expression persisted after blockade of Hh signalling (Fig. 3B). Pre-adaxial *myod* mRNA  
204 was ablated by *cyA*-treatment of embryos injected with *fgf4+fgf8a* MO (Fig. 3B). Control  
205 injection of *fgf4+fgf8a* MOs into vehicle-treated embryos did not reduce *myod* mRNA  
206 accumulation, confirming that Hh can initiate trunk adaxial *myod* expression (Fig. 3B).  
207 Thus, expression of Fgf4 and Fgf8a in the shield, CNH and posterior notochord, provides a  
208 spatiotemporal cue for pre-adaxial myogenic initiation in the tailbud.

209 To test the ability of Fgfs to promote myogenesis further, we generated ectopic Fgf signals  
210 by injection of *fgf4* or *fgf6a* mRNA into wild type embryos, and analysed *myf5* and *myod*  
211 mRNA at 80% epiboly (Fig. 3C). Both *myod* and *myf5* mRNAs were upregulated in more  
212 ventral regions at levels comparable to those in adaxial cells of control embryos, despite  
213 the absence of Hh signalling in these regions. Over-expression of *fgf4* mRNA upregulated  
214 *myf5* mRNA in an initially uniform band around the embryo that extended towards the  
215 animal pole for a distance similar to that of *myod* mRNA in adaxial cells of controls (Fig.  
216 3C). *Myod* was less easily induced, but in similar regions of the mesoderm. The posterior  
217 notochord and shield still lacked MRFs and appeared wider. However, this dorsalmost  
218 tissue was not enlarged; the number of notochord cells scored by DAPI stain appeared  
219 normal (data not shown). The embryos became ovoid, with a constriction around the germ  
220 ring that appeared to stretch and broaden the notochord. There was a positive correlation  
221 between the extent of *myf5* and *myod* mRNA up-regulation and the extent of deformation  
222 towards egg-shape. *AplnrB* mRNA persisted in animal regions of the mesoderm, but was  
223 downregulated where *myf5* mRNA was induced nearer to the margin, revealing that



224 anterior/cranial mesoderm is present but resistant to Fgf-driven MRF induction and *aplnrb*  
225 suppression (Fig. 3D). Thus, Fgf4 dorsalized the embryo, converting the entire posterior  
226 paraxial and ventral mesoderm to a myogenic profile with some regions expressing only  
227 *myf5* and others expressing also *myod*, particularly around the germ ring. Fgf6a  
228 overexpression also induced ectopic MRFs in cells around the germ ring, which then  
229 appeared to cluster (Fig. 3C). Taken together, these data show that posterior/dorsal Fgf  
230 signals initiate MRF expression in both pre-adaxial slow and paraxial fast muscle  
231 precursors in pre-somitic mesoderm.

232

### 233 ***MRFs are initially induced by fgfs, tbxta and tbx16***

234 Zebrafish Tbx genes, including *tbxta* and *tbx16* (formerly called *no tail (ntla)* and *spadetail*  
235 (*spt*), respectively), are potentially important mediators of Fgf signalling in gastrulating  
236 embryos (Amaya et al., 1993; Griffin et al., 1995; Smith et al., 1991; Sun et al., 1999).  
237 *Tbx16* is suppressed by a dominant negative Fgf receptor (FgfR) (Griffin et al., 1998).  
238 However, whether *tbxta* and *tbx16* activities are altered by SU5402 treatment, which also  
239 blocks FgfR function, is unclear (Rentzsch et al., 2004; Rhinn et al., 2005). Wild type  
240 embryos at 30% epiboly were therefore exposed to SU5402 and subsequently fixed at  
241 80% epiboly or 6ss to investigate expression of *tbxta* and *tbx16* (Fig. 4A). Compared to  
242 controls, embryos treated with 10  $\mu$ M SU5402 showed diminished *tbxta* expression in  
243 notochord and less *tbxta* and *tbx16* in the germ ring, particularly in dorsal paraxial regions.  
244 At 6ss, expression of *tbxta* and *tbx16* was mildly reduced in tailbud by 10  $\mu$ M SU5402 (Fig.  
245 4A). When the concentration of SU5402 was increased to 30  $\mu$ M, expression of *tbxta* and  
246 *tbx16* was abolished throughout the trunk (Fig. 4A). Thus, Fgf-like signalling is required for  
247 normal Tbx gene expression in the mesoderm.

248 *Tbxta* is required for normal *myf5* and *myod* expression in posterior regions during tailbud  
249 outgrowth, partly due to loss of Hh signalling from the missing notochord (Coutelle et al.,  
250 2001; Weinberg et al., 1996). To test whether Tbx genes are required for MRF expression  
251 at earlier stages, each Tbx gene was knocked down and MRF and Fgf expression  
252 analysed at 80% epiboly. *Tbxta* knockdown reduced expression of dorsal midline Fgfs,  
253 ablated *myod* mRNA and reduced *myf5* mRNA accumulation in pre-adaxial cells to the  
254 level in paraxial regions (Fig. 4B). However, *Tbxta* knockdown had little effect on either  
255 *fgf8a* or *myf5* mRNAs in more lateral paraxial mesoderm or the germ ring (Fig. 4B). This

256 correlation raised the possibility (addressed below) that *Tbx16* may drive MRF expression  
257 through induction of Fgf expression. *Tbx16* knockdown, on the other hand, ablated pre-  
258 adaxial *myod* mRNA and reduced both pre-adaxial and paraxial *myf5* mRNA without  
259 reduction of Fgf expression (Fig. 4B). Indeed, both germ ring *fgf8a* mRNA at 80% epiboly  
260 and dorsal midline *fgf3*, *fgf4* and *fgf8a* mRNAs in the tailbud at 6ss appeared increased  
261 (Fig. 4B), as previously described (Warga et al., 2013). As *Tbx16* expression persists in  
262 *tbxta* mutants (Amack et al., 2007; Griffin et al., 1998), these data raise the possibility that  
263 *Tbx16* is required to mediate the action of Fgf signals on myogenesis.

264 *Tbx16* null mutants show a failure of convergent migration of mesodermal cells into the  
265 paraxial region (Ho and Kane, 1990; Molven et al., 1990) which, by reducing mesodermal  
266 cells flanking the CNH, may contribute to the reduction in MRF mRNAs observed at 80%  
267 epiboly. Nevertheless, *tbx16* mutants generate enough paraxial mesoderm that reduced  
268 numbers of both paraxial fast muscle and adaxially-derived slow muscle fibres arise after  
269 Hh signalling commences (Honjo and Eisen, 2005; Weinberg et al., 1996). To investigate  
270 whether *Tbx16* is required for initial induction of *myf5* and/or *myod* expression in pre-  
271 adaxial cells, we titrated MO to reduce *tbx16* function to a level that did not prevent  
272 accumulation of significant numbers of trunk mesodermal cells and examined *myf5* and  
273 *myod* expression at 6ss (Fig. 4C). In *tbx16* morphants, *myod* mRNA was readily detected  
274 in adaxial cells adjacent to notochordal Hh expression (Fig. 4C, cyan arrowheads).  
275 Treatment of these *tbx16* morphants with cyA to block Hh signalling, however, completely  
276 ablated adaxial *myod* expression, leaving only weak *myod* in paraxial somitic fast muscle  
277 precursors (Fig. 4C, cyan and purple arrowheads). In contrast, treatment of control  
278 embryos with cyA left pre-adaxial *myod* mRNA intact (Fig. 4C, black arrowheads). These  
279 findings show that Fgf-driven pre-adaxial *myod* expression flanking the CNH requires  
280 *Tbx16* function.

281 Adaxial *myf5* expression also requires *Tbx16*. *Tbx16* knockdown reduces *myf5* mRNA  
282 accumulation in the posterior tailbud, and also diminishes the upregulation of *myf5* mRNA  
283 in pre-adaxial and adaxial cells (Fig. 4C, orange arrowheads). Addition of cyA to *tbx16*  
284 morphants has little further effect on *myf5* expression (Fig. 4C, white arrowheads). In  
285 contrast, cyA treatment alone reduces adaxial *myf5* mRNA in anterior PSM, but does not  
286 affect the *myf5* up-regulation in pre-adaxial cells or tailbud *myf5* expression (Fig. 4C, white  
287 arrowheads). Additional knockdown of *Tbx16* in cyA-treated embryos prevents pre-adaxial  
288 *myf5* up-regulation (Fig. 4C, white arrowheads). Thus, *Tbx16* is required for Fgf to up-

289 regulate both *myf5* and *myod* in pre-adaxial cells.

290 Both pre-adaxial and anterior PSM adaxial *myod* expression were also absent in *tbxta*  
291 morphants, but recovered in somites, again due to midline-derived Hh signalling (Fig. 4C  
292 and (Coutelle et al., 2001)). In marked contrast, *Tbxta* knockdown up-regulated *myf5*  
293 mRNA in tailbud (Fig. 4C, asterisk), presumably reflecting loss of tailbud stem cells that  
294 lack *myf5* mRNA. Taken together, the data strongly suggest that *tbx16* is required for  
295 midline-derived Fgf signals to induce *myod* and up-regulate *myf5* in pre-adaxial cells in  
296 tailbud. In contrast, the loss of MRF expression in *tbxta* mutants could be simply  
297 explained by loss of midline-derived Fgf signals, and/or might require some other *Tbxta*-  
298 dependent process.

299

### 300 ***Myf5 and myod induction by Fgf signalling require Tbx16***

301 To test rigorously the idea that *Tbx16* is required for Fgf to induce MRFs, *fgf4* mRNA was  
302 injected into embryos from a *tbx16* heterozygote incross. Whereas *Fgf4* up-regulated  
303 *myf5* and *myod* mRNAs all around the germ ring in siblings, in sequence-genotyped *tbx16*  
304 mutant embryos no up-regulation was detected (Fig. 5A). It is clear that mesoderm was  
305 present in *tbx16* mutants because the mRNAs encoding *Aplnr*, *Tbxta*, *Tbx16* and *Tbx16*-  
306 like (formerly *Tbx6*-like) are present in *tbx16* mutants (Fig. S4; (Griffin et al., 1998; Morrow  
307 et al., 2017)). The effect of *Fgf4* does not act by radically altering *tbxta* or *tbx16* gene  
308 expression (Fig. 5B). Thus, *Tbx16* is required for Fgf-driven expression of MRFs in pre-  
309 somitic mesoderm.

310

### 311 ***Tbx16 requires Fgf-like signalling to rescue myf5 and myod expression***

312 The results so far show that *tbx16* function is necessary for Fgf to induce *myf5* and *myod*  
313 (Fig. 5A). To determine if increased *Tbx16* activity is sufficient to induce MRFs, *Tbx16*  
314 was overexpressed. Injection of *tbx16* mRNA caused ectopic expression of both *myf5* and  
315 *myod* in the germ marginal zone (Fig. 5C). Notably, *Tbx16* over-expression induced *myf5*  
316 mRNA in a much broader region than *myod* mRNA.

317 We next wanted to determine whether *Tbx16* could induce expression of *myf5* or *myod* in  
318 the absence of Fgf signalling. Exposure to high dose SU5402, which down-regulates  
319 endogenous *tbx16* and *tbxta* mRNA (Fig. 4A), prevented MRF induction by injection of

320 *tbx16* mRNA (Fig. 5D). Nevertheless, when *tbx16* mRNA was injected into low dose  
321 (10  $\mu$ M) SU5402-treated embryos, which normally have reduced MRF expression, the  
322 level of *myf5* and *myod* mRNAs was rescued (Fig. 5C). However, *tbx16* mRNA was less  
323 effective at ectopic MRF induction in the presence of SU5402 (Fig. 5C). These results  
324 demonstrate that Fgf signalling cooperates with Tbx16 activity in inducing expression of  
325 *myf5* and *myod* in pre-axial cells at gastrulation stages. Moreover, Tbx16 requires Fgf-  
326 like signals to induce MRF gene expression.

327

### 328 ***Myf5 and Myod are direct transcriptional targets of Tbx16***

329 In order to determine further the regulatory relationship between Tbx16 and *myf5* and  
330 *myod* we interrogated ChIP-seq experiments for endogenous Tbx16 on 75-85% epiboly  
331 stage embryos (see Materials and Methods)(Nelson et al., 2017). Our analyses revealed  
332 a highly significant peak at -80 kb upstream (*myf5* Distal Element, 5DE) and two binding  
333 peaks proximal to *myf5* (Proximal Elements, 5PE1,5PE3) (Fig. 6A,B and Supplementary  
334 Table S3). To determine which of these binding events are likely to be functionally  
335 important we further cross-referenced the data with published histone modification ChIP-  
336 seq data (Bogdanovic et al., 2012). The 5DE and 5PE1 peaks overlapped significant  
337 H3K27ac and H3K4me1 peaks (Table S3), suggesting these are likely to be functionally  
338 active enhancers. Tbx16 ChIP-qPCR confirmed the validity of the 5DE and 5PE1 ChIP-  
339 seq peaks (Fig. 6C). These putative enhancers are likely to regulate *myf5*, the promoter of  
340 which has a H3K4me3 mark, rather than the adjacent *myf6* gene, which is not expressed  
341 at 80% epiboly and does not have a H3K4me3 mark. Comparison of the sequences under  
342 each peak to genomic regions adjacent to the *myf5* gene in other fish species revealed  
343 significant conservation (Figs 6A,B). Of particular note was the conservation of 5DE in  
344 medaka and stickleback, while 5PE1 showed notable conservation in medaka and fugu  
345 (Figs 6A,B). Thus, ChIP-seq peaks corresponding to histone marks indicative of enhancer  
346 activity suggest evolutionarily conserved mechanisms of *myf5* regulation in fish.

347 We next tested whether Tbx16 is able to positively regulate *myf5* directly. To do this we  
348 used a hormone-inducible system to activate Tbx16 in the presence of cycloheximide,  
349 followed by *in situ* hybridization (Kolm and Sive, 1995; Martin and Kimelman, 2008).  
350 Briefly, mRNA corresponding to Tbx16 fused to the hormone binding domain of  
351 glucocorticoid receptor (GR) was overexpressed in wild-type embryos. The resulting

352 protein is held in the cytoplasm until dexamethasone (DEX) stimulates GR nuclear  
353 translocation. In the presence of the translation inhibitor cycloheximide (CHD), increased  
354 nuclear Tbx16 is expected mosaically to induce only direct targets of Tbx16 (Fig. 6D).  
355 Ectopic expression of *myf5* around the germ ring was observed upon Tbx16 activation  
356 (Fig. 6E). Moreover, using the same approach, induction of *myod* mRNA indicated that  
357 *myod* is also a direct target of Tbx16 (Fig. 6E). *Myod* mRNA up-regulation was, however,  
358 restricted to a narrow ectopic domain flanking the base of the notochord, indicating that  
359 *myod* expression is under additional Tbx16-independent constraints compared to *myf5*  
360 (Fig. 6E). Interestingly, across the set of CHD+DEX-treated embryos, ectopic *myf5* mRNA  
361 was induced to a higher level in a similar region to ectopic *myod* mRNA than elsewhere,  
362 suggesting that Tbx16 was able to induce two aspects of pre-adaxial character directly in  
363 this region of the embryo. To confirm this result, we tested whether *Myf5* is required for  
364 Tbx16 to induce *myod* expression. When *tbx16* mRNA was injected into *myf5* mutant or  
365 heterozygote embryos, ectopic *myod* mRNA was observed flanking the base of the  
366 notochord in about 50% of embryos, but appeared more readily induced in wild type  
367 siblings (Fig. 6F). Thus, Tbx16 is necessary for MRF expression and can induce both  
368 *myf5* and *myod* independently, so long as Fgf signalling is active. In summary, Tbx16  
369 directly induces MRF expression in gastrulating mesoderm and is particularly potent in the  
370 pre-adaxial region that normally retains high Tbx16 expression.

371

### 372 ***Tbx16 is essential for pre-adaxial but not paraxial myogenesis***

373 Whereas the entire paraxial PSM expresses *myf5*, pre-adaxial cells upregulate *myf5* and  
374 are the first cells to express *myod*. *Tbx16* and *Tbx16* have similar DNA binding recognition  
375 sequences (Garnett et al., 2009; Nelson et al., 2017). Congruently, we find a prominent  
376 *Tbx16* ChIP-seq peak at the 5DE -80 kb site upstream of *myf5*, and minor peaks at the  
377 proximal sites (Fig. 6A,B; Table S3). Because of the role of *Tbx16* and *Tbx16* in *myod*  
378 expression we also examined the *myod* locus for Tbx protein binding. We found multiple  
379 sites occupied by *Tbx16* and *Tbx16* either individually or in combination (Table S3).  
380 Notably, only one site (DDE3) displayed strongly significant H3K4me1 and H3K27ac  
381 peaks and this was only occupied by *Tbx16* and not by *Tbx16* (Fig. S5 and Table S3).  
382 However, an additional site (DDE1) showed significant occupancy by *Tbx16* and *Tbx16*  
383 concurrent with H3K4me1 but not H3K27ac (Fig. S5; Table S3). In spite of the absence of  
384 a significant H3K27ac mark, this peak may be important to *Tbx16* regulation of *myod*.

385 These findings indicate that differential direct binding of *Tbx16* and *Tbx16* may control both  
386 *myf5* and *myod* expression at the inception of skeletal myogenesis.

387 Is *Tbx16* also required for MRF expression in response to Fgf? Over-expression of Fgf4 in  
388 *tbx16* mutants successfully induced *myf5* mRNA and suppressed *aplnrb* mRNA widely in  
389 the posterior mesoderm except in a widened dorsal midline region, showing that the  
390 introduced Fgf4 was active (Fig. 7A,B). However, *myod* expression was not rescued in  
391 *tbx16* mutants in the dorsal pre-adaxial region of Fgf4-injected embryos, or elsewhere  
392 around the germ ring (Fig. 7A). Moreover, even an increased dose of 225 pg *fgf4*  
393 mRNA/embryo failed to rescue *myod* mRNA in *tbx16* mutants. Importantly, *tbx16*  
394 heterozygotes showed significantly less extensive induction of *myod* mRNA in response to  
395 Fgf4 than did their wt siblings ( $p=0.0001$   $X^2$ -test; Fig. 7C and Table S4). Therefore, *Tbx16*  
396 is essential for *myod* induction in pre-adaxial cells independent of its role in promoting  
397 expression of midline Fgfs.

398 Although Fgf4-injection did not radically alter the location of *tbx16* or *tbx16* mRNA (Fig. 5B),  
399 we noticed that the higher level of *tbx16* mRNA in adaxial compared to paraxial cells was  
400 not obvious in Fgf4-injected wt embryos, with high levels present at all dorsoventral  
401 locations, presumably because pre-adaxial character was induced widely in posterior  
402 mesoderm (Fig. 5B). Nevertheless, as Fgf4-injection into *tbx16* mutants induced *myf5* but  
403 not *myod* mRNA (Fig. 7A), it seems *Tbx16* is essential to progress from *myf5* to *myod*  
404 expression.

405 Two hypotheses could explain the lack of *myod* expression in Fgf4-injected *tbx16* mutants.  
406 First, despite the apparent lack of *Tbx16* protein in adaxial cells (Ochi et al., 2008; Schulte-  
407 Merker et al., 1994a), *Tbx16* might act directly on *myod*. Alternatively, Fgf-driven *Tbx16*  
408 activity might act indirectly in pre-adaxial cells to upregulate *Tbx16* and thereby drive *myod*  
409 expression. We therefore examined the ability of Fgf4 to upregulate *Tbx16* in *tbx16*  
410 mutants. Fgf4 enhanced *tbx16* mRNA throughout the posterior mesoderm in siblings, with  
411 the exception of the widened notochordal tissue that contained nuclear *Tbx16* protein and  
412 failed to upregulate MRFs (Figs 7D, 8D). In *tbx16* mutants, Fgf4 also enhanced *tbx16*  
413 mRNA in the ventral mesoderm, but a broader dorsal region did not express *tbx16*.  
414 Moreover, the level of *tbx16* mRNA appeared lower than in siblings (Fig. 7D). Thus, Fgf4-  
415 injected *tbx16* mutants lack both *Tbx16* and *Tbx16* upregulation in pre-adaxial cells. We  
416 conclude that Fgf-driven induction of lateral myogenic tissue requires *Tbx16*, but not  
417 *Tbx16*. In contrast, induction of pre-adaxial character, marked by upregulated *myf5* and

418 *myod* mRNAs, requires both Tbx genes.

419

#### 420 ***Fgf action on Tbx16 suppresses dorsoposterior axial fate***

421 Finally, we examined the wider effect of Fgf signalling when skeletal muscle cannot form in  
422 the absence of *tbx16* function. Excess Fgf action in embryos causes gross patterning  
423 defects (Kimelman and Kirschner, 1987; Slack et al., 1987). When Fgf4 was over-  
424 expressed in *tbx16* heterozygote in-cross lays, some anterior mesodermal tissue formed  
425 and head tissues such as eye and brain were apparent, but trunk and tail mesoderm was  
426 grossly deficient (Fig. 8A). In siblings over-expressing Fgf4, some muscle was formed and  
427 truncated embryos were observed to twitch at 24 hpf. In contrast, no muscle was detected  
428 in *tbx16* mutants (Fig. 8B). Moreover, the residual expression of mutant *tbx16* mRNA at  
429 90% epiboly observed in un-injected embryos was lost upon Fgf4 over-expression (Fig.  
430 8C). This suggests that the cells with tailbud character normally accumulating in *tbx16*  
431 mutants were missing. Instead, widespread expression of Tbx16 protein in nuclei far from  
432 the germ ring suggested that the entire posterior (but not anterior) mesoderm had  
433 converted to notochord, the most dorsal posterior mesoderm fate (Fig. 8D). Indeed, *shha*  
434 mRNA, a marker of notochord, was found to be broadly upregulated around the embryo in  
435 *tbx16* mutant embryos injected with Fgf4 mRNA, but not in their siblings (Fig. 8E). The  
436 data suggest that Fgf4 drives early involution of all posterior mesoderm precursors, leaving  
437 none to form a tailbud. In the presence of Tbx16, Fgf4 also dorsalizes most involuted  
438 mesoderm to muscle, whereas, in the absence of Tbx16, Fgf4 converts most of the  
439 mesoderm to notochord.

440

#### 441 **Discussion**

442 The current work contains four main findings. First, that Tbx16 directly binds and activates  
443 *myf5* regulatory elements to initiate skeletal myogenesis. Second, that Fgf signalling acts  
444 through Tbx16 to drive the initial myogenic events in the adaxial cell lineage that  
445 subsequently require Hh signalling to complete myogenesis. Third, that Tbx16, the dorsal-  
446 most/posterior Tbx factor, binds directly to *myod* regulatory elements and also promotes  
447 dorsal midline expression of Fgfs that subsequently cooperate to drive dorsal myogenesis  
448 through Tbx16. Fourth, that Fgf action through Tbx16 suppresses the dorsoposterior axial  
449 fate induced by Tbx16. Overall, Tbx transcription factors provide a crucial link between

450 mesoderm induction and the initiation of myogenesis, which has profound implications for  
451 understanding the evolution of vertebrates.

#### 452 ***Tbx genes and myogenesis***

453 Building on previous evidence that Tbx16 up-regulates *myf5* mRNA level (Garnett et al.,  
454 2009; Mueller et al., 2010), our findings that Tbx16 protein binds to DNA elements near the  
455 *myf5* and *myod* genes, is required for their expression, and can induce them in the  
456 absence of protein synthesis show that these MRF genes are direct targets of Tbx16. We  
457 also present evidence that *myf5* and *myod* are direct targets of Tbx16, including the  
458 presence of several significant Tbx16 ChIP-seq peaks and the requirement for Tbx16 to  
459 transduce Fgf4-mediated signalling into *myod* expression. As MRF gene activity,  
460 principally that of *myf5* and *myod*, drives commitment to skeletal myogenesis in  
461 vertebrates, our findings place Tbx protein activity at the base of skeletal myogenesis in  
462 zebrafish.

463 Before myogenesis, Tbx16 is required for migration of most trunk PSM cells away from the  
464 'maturation zone' immediately after their involution (Griffin and Kimelman, 2002; Row et  
465 al., 2011). Our analysis of *aplnrb*-expressing mesodermal cells shows that most anterior  
466 (i.e. head) and posterior ventral (i.e. ventral trunk) mesoderm involution and migration  
467 occurs normally in both *tbx16* and *tbxta* mutants. Indeed, some PSM is formed in *tbx16*  
468 mutants and goes on to make small amounts of muscle (Amacher et al., 2002; Kimmel et  
469 al., 1989). PSM formation is more severely lacking in *tbx16;tbxta* or *tbx16;tbx16l* double  
470 mutants (Amacher et al., 2002; Griffin et al., 1998; Morrow et al., 2017; Nelson et al.,  
471 2017) or after knockdown of Tbx16 in *tbxta* mutant (Martin and Kimelman, 2008).  
472 Cooperation of Tbx proteins in PSM formation also occurs in *Xenopus tropicalis* (Gentsch  
473 et al., 2013). It is likely, therefore, that all PSM formation and its accompanying *myf5*  
474 expression requires Tbx proteins. Our findings also help to explain the observation that  
475 *tbx16* mutants have increased pronephric mesoderm (Warga et al., 2013). As Tbx16 is  
476 required for direct induction of *myf5* and for *pcdh8*, *msgn1*, *mespaa* and *tbx6* expression in  
477 PSM (Fior et al., 2012; Goering et al., 2003; Griffin and Kimelman, 2002; Lee et al., 2009;  
478 Morrow et al., 2017; Yamamoto et al., 1998), the data support previous proposals  
479 (Amacher and Kimmel, 1998; Griffin and Kimelman, 2002) of a role for Tbx16 in promotion  
480 of the earliest step in PSM formation, *en route* to myogenesis. These early actions of  
481 Tbx16 and Tbx16 proteins have previously masked their direct myogenic actions in  
482 mutants.



483 To identify likely MRF enhancers at 80% epiboly, we have largely restricted our focus to  
484 robust *Tbx16* and *Tbx16a* ChIP-seq peaks that are co-incident with established histone  
485 marks indicative of active enhancers, H3K4me1 and H3K27ac. This approach is driven by  
486 the accepted knowledge that most transcription factor binding events are unlikely to be  
487 functionally important, and the availability of stage-matched ChIP-seq data for these  
488 established histone marks (Bogdanovic et al., 2012). However, there is an increasing  
489 realization that comprehensive identification of enhancers will require a more complete  
490 analysis of further histone acetylation events, as evidenced by the recent discovery of  
491 functional enhancers marked by H3K122ac, H3K64ac and/or H4K16ac, many of which  
492 lack H3K27ac (Pradeepa, 2017; Pradeepa et al., 2016). It is therefore possible that  
493 additional *Tbx16* and *Tbx16a* ChIP-seq peaks beyond 5DE, 5PE1 and DDE3 may mark  
494 functionally important enhancers regulating *myf5* and *myod*. Of particular note is DDE1, -  
495 31kb upstream of *myod*, which is colocalized with a significant H3K4me1 mark, though not  
496 H3K27ac. Given that *myod* expression is restricted to a minor cell population at 80%  
497 epiboly, we further note that the probability of detecting significant histone marks specific  
498 to this population is low due to averaging of signal across whole embryos. It is therefore  
499 possible that the ChIP-seq peak common to *Tbx16* and *Tbx16a* at the DDE1 -31kb upstream  
500 of *myod* is functionally important. Given that *Tbx16* is essential for *myod* expression in  
501 pre-adaxial cells and can upregulate *myod* in the absence of protein synthesis, this ChIP-  
502 seq peak may represent a key region mediating this direct transcriptional activation. Our  
503 data argue that once posterior (i.e. trunk) mesoderm forms, Tbx proteins are still required  
504 for MRF expression and normal myogenesis. Hh signalling from notochord acts to  
505 maintain adaxial MRF expression in wild type and, if Tbx-driven initiation fails, Hh can  
506 initiate *myod* and up-regulate *myf5* expression, thereby driving slow myogenesis (Blagden  
507 et al., 1997; Coutelle et al., 2001; Du et al., 1997). When Hh signalling is prevented, the  
508 small amount of trunk PSM that forms in *tbx16* mutants activates low level *myf5* but not  
509 *myod* mRNA expression, showing that *Tbx16* is essential for initial pre-adaxial *myod*  
510 transcription. *Tbx16a* mutants also fail to initiate pre-adaxial *myod* expression. Conversely,  
511 overexpression of *Tbx16* directly induces ectopic *myf5* mRNA, but only in mesoderm and  
512 preferentially in dorsal mesoderm at somite levels. In contrast, *Tbx16*-induced ectopic  
513 *myod* expression is restricted to a narrower mesodermal region flanking the pre-adaxial  
514 cells, likely due to the restricted expression of *smarcd3* in this region (Ochi et al., 2008).  
515 Nevertheless, *myod* is induced by *Tbx16* in the absence of *Myf5*, probably through direct  
516 binding to regulatory elements in the *myod* locus. It is thus likely that changes in

517 chromatin structure in the *myf5* and *myod* loci that accompany posterior mesoderm  
518 formation facilitate Tbx16 access to its binding sites in these MRF genes.

519 In considering the role of Tbx genes in myogenesis it is essential to distinguish adaxial and  
520 paraxial myogenesis, which give rise to different kinds of muscle with distinct timing  
521 (Blagden et al., 1997; Devoto et al., 1996). Paraxial PSM cells form fast muscle after  
522 somitogenesis. As posterior mesoderm involutes during gastrulation, *myf5* expression is  
523 initiated throughout the PSM. Tbx16, but not Tbx16, is required for almost all this low level  
524 *myf5* expression. In wild type, *myf5* mRNA then declines as paraxial PSM matures before  
525 being up-regulated once more as somite borders form (Coutelle et al., 2001). In contrast,  
526 pre-adaxial cells require both Tbx16 and Tbx16 function for up-regulation of *myf5* and  
527 initiation of *myod* expression, and then commence terminal differentiation within the PSM  
528 to generate slow muscle. Thus, distinct Tbx proteins are required for normal adaxial and  
529 paraxial myogenesis.

530 Our data show that Tbx16 is a key direct regulator of MRFs required for myogenesis. The  
531 expression of low levels of *myf5* mRNA and formation of small amounts of both fast and  
532 slow muscle in the trunk and more in the tail in *tbx16* mutants are likely driven by Tbx16  
533 (formerly known as Tbx6 and then Tbx6) (Morrow et al., 2017). Morrow et al (2017) show  
534 that tail somite formation is prevented and tail *myod* mRNA diminished in the *tbx16;tbx16*  
535 double mutant, but that *myod* mRNA expression continues at a reduced level throughout  
536 the axis at 24 hpf. Our data argue that the residual *myod* mRNA is in adaxially-derived  
537 slow muscle induced by Hh signalling.

538 Other Tbx proteins may directly regulate myogenic initiation in other body regions and  
539 species, as many Tbx proteins bind similar DNA motifs (Papaioannou, 2014). For  
540 example, Tbx1, Tbx5a and Tbx20 are required in anterior mesoderm for patterning of  
541 cranial and cardiac muscles (Knight et al., 2008; Lu et al., 2017; Valasek et al., 2011).  
542 *Tbx4/5a* gene function is required for limb muscle patterning, at least partially non-cell  
543 autonomously (Don et al., 2016; Hasson et al., 2010; Valasek et al., 2011). Tbx6  
544 suppresses myogenesis in anterior PSM indirectly through *Mesp-b* (Windner et al., 2015),  
545 but direct actions of Tbx6 could also regulate myogenesis. The increasing complexity of  
546 Tbx gene diversity during vertebrate evolution may have permitted muscle diversification.

547

548 ***Fgf and myogenesis***

549 Tbx16 is required for Fgf signalling to induce *myf5* (Fig. 5A). In its absence, Fgf drives all  
550 posterior mesoderm to a notochord-like fate (Fig. 8D,E), probably via activation of *Tbxta*.  
551 It has long been known that Fgf signalling is required for expression of *myf5* in the paraxial  
552 PSM of the tailbud (Groves et al., 2005). Here we show Fgf is also required for the earliest  
553 *myf5* expression in invaginating trunk mesoderm and for the initiation of *myf5* and *myod*  
554 expression in pre-adaxial cells destined to form the slow muscle of anterior somites. Our  
555 data also extend the evidence that this MRF expression is subsequently maintained, as  
556 the shield/tailbud-derived sources of Fgf recede from the adaxial cells, by Hh signalling  
557 from the maturing notochord (Coutelle et al., 2001; Osborn et al., 2011). The finding that  
558 the latter Fgf action is restricted to trunk (as opposed to tail) somites is consistent with a)  
559 the dwindling level of Fgf mRNAs in the chordoneural hinge as tailbud outgrowth slows  
560 becoming insufficient to promote MRF expression and b) the unresolved issue of Hh-  
561 independent initiation of MRF expression in the anteriormost somites of murine  
562 *smoothened* mutants (Zhang et al., 2001). We suggest this MRF expression is Fgf-  
563 triggered in mouse, as in zebrafish.

564 *Tbxta* is also required for normal Fgf signalling from the midline to promote pre-adaxial  
565 myogenesis. One likely reason is the loss of *fgf4*, *fgf6a*, *fgf8a*, *fgf8b* and possibly *fgf3*  
566 expression in the dorsal midline chordoneural hinge region in *tbxta* mutants (Fig.  
567 3B)(Draper et al., 2003). When Fgf4 or Fgf6a is artificially over-expressed in *tbxta*  
568 mutants, *myf5* is readily induced. This suggests that *Tbxta* protein is not required cell  
569 autonomously to drive *myf5* expression, even though our data reveal that *Tbxta* binds in  
570 vivo to similar regions of the *myf5* locus to those bound by Tbx16. In marked contrast,  
571 *Tbxta* is essential for over-expressed Fgf to induce *myod*. Interestingly, ChIP shows *Tbxta*  
572 binding sites upstream of *myod* that are preferentially bound by *Tbxta*, suggesting that  
573 *Tbxta* binding to one or more of these sites directly activates *myod* in response to Fgf. We  
574 note, however, that *Tbxta* protein is immunodetectable in notochord and germ ring cells  
575 but barely in *myod*-expressing pre-adaxial cells (Hammerschmidt and Nüsslein-Volhard,  
576 1993; Odenthal et al., 1996; Schulte-Merker et al., 1994a). Nevertheless, as pre-adaxial  
577 cells have recently expressed *Tbxta* during their involution, it is possible that functionally  
578 significant protein could remain bound at the *myod* locus. Transient persistence of *Tbxta*  
579 is consistent with the downregulation of *myod* mRNA in more anterior adaxial cells in the  
580 absence of Hh signals. An alternative hypothesis of indirect regulation of *myod* by Fgf  
581 through another transcription factor, such as Tbx16, is also tenable, and is supported by

582 the reduced upregulation of *tbx16* observed in *tbxta* mutants injected with Fgf4 (Fig. 7D).  
583 We show that *Myf5* is not an indirect mediator of Fgf signalling on *myod*, despite its Fgf  
584 sensitivity.

585 Our data argue strongly that Fgf signalling not only promotes *tbx16* expression, but also  
586 enhances the activity of the Tbx16 protein. The MRF inducing activity of Tbx16 is  
587 suppressed by inhibition of Fgf signalling (Fig. 5C,D). Bearing in mind the existence of  
588 Tbx16l and *Tbxta*, this result is consistent with the finding that *tbxta* or *tbx16* mutation  
589 sensitizes embryos to Fgf inhibition (Griffin and Kimelman, 2003). As Tbx16  
590 overexpression can expand PSM fates and reverse the effect of partial Fgf inhibition, a  
591 primary role of Fgf signalling is to cooperate with Tbx16 to drive expression of its target  
592 genes, including *myf5*. This understanding provides mechanistic insight into how the  
593 effects of Fgf on gastrulation movements and histogenesis are separated, as originally  
594 proposed (Amaya et al., 1993). Interestingly, Tbx16 over-expression rescues *myf5* mRNA  
595 preferentially on the dorsal side of the embryo, suggesting that BMP and/or other signals  
596 continue to suppress PSM fates ventrally, and thus that Tbx16 does not act by simply  
597 suppressing the inhibitory effect of such signals.

598 We find that in the absence of *Tbxta*, Fgf acts to induce Tbx16 in ventral regions (Fig. 7D).  
599 The data are fully consistent with a positive feedback loop between *Tbxta* and Fgf  
600 signalling to maintain tailbud character and notochord formation. Additionally, our findings  
601 suggest that Tbx16 acts in competition with *Tbxta* to suppress tailbud/notochord fate and  
602 Fgf expression and to promote paraxial fates. Ochi et al. (2008) have suggested *Tbxta*  
603 and Fgf work through Smarcd3 (Baf60c) to permit pre-adaxial cell formation. It is striking  
604 that the requirement for both *Tbxta* and Tbx16 proteins to generate adaxial cells located  
605 between the axial and paraxial regions triggers the first step of diversification of muscle to  
606 slow and fast contractile character.

607

### 608 ***Evolution of vertebrates***

609 From an evolutionary perspective, our findings yield a number of important insights. As  
610 efficient motility is a key feature of animals, and efficient sarcomeric muscle is found  
611 throughout triploblasts, it is likely that mesodermal striated muscle existed in the common  
612 ancestor of deuterostomes and protostomes. It is widely thought that chordates evolved  
613 from an early deuterostome consisting of a segmented 'branchial basket', possibly

614 attached to a propulsive segmented pre-anal trunk. There is consensus that subsequent  
615 appearance of neural crest, notochord and a post-anal tail were significant evolutionary  
616 steps for chordates (Gee, 2018). Already in cephalochordates at least two kinds of  
617 striated muscle had evolved in anterior somites (Devoto et al., 2006; Lacalli, 2002). Our  
618 evidence that initiation of both slow and fast myogenesis in the most anterior trunk is  
619 driven by Fgf/Tbx signalling indicates that a major function of this early mesodermal  
620 inducer was induction of trunk striated myogenesis, which may constitute an ancestral  
621 chordate character. Once Hh is expressed in maturing midline tissues, it triggers terminal  
622 differentiation of muscle precursors into functional muscle through a positive feedback loop  
623 (Coutelle et al., 2001; Osborn et al., 2011). Parallel diversification of neural tube cells,  
624 also regulated by Hh (Placzek and Briscoe, 2018), may have generated matching  
625 motoneural and muscle fibre populations that enhanced organismal motility. With the  
626 evolution of a *tbxta*-dependent tailbud destined to make the post-anal tail, our data  
627 suggest that weakening Fgf signalling continued to induce *myf5* expression and paraxial  
628 mesoderm character through *tbx16*, but was insufficient to induce adaxial myogenesis.  
629 The presence of Hh and *Tbxta* plus *Tbx16*, however, ensure that adaxial slow muscle is  
630 initiated in the zebrafish tail.

631 In the anterior somites of amniotes, as in zebrafish, Hh signalling maintains, rather than  
632 initiates *myf5* expression (Zhang et al., 2001). In more posterior somites of zebrafish,  
633 *Xenopus* and amniote, Hh signalling drives *myf5* initiation (Borycki et al., 1999; Grimaldi et  
634 al., 2004). Compared to zebrafish, however, murine *Myf5* induction is further delayed until  
635 after somitogenesis, when *Gli3* repressive signals in PSM have diminished (McDermott et  
636 al., 2005). In mouse, *Brachyury/Tbxt* is required for myogenesis and binds 20 kb  
637 downstream of *Myod*, but does not obviously control its expression (Lolas et al., 2014). No  
638 clear orthologue of *tbx16* exists in mammals, although it clusters by sequence with *Tbx6*  
639 genes. In mammals, *Tbx6* suppresses neurogenesis in posterior paraxial mesoderm,  
640 suggesting that additional mechanisms have evolved that suppress early pre-somitic *Myf5*  
641 expression (Chapman and Papaioannou, 1998). Indeed, possible low level *Myf5*  
642 expression in PSM has long been a source of controversy (George-Weinstein et al., 1996;  
643 Gerhart et al., 2004). Thus, there has been diversification in how these Tbx genes  
644 regulate somitic myogenesis.

645 Our data suggest that regions required for *myf5* expression are located ~80 kb upstream  
646 of the transcription start site. A BAC transgenic encompassing 80 kb upstream and 76 kb

647 downstream of *myf5* has been shown to drive GFP expression in muscle (Chen et al.,  
648 2007). However, analysis of shorter constructs has been confounded by cloning artefacts  
649 (Chen et al., 2001; Chen et al., 2007), leaving understanding of elements driving specific  
650 aspects of zebrafish *myf5* expression unclear. Murine *Myf5* is also regulated by many  
651 distant elements (Buckingham and Rigby, 2014). Similarly, we observe Tbx binding peaks  
652 far upstream of zebrafish *myod*. Upstream elements are known to initiate murine *Myod*  
653 expression in some embryonic regions, but whether these elements drive the earliest  
654 myotomal regulation of *Myod* is unknown (Chen and Goldhamer, 2004). The extent to  
655 which similar transcription factors act through similar binding elements to initiate MRF  
656 expression and myogenesis across vertebrates remains to be determined.

657 The ancestral situation seems clearer. In amphioxus, *Tbx6/16* is expressed in tailbud and  
658 PSM (Belgacem et al., 2011). In *Ciona*, knockdown of *Tbx6b/c/d* leads to reduced *MyoD*  
659 expression, loss of muscle and paralysis (Imai et al., 2006). In *Xenopus*, both *Tbx6* and  
660 *VegT* are implicated in early myogenesis (Callery et al., 2010; Fukuda et al., 2010; Tazumi  
661 et al., 2010), although some mechanisms may differ from those in zebrafish (Maguire et  
662 al., 2012). By adding our zebrafish findings, we show that in all major chordate groups  
663 Tbx-dependent gene regulation is central to skeletal myogenesis. The conserved  
664 involvement, yet divergent detail, of how *Tbx16*, *Tbx6* and *Tbxt* genes regulate somitic  
665 myogenic diversity along the body axis are consistent with selective pressures on these  
666 duplicated Tbx gene families playing a key role in the diversification of myogenesis in the  
667 vertebrate trunk and tail, characters that gave chordates their predatory advantage.

668

669

670

## 671 **Materials and methods**

### 672 ***Zebrafish lines and maintenance***

673 Mutant lines *fgf8a*<sup>ti282a</sup> (Reifers et al., 1998), *noto*<sup>n1</sup> (Talbot et al., 1995), *smo*<sup>b641</sup> (Barresi et  
674 al., 2000), *tbxta*<sup>b195</sup> and *tbx16*<sup>b104</sup> (Griffin et al., 1998) are likely nulls and were maintained  
675 on King's wild type background. Staging and husbandry were as described previously  
676 (Westerfield, 2000). All experiments were performed under licences awarded under the UK  
677 Animal (Scientific Procedures) Act 1986 and subsequent modifications.

678

### 679 ***In situ mRNA hybridization and immunohistochemistry***

680 In situ mRNA hybridization for *myf5* and *myod* was as described previously (Hinits et al.,  
681 2009). Additional probes were *fgf3* (Maroon et al., 2002), *fgf4* (IMAGE: 6790533), *fgf6a*  
682 (Thisse and Thisse, 2005), *fgf8a* (Reifers et al., 1998), *tbxta* (Schulte-Merker et al., 1994b)  
683 and *tbx16* (Griffin et al., 1998; Ruvinsky et al., 1998). Anti-Ntla immunostaining was  
684 performed after in situ hybridization using rabbit anti-Ntla (Schulte-Merker et al., 1992,  
685 1:2000) and Horse anti-rabbit IgG-HRP conjugated (Vector Laboratories, Inc.)

686

### 687 ***Embryo Manipulations***

688 Embryos were injected with MOs (GeneTools LLC) as indicated in Table S2 to *fgf3*, *fgf4*,  
689 *fgf6a*, *fgf8a*, *tbxta* (Feldman and Stemple, 2001) or *tbx16* (Bisgrove et al., 2005). Controls  
690 were vehicle or irrelevant mismatch MO. Cyclopamine (100 µM in embryo medium),  
691 SU5402 (at indicted concentration in embryo medium) and vehicle control were added at  
692 30% epiboly to embryos whose chorions had been punctured with a 30G hypodermic  
693 needle. A PCR product of *fgf4* (IMAGE: 6790533) was cloned (primers in Table S2) into  
694 the SacI/SalI sites of pβUT3 to make mRNA for over-expression. 100-220 pg *fgf4* mRNA  
695 (made with messageMachine), 50 pg *fgf6a* mRNA, 150 pg *tbx16* mRNA (Griffin et al.,  
696 1998) or 150 pg *tbx16*-GR mRNA (Jahangiri et al., 2012) were injected into 1-cell-stage  
697 embryos. For hormone-inducible Tbx16 activation, embryos were treated with 10 µg/ml  
698 final concentration of cycloheximide two hours prior to collection at 75-80% epiboly. After  
699 30 min, 20 µM dexamethasone was added for the remaining 1.5 h.

700

### 701 ***Chromatin immunoprecipitation and sequencing (ChIP-seq) and ChIP-qPCR***

702 ChIP-seq data was analysed as reported previously (Nelson et al., 2017). ChIP-qPCR  
703 experiments were performed as previously reported (Jahangiri et al., 2012) using the  
704 primers in Table S2.

705

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712

## 713 **References**

- 714 Amacher, S.L., Draper, B.W., Summers, B.R., Kimmel, C.B., 2002. The zebrafish T-box  
715 genes no tail and spadetail are required for development of trunk and tail mesoderm  
716 and medial floor plate. *Development* 129, 3311-3323.
- 717 Amacher, S.L., Kimmel, C.B., 1998. Promoting notochord fate and repressing muscle  
718 development in zebrafish axial mesoderm. *Development* 125, 1397-1406.
- 719 Amack, J.D., Wang, X., Yost, H.J., 2007. Two T-box genes play independent and  
720 cooperative roles to regulate morphogenesis of ciliated Kupffer's vesicle in zebrafish.  
721 *Dev Biol* 310, 196-210.
- 722 Amaya, E., Stein, P.A., Musci, T.J., Kirschner, M.W., 1993. FGF signalling in the early  
723 specification of mesoderm in *Xenopus*. *Development* 118, 477-487.
- 724 Arenas-Mena, C., 2013. Brachyury, Tbx2/3 and sall expression during embryogenesis of  
725 the indirectly developing polychaete *Hydroides elegans*. *The International journal of*  
726 *developmental biology* 57, 73-83.
- 727 Barresi, M.J., Stickney, H.L., Devoto, S.H., 2000. The zebrafish slow-muscle-omitted gene  
728 product is required for Hedgehog signal transduction and the development of slow  
729 muscle identity. *Development* 127, 2189-2199.
- 730 Beattie, C.E., Hatta, K., Halpern, M.E., Liu, H., Eisen, J.S., Kimmel, C.B., 1997. Temporal  
731 separation in the specification of primary and secondary motoneurons in zebrafish.  
732 *Dev Biol* 187, 171-182.
- 733 Belgacem, M.R., Escande, M.L., Escrava, H., Bertrand, S., 2011. Amphioxus Tbx6/16 and  
734 Tbx20 embryonic expression patterns reveal ancestral functions in chordates. *Gene*  
735 *Expr Patterns* 11, 239-243.
- 736 Bisgrove, B.W., Snarr, B.S., Emrazian, A., Yost, H.J., 2005. Polaris and Polycystin-2 in  
737 dorsal forerunner cells and Kupffer's vesicle are required for specification of the  
738 zebrafish left-right axis. *Dev Biol* 287, 274-288.
- 739 Blagden, C.S., Currie, P.D., Ingham, P.W., Hughes, S.M., 1997. Notochord induction of  
740 zebrafish slow muscle mediated by Sonic Hedgehog. *Genes Dev* 11, 2163-2175.
- 741 Bogdanovic, O., Fernandez-Minan, A., Tena, J.J., de la Calle-Mustienes, E., Hidalgo, C.,  
742 van Kruysbergen, I., van Heeringen, S.J., Veenstra, G.J., Gomez-Skarmeta, J.L.,  
743 2012. Dynamics of enhancer chromatin signatures mark the transition from  
744 pluripotency to cell specification during embryogenesis. *Genome research* 22, 2043-  
745 2053.



- 746 Borycki, A.G., Brunk, B., Tajbakhsh, S., Buckingham, M., Chiang, C., Emerson, C.P., Jr.,  
747 1999. Sonic hedgehog controls epaxial muscle determination through Myf5 activation.  
748 Development 126, 4053-4063.
- 749 Bouldin, C.M., Manning, A.J., Peng, Y.H., Farr, G.H., 3rd, Hung, K.L., Dong, A., Kimelman,  
750 D., 2015. Wnt signaling and *tbx16* form a bistable switch to commit bipotential  
751 progenitors to mesoderm. Development 142, 2499-2507.
- 752 Brunet, T., Lauri, A., Arendt, D., 2015. Did the notochord evolve from an ancient axial  
753 muscle? The axochord hypothesis. Bioessays 37, 836-850.
- 754 Buckingham, M., Rigby, P.W., 2014. Gene regulatory networks and transcriptional  
755 mechanisms that control myogenesis. Dev Cell 28, 225-238.
- 756 Callery, E.M., Thomsen, G.H., Smith, J.C., 2010. A divergent *Tbx6*-related gene and *Tbx6*  
757 are both required for neural crest and intermediate mesoderm development in  
758 *Xenopus*. Dev Biol 340, 75-87.
- 759 Chapman, D.L., Papaioannou, V.E., 1998. Three neural tubes in mouse embryos with  
760 mutations in the T-box gene *Tbx6*. Nature 391, 695-697.
- 761 Chen, J.C., Goldhamer, D.J., 2004. The core enhancer is essential for proper timing of  
762 *MyoD* activation in limb buds and branchial arches. Dev Biol 265, 502-512.
- 763 Chen, Y.H., Lee, W.C., Liu, C.F., Tsai, H.J., 2001. Molecular structure, dynamic  
764 expression, and promoter analysis of zebrafish (*Danio rerio*) *myf-5* gene. Genesis 29,  
765 22-35.
- 766 Chen, Y.H., Wang, Y.H., Chang, M.Y., Lin, C.Y., Weng, C.W., Westerfield, M., Tsai, H.J.,  
767 2007. Multiple upstream modules regulate zebrafish *myf5* expression. BMC  
768 developmental biology 7, 1.
- 769 Chiba, S., Jiang, D., Satoh, N., Smith, W.C., 2009. Brachyury null mutant-induced defects  
770 in juvenile ascidian endodermal organs. Development 136, 35-39.
- 771 Coutelle, O., Blagden, C.S., Hampson, R., Halai, C., Rigby, P.W., Hughes, S.M., 2001.  
772 Hedgehog signalling is required for maintenance of *myf5* and *myoD* expression and  
773 timely terminal differentiation in zebrafish adaxial myogenesis. Dev Biol 236, 136-150.
- 774 Devoto, S.H., Melancon, E., Eisen, J.S., Westerfield, M., 1996. Identification of separate  
775 slow and fast muscle precursor cells in vivo, prior to somite formation. Development  
776 122, 3371-3380.
- 777 Devoto, S.H., Stoiber, W., Hammond, C.L., Steinbacher, P., Haslett, J.R., Barresi, M.J.,  
778 Patterson, S.E., Adiarte, E.G., Hughes, S.M., 2006. Generality of vertebrate  
779 developmental patterns: evidence for a dermomyotome in fish. Evol Dev 8, 101-110.
- 780 Dietrich, S., Schubert, F.R., Gruss, P., Lumsden, A., 1999. The role of the notochord for  
781 epaxial myotome formation in the mouse. Cell Mol Biol (Noisy-le-grand) 45, 601-616.
- 782 Don, E.K., de Jong-Curtain, T.A., Doggett, K., Hall, T.E., Heng, B., Badrock, A.P., Winnick,  
783 C., Nicholson, G.A., Guillemin, G.J., Currie, P.D., Hesselson, D., Heath, J.K., Cole,  
784 N.J., 2016. Genetic basis of hindlimb loss in a naturally occurring vertebrate model.  
785 Biology open 5, 359-366.
- 786 Draper, B.W., Stock, D.W., Kimmel, C.B., 2003. Zebrafish *fgf24* functions with *fgf8* to  
787 promote posterior mesodermal development. Development 130, 4639-4654.
- 788 Du, S.J., Devoto, S.H., Westerfield, M., Moon, R.T., 1997. Positive and negative regulation  
789 of muscle cell identity by members of the *hedgehog* and *TGF- $\beta$*  gene families. Journal  
790 of Cell Biology 139, 145-156.
- 791 Feldman, B., Stemple, D.L., 2001. Morpholino phenocopies of *sqt*, *oep*, and *ntl* mutations.  
792 Genesis 30, 175-177.
- 793 Fior, R., Maxwell, A.A., Ma, T.P., Vezzano, A., Moens, C.B., Amacher, S.L., Lewis, J.,  
794 Saude, L., 2012. The differentiation and movement of presomitic mesoderm progenitor  
795 cells are controlled by Mesogenin 1. Development 139, 4656-4665.

- 796 Fukuda, M., Takahashi, S., Haramoto, Y., Onuma, Y., Kim, Y.J., Yeo, C.Y., Ishiura, S.,  
797 Asashima, M., 2010. Zygotic VegT is required for *Xenopus* paraxial mesoderm  
798 formation and is regulated by Nodal signaling and Eomesodermin. *The International*  
799 *journal of developmental biology* 54, 81-92.
- 800 Furthauer, M., Reifers, F., Brand, M., Thisse, B., Thisse, C., 2001. *sprouty4* acts in vivo as  
801 a feedback-induced antagonist of FGF signaling in zebrafish. *Development* 128, 2175-  
802 2186.
- 803 Garnett, A.T., Han, T.M., Gilchrist, M.J., Smith, J.C., Eisen, M.B., Wardle, F.C., Amacher,  
804 S.L., 2009. Identification of direct T-box target genes in the developing zebrafish  
805 mesoderm. *Development* 136, 749-760.
- 806 Gee, H., 2018. *Across the Bridge: Understanding the Origin of the Vertebrates*. The  
807 University of Chicago Press, London.
- 808 Gentsch, G.E., Owens, N.D., Martin, S.R., Piccinelli, P., Faial, T., Trotter, M.W., Gilchrist,  
809 M.J., Smith, J.C., 2013. In vivo T-box transcription factor profiling reveals joint  
810 regulation of embryonic neuromesodermal bipotency. *Cell reports* 4, 1185-1196.
- 811 George-Weinstein, M., Gerhart, J., Reed, R., Flynn, J., Callihan, B., Mattiacci, M., Miehle,  
812 C., Foti, G., Lash, J.W., Weintraub, H., 1996. Skeletal myogenesis: the preferred  
813 pathway of chick embryo epiblast cells in vitro. *Dev Biol* 173, 279-291.
- 814 Gerhart, J., 2001. Evolution of the organizer and the chordate body plan. *The International*  
815 *journal of developmental biology* 45, 133-153.
- 816 Gerhart, J., Neely, C., Stewart, B., Perlman, J., Beckmann, D., Wallon, M., Knudsen, K.,  
817 George-Weinstein, M., 2004. Epiblast cells that express MyoD recruit pluripotent cells  
818 to the skeletal muscle lineage. *J Cell Biol*.
- 819 Goering, L.M., Hoshijima, K., Hug, B., Bisgrove, B., Kispert, A., Grunwald, D.J., 2003. An  
820 interacting network of T-box genes directs gene expression and fate in the zebrafish  
821 mesoderm. *Proc Natl Acad Sci U S A* 100, 9410-9415.
- 822 Griffin, K., Patient, R., Holder, N., 1995. Analysis of FGF function in normal and *no tail*  
823 zebrafish embryos reveals separate mechanisms for formation of the trunk and tail.  
824 *Development* 121, 2983-2994.
- 825 Griffin, K.J., Amacher, S.L., Kimmel, C.B., Kimelman, D., 1998. Molecular identification of  
826 *spadetail*: regulation of zebrafish trunk and tail mesoderm formation by T-box genes.  
827 *Development* 125, 3379-3388.
- 828 Griffin, K.J., Kimelman, D., 2002. One-Eyed Pinhead and Spadetail are essential for heart  
829 and somite formation. *Nat Cell Biol* 4, 821-825.
- 830 Griffin, K.J., Kimelman, D., 2003. Interplay between FGF, one-eyed pinhead, and T-box  
831 transcription factors during zebrafish posterior development. *Dev Biol* 264, 456-466.
- 832 Grimaldi, A., Tettamanti, G., Martin, B.L., Gaffield, W., Pownall, M.E., Hughes, S.M., 2004.  
833 Hedgehog regulation of superficial slow muscle fibres in *Xenopus* and the evolution of  
834 tetrapod trunk myogenesis. *Development* 131, 3249-3262.
- 835 Groves, J.A., Hammond, C.L., Hughes, S.M., 2005. *Fgf8* drives myogenic progression of a  
836 novel lateral fast muscle fibre population in zebrafish. *Development* 132, 4211-4222.
- 837 Haeussler, M., Zweig, A.S., Tyner, C., Speir, M.L., Rosenbloom, K.R., Raney, B.J., Lee,  
838 C.M., Lee, B.T., Hinrichs, A.S., Gonzalez, J.N., Gibson, D., Diekhans, M., Clawson, H.,  
839 Casper, J., Barber, G.P., Haussler, D., Kuhn, R.M., Kent, W.J., 2019. The UCSC  
840 Genome Browser database: 2019 update. *Nucleic Acids Res* 47, D853-D858.
- 841 Halpern, M.E., Ho, R.K., Walker, C., Kimmel, C.B., 1993. Induction of muscle pioneers and  
842 floor plate is distinguished by the zebrafish *no tail* mutation. *Cell* 75, 99-111.
- 843 Halpern, M.E., Thisse, C., Ho, R.K., Thisse, B., Riggleman, B., Trevarrow, B., Weinberg,  
844 E.S., Postlethwait, J.H., Kimmel, C.B., 1995. Cell-autonomous shift from axial to

- 845 paraxial mesodermal development in zebrafish floating head mutants. *Development*  
846 121, 4257-4264.
- 847 Hammerschmidt, M., Nüsslein-Volhard, C., 1993. The expression of a zebrafish gene  
848 homologous to *Drosophila* snail suggests a conserved function in invertebrate and  
849 vertebrate gastrulation. *Development* 119, 1107-1118.
- 850 Hasson, P., DeLaurier, A., Bennett, M., Grigorieva, E., Naiche, L.A., Papaioannou, V.E.,  
851 Mohun, T.J., Logan, M.P., 2010. *Tbx4* and *tbx5* acting in connective tissue are  
852 required for limb muscle and tendon patterning. *Dev Cell* 18, 148-156.
- 853 Hinits, Y., Osborn, D.P., Hughes, S.M., 2009. Differential requirements for myogenic  
854 regulatory factors distinguish medial and lateral somitic, cranial and fin muscle fibre  
855 populations. *Development* 136, 403-414.
- 856 Hinits, Y., Williams, V.C., Sweetman, D., Donn, T.M., Ma, T.P., Moens, C.B., Hughes,  
857 S.M., 2011. Defective cranial skeletal development, larval lethality and  
858 haploinsufficiency in *Myod* mutant zebrafish. *Dev Biol* 358, 102-112.
- 859 Hirsinger, E., Stellabotte, F., Devoto, S.H., Westerfield, M., 2004. Hedgehog signaling is  
860 required for commitment but not initial induction of slow muscle precursors. *Dev Biol*  
861 275, 143-157.
- 862 Ho, R.K., Kane, D.A., 1990. Cell-autonomous action of zebrafish *spt-1* mutation in specific  
863 mesodermal precursors. *Nature* 348, 728-730.
- 864 Honjo, Y., Eisen, J.S., 2005. Slow muscle regulates the pattern of trunk neural crest  
865 migration in zebrafish. *Development* 132, 4461-4470.
- 866 Imai, K.S., Levine, M., Satoh, N., Satou, Y., 2006. Regulatory blueprint for a chordate  
867 embryo. *Science* 312, 1183-1187.
- 868 Isaacs, H.V., Deconinck, A.E., Pownall, M.E., 2007. FGF4 regulates blood and muscle  
869 specification in *Xenopus laevis*. *Biology of the cell / under the auspices of the*  
870 *European Cell Biology Organization* 99, 165-173.
- 871 Itoh, N., Konishi, M., 2007. The zebrafish *fgf* family. *Zebrafish* 4, 179-186.
- 872 Jahangiri, L., Nelson, A.C., Wardle, F.C., 2012. A cis-regulatory module upstream of  
873 *deltaC* regulated by *Ntla* and *Tbx16* drives expression in the tailbud, presomitic  
874 mesoderm and somites. *Dev Biol* 371, 110-120.
- 875 Kimelman, D., Kirschner, M., 1987. Synergistic induction of mesoderm by FGF and TGF-  
876 beta and the identification of an mRNA coding for FGF in the early *Xenopus* embryo.  
877 *Cell* 51, 869-877.
- 878 Kimmel, C.B., Kane, D.A., Walker, C., Warga, R.M., Rothman, M.B., 1989. A mutation that  
879 changes cell movement and cell fate in the zebrafish embryo. *Nature* 337, 358-362.
- 880 Kispert, A., Herrmann, B.G., Leptin, M., Reuter, R., 1994. Homologs of the mouse  
881 *Brachyury* gene are involved in the specification of posterior terminal structures in  
882 *Drosophila*, *Tribolium*, and *Locusta*. *Genes Dev* 8, 2137-2150.
- 883 Knight, R.D., Mebus, K., Roehl, H.H., 2008. Mandibular arch muscle identity is regulated  
884 by a conserved molecular process during vertebrate development. *J Exp Zool B Mol*  
885 *Dev Evol* 310, 355-369.
- 886 Kolm, P.J., Sive, H.L., 1995. Efficient hormone-inducible protein function in *Xenopus*  
887 *laevis*. *Dev Biol* 171, 267-272.
- 888 Krauss, S., Concordet, J.-P., Ingham, P.W., 1993. A functionally conserved homologue of  
889 the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity  
890 in zebrafish embryos. *Cell* 75, 1431-1444.
- 891 Kudoh, T., Tsang, M., Hukriede, N.A., Chen, X., Dedekian, M., Clarke, C.J., Kiang, A.,  
892 Schultz, S., Epstein, J.A., Toyama, R., Dawid, I.B., 2001. A gene expression screen in  
893 zebrafish embryogenesis. *Genome research* 11, 1979-1987.

- 894 Lacalli, T.C., 2002. The dorsal compartment locomotory control system in amphioxus  
895 larvae. *J Morphol* 252, 227-237.
- 896 Lee, H.C., Tseng, W.A., Lo, F.Y., Liu, T.M., Tsai, H.J., 2009. FoxD5 mediates anterior-  
897 posterior polarity through upstream modulator Fgf signaling during zebrafish  
898 somitogenesis. *Dev Biol* 336, 232-245.
- 899 Lewis, K.E., Currie, P.D., Roy, S., Schauerte, H., Haffter, P., Ingham, P.W., 1999. Control  
900 of muscle cell-type specification in the zebrafish embryo by Hedgehog signalling. *Dev*  
901 *Biol* 216, 469-480.
- 902 Lolas, M., Valenzuela, P.D., Tjian, R., Liu, Z., 2014. Charting Brachyury-mediated  
903 developmental pathways during early mouse embryogenesis. *Proc Natl Acad Sci U S*  
904 *A* 111, 4478-4483.
- 905 Lu, F., Langenbacher, A., Chen, J.N., 2017. Tbx20 drives cardiac progenitor formation and  
906 cardiomyocyte proliferation in zebrafish. *Dev Biol* 421, 139-148.
- 907 Maguire, R.J., Isaacs, H.V., Pownall, M.E., 2012. Early transcriptional targets of MyoD link  
908 myogenesis and somitogenesis. *Dev Biol* 371, 256-268.
- 909 Manning, A.J., Kimelman, D., 2015. Tbx16 and Msgn1 are required to establish directional  
910 cell migration of zebrafish mesodermal progenitors. *Dev Biol*.
- 911 Maroon, H., Walshe, J., Mahmood, R., Kiefer, P., Dickson, C., Mason, I., 2002. Fgf3 and  
912 Fgf8 are required together for formation of the otic placode and vesicle. *Development*  
913 129, 2099-2108.
- 914 Martin, B.L., Kimelman, D., 2008. Regulation of canonical Wnt signaling by Brachyury is  
915 essential for posterior mesoderm formation. *Dev Cell* 15, 121-133.
- 916 McDermott, A., Gustafsson, M., Elsam, T., Hui, C.C., Emerson, C.P., Jr., Borycki, A.G.,  
917 2005. Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle  
918 formation. *Development* 132, 345-357.
- 919 Melby, A.E., Warga, R.M., Kimmel, C.B., 1996. Specification of cell fates at the dorsal  
920 margin of the zebrafish gastrula. *Development* 122, 2225-2237.
- 921 Molven, A., Wright, C.V., Bremiller, R., De Robertis, E.M., Kimmel, C.B., 1990. Expression  
922 of a homeobox gene product in normal and mutant zebrafish embryos: evolution of the  
923 tetrapod body plan. *Development* 109, 279-288.
- 924 Morrow, Z.T., Maxwell, A.M., Hoshijima, K., Talbot, J.C., Grunwald, D.J., Amacher, S.L.,  
925 2017. tbx6l and tbx16 are redundantly required for posterior paraxial mesoderm  
926 formation during zebrafish embryogenesis. *Dev Dyn* 246, 759-769.
- 927 Mueller, R.L., Huang, C., Ho, R.K., 2010. Spatio-temporal regulation of Wnt and retinoic  
928 acid signaling by tbx16/spadetail during zebrafish mesoderm differentiation. *BMC*  
929 *Genomics* 11, 492.
- 930 Münsterberg, A.E., Kitajewski, J., Bumcrot, D.A., McMahon, A.P., Lassar, A.B., 1995.  
931 Combinatorial signalling by sonic hedgehog and Wnt family members induces  
932 myogenic bHLH gene expression in the somite. *Genes and Development* 9, 2911-  
933 2922.
- 934 Nelson, A.C., Cutty, S.J., Gasiunas, S.N., Deplae, I., Stemple, D.L., Wardle, F.C., 2017. In  
935 Vivo Regulation of the Zebrafish Endoderm Progenitor Niche by T-Box Transcription  
936 Factors. *Cell reports* 19, 2782-2795.
- 937 Nikaido, M., Kawakami, A., Sawada, A., Furutani-Seiki, M., Takeda, H., Araki, K., 2002.  
938 Tbx24, encoding a T-box protein, is mutated in the zebrafish somite-segmentation  
939 mutant fused somites. *Nature genetics* 31, 195-199.
- 940 Ochi, H., Hans, S., Westerfield, M., 2008. Smarcd3 regulates the timing of zebrafish  
941 myogenesis onset. *J Biol Chem* 283, 3529-3536.
- 942 Odenthal, J., Haffter, P., Vogelsang, E., Brand, M., Van Eeden, F.J.M., Furutani-Seiki, M.,  
943 Granato, M., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh,

- 944 R.N., Mullins, M.C., Warga, R.M., Allende, M.L., Weinberg, E.S., Nusslein-Volhard, C.,  
945 1996. Mutations affecting the formation of the notochord in the zebrafish, *Danio rerio*.  
946 *Development* 123, 103-115.
- 947 Osborn, D.P., Li, K., Hinitz, Y., Hughes, S.M., 2011. Cdkn1c drives muscle differentiation  
948 through a positive feedback loop with MyoD. *Dev Biol* 350, 464-475.
- 949 Papaioannou, V.E., 2014. The T-box gene family: emerging roles in development, stem  
950 cells and cancer. *Development* 141, 3819-3833.
- 951 Placzek, M., Briscoe, J., 2018. Sonic hedgehog in vertebrate neural tube development.  
952 *International Journal of Developmental Biology* 62, 225-234.
- 953 Pradeepa, M.M., 2017. Causal role of histone acetylations in enhancer function.  
954 *Transcription* 8, 40-47.
- 955 Pradeepa, M.M., Grimes, G.R., Kumar, Y., Olley, G., Taylor, G.C., Schneider, R.,  
956 Bickmore, W.A., 2016. Histone H3 globular domain acetylation identifies a new class  
957 of enhancers. *Nature genetics* 48, 681-686.
- 958 Reifers, F., Bohli, H., Walsh, E.C., Crossley, P.H., Stainier, D.Y., Brand, M., 1998. *Fgf8* is  
959 mutated in zebrafish *acerebellar (ace)* mutants and is required for maintenance of  
960 midbrain-hindbrain boundary development and somitogenesis. *Development* 125,  
961 2381-2395.
- 962 Rentzsch, F., Bakkers, J., Kramer, C., Hammerschmidt, M., 2004. Fgf signaling induces  
963 posterior neuroectoderm independently of Bmp signaling inhibition. *Dev Dyn* 231, 750-  
964 757.
- 965 Rhinn, M., Lun, K., Luz, M., Werner, M., Brand, M., 2005. Positioning of the midbrain-  
966 hindbrain boundary organizer through global posteriorization of the neuroectoderm  
967 mediated by Wnt8 signaling. *Development* 132, 1261-1272.
- 968 Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A.,  
969 Tanabe, Y., Placzek, M., Edlund, T., Jessell, T.M., Dodd, J., 1994. Floor plate and  
970 motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the  
971 notochord. *Cell* 76, 761-775.
- 972 Row, R.H., Maitre, J.L., Martin, B.L., Stockinger, P., Heisenberg, C.P., Kimelman, D.,  
973 2011. Completion of the epithelial to mesenchymal transition in zebrafish mesoderm  
974 requires Spadetail. *Dev Biol* 354, 102-110.
- 975 Rudnicki, M.A., Schnegelsberg, P.N., Stead, R.H., Braun, T., Arnold, H.H., Jaenisch, R.,  
976 1993. MyoD or Myf-5 is required for the formation of skeletal muscle. *Cell* 75, 1351-  
977 1359.
- 978 Ruvinsky, I., Silver, L.M., Ho, R.K., 1998. Characterization of the zebrafish *tbx16* gene and  
979 evolution of the vertebrate T-box family. *Development genes and evolution* 208, 94-99.
- 980 Satoh, N., Tagawa, K., Takahashi, H., 2012. How was the notochord born? *Evolution &*  
981 *Development* 14, 56-75.
- 982 Schulte-Merker, S., Hammerschmidt, M., Beuchle, D., Cho, K.W., De Robertis, E.M.,  
983 Nüsslein-Volhard, C., 1994a. Expression of zebrafish *gooseoid* and *no tail* gene  
984 products in wild-type and mutant *no tail* embryos. *Development* 120, 843-852.
- 985 Schulte-Merker, S., van Eeden, F.J.M., Halpern, M.E., Kimmel, C.B., Nüsslein-Volhard, C.,  
986 1994b. *no tail (ntl)* is the zebrafish homologue of the mouse *T (Brachyury)* gene.  
987 *Development* 120, 1009-1015.
- 988 Showell, C., Binder, O., Conlon, F.L., 2004. T-box genes in early embryogenesis. *Dev Dyn*  
989 229, 201-218.
- 990 Slack, J.M., Darlington, B.G., Heath, J.K., Godsave, S.F., 1987. Mesoderm induction in  
991 early *Xenopus* embryos by heparin-binding growth factors. *Nature* 326, 197-200.

- 992 Smith, J.C., Price, B.M., Green, J.B., Weigel, D., Herrmann, B.G., 1991. Expression of a  
993 *Xenopus* homolog of Brachyury (T) is an immediate-early response to mesoderm  
994 induction. *Cell* 67, 79-87.
- 995 Sun, X., Meyers, E.N., Lewandoski, M., Martin, G.R., 1999. Targeted disruption of *Fgf8*  
996 causes failure of cell migration in the gastrulating mouse embryo. *Genes Dev* 13,  
997 1834-1846.
- 998 Talbot, W.B., Trevarrow, B., Halpern, M.E., Melby, A.E., Farr, G., Postlethwait, T.J.,  
999 Kimmel, C.B., Kimelman, D., 1995. A homeobox gene essential for zebrafish  
1000 notochord development. *Nature* 378, 150-157.
- 1001 Taylor, M.V., Hughes, S.M., 2017. *Mef2* and the skeletal muscle differentiation program.  
1002 *Semin Cell Dev Biol* 72, 33-44.
- 1003 Tazumi, S., Yabe, S., Uchiyama, H., 2010. Paraxial T-box genes, *Tbx6* and *Tbx1*, are  
1004 required for cranial chondrogenesis and myogenesis. *Dev Biol* 346, 170-180.
- 1005 Thisse, B., Thisse, C., 2005. Functions and regulations of fibroblast growth factor signaling  
1006 during embryonic development. *Dev Biol* 287, 390-402.
- 1007 Valasek, P., Theis, S., DeLaurier, A., Hinitz, Y., Luke, G.N., Otto, A.M., Minchin, J., He, L.,  
1008 Christ, B., Brooks, G., Sang, H., Evans, D.J., Logan, M., Huang, R., Patel, K., 2011.  
1009 Cellular and molecular investigations into the development of the pectoral girdle. *Dev*  
1010 *Biol* 357, 108-116.
- 1011 van Eeden, F.J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P.,  
1012 Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins,  
1013 M.C., Odenthal, J., Warga, R.M., Allende, M.L., Weinberg, E.S., Nüsslein-Volhard, C.,  
1014 1996. Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*.  
1015 *Development* 123, 153-164.
- 1016 van Eeden, F.J., Holley, S.A., Haffter, P., Nüsslein-Volhard, C., 1998. Zebrafish  
1017 segmentation and pair-rule patterning. *Dev Genet* 23, 65-76.
- 1018 Warga, R.M., Mueller, R.L., Ho, R.K., Kane, D.A., 2013. Zebrafish *Tbx16* regulates  
1019 intermediate mesoderm cell fate by attenuating Fgf activity. *Dev Biol* 383, 75-89.
- 1020 Weinberg, E.S., Allende, M.L., Kelly, C.S., Abdelhamid, A., Murakami, T., Andermann, P.,  
1021 Doerre, O.G., Grunwald, D.J., Riggleman, B., 1996. Developmental regulation of  
1022 zebrafish *MyoD* in wild-type, no tail and spadetail embryos. *Development* 122, 271-  
1023 280.
- 1024 Westerfield, M., 2000. *The Zebrafish Book - A guide for the laboratory use of zebrafish*  
1025 (*Danio rerio*). University of Oregon Press.
- 1026 White, P.H., Farkas, D.R., McFadden, E.E., Chapman, D.L., 2003. Defective somite  
1027 patterning in mouse embryos with reduced levels of *Tbx6*. *Development* 130, 1681-  
1028 1690.
- 1029 Wilkinson, D.G., Peters, G., Dickson, C., McMahon, A.P., 1988. Expression of the FGF-  
1030 related proto-oncogene *int-2* during gastrulation and neurulation in the mouse. *EMBO*  
1031 *J* 7, 691-695.
- 1032 Windner, S.E., Bird, N.C., Patterson, S.E., Doris, R.A., Devoto, S.H., 2012. *Fss/Tbx6* is  
1033 required for central dermomyotome cell fate in zebrafish. *Biology open* 1, 806-814.
- 1034 Windner, S.E., Doris, R.A., Ferguson, C.M., Nelson, A.C., Valentin, G., Tan, H., Oates,  
1035 A.C., Wardle, F.C., Devoto, S.H., 2015. *Tbx6*, *Mesp-b* and *Ripply1* regulate the onset  
1036 of skeletal myogenesis in zebrafish. *Development* 142, 1159-1168.
- 1037 Woollard, A., Hodgkin, J., 2000. The *Caenorhabditis elegans* fate-determining gene *mab-9*  
1038 encodes a T-box protein required to pattern the posterior hindgut. *Genes Dev* 14, 596-  
1039 603.

- 1040 Yamamoto, A., Amacher, S.L., Kim, S.H., Geissert, D., Kimmel, C.B., De Robertis, E.M.,  
1041 1998. Zebrafish paraxial protocadherin is a downstream target of spadetail involved in  
1042 morphogenesis of gastrula mesoderm. *Development* 125, 3389-3397.
- 1043 Yamauchi, H., Miyakawa, N., Miyake, A., Itoh, N., 2009. Fgf4 is required for left-right  
1044 patterning of visceral organs in zebrafish. *Dev Biol* 332, 177-185.
- 1045 Yasuoka, Y., Shinzato, C., Satoh, N., 2016. The Mesoderm-Forming Gene brachyury  
1046 Regulates Ectoderm-Endoderm Demarcation in the Coral *Acropora digitifera*. *Current*  
1047 *Biology* 26, 2885-2892.
- 1048 Yin, J., Lee, R., Ono, Y., Ingham, P.W., Saunders, T.E., 2018. Spatiotemporal  
1049 Coordination of FGF and Shh Signaling Underlies the Specification of Myoblasts in the  
1050 Zebrafish Embryo. *Dev Cell* 46, 735-750 e734.
- 1051 Zeng, X.X., Wilm, T.P., Sepich, D.S., Solnica-Krezel, L., 2007. Apelin and its receptor  
1052 control heart field formation during zebrafish gastrulation. *Dev Cell* 12, 391-402.
- 1053 Zhang, X.M., Ramalho-Santos, M., McMahon, A.P., 2001. Smoothed mutants reveal  
1054 redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the  
1055 mouse node. *Cell* 105, 781-792.

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1060 **Fig. 1. Inhibition of Fgf signalling blocks initial induction of *myod* and *myf5***  
1061 **expression**

1062 *In situ* mRNA hybridization for *myod* and *myf5* in control untreated, cyclopamine-treated  
1063 (cyA, 100  $\mu$ M), SU5402-treated and double SU5402- and cyA-treated wild type or mutant  
1064 embryos, shown in dorsal view, anterior to top. **A.** Adaxial (arrows) and paraxial *myod* and  
1065 *myf5* mRNAs are lost upon SU5402-treatment (60  $\mu$ M) from 30% to 80 or 90% epiboly  
1066 (dashes indicate approximate position of germ ring) but are unaffected by cyA treatment.  
1067 The anterior mesoderm marker *aplnrb* is normally down-regulated in paraxial presomitic  
1068 cells expressing *myf5* (white dashes) and up-regulated in adaxial cells (arrow). Both  
1069 changes were absent after SU5402-treatment. Schematics illustrate the location of  
1070 equivalent cell types at two successive stages. mpcs = muscle precursor cells, CNH =  
1071 chordoneural hinge (hatched), PSM = presomitic mesoderm (brackets). **B.** *Smo*<sup>b641</sup>  
1072 mutants retain pre-adaxial *myod* mRNA at 6ss even after cyA treatment, but lack pre-  
1073 adaxial *myod* mRNA at 15ss. *Ptc1* mRNA downregulation shows that both *smo* mutation  
1074 and cyA treatment (shown after longer colour reaction) fully block Hh signalling throughout  
1075 the axis. Bars: 50  $\mu$ m.

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1077

1078 **Fig. 2. Successive Fgf and Hh signals drive trunk slow myogenesis**

1079 Immunodetection of slow MyHC in *shha* and *noto* mutants. **A.** Reduction in slow fibres in  
1080 *shha* mutants is greater in tail than in trunk. Insets show individual fibres magnified.  
1081 Upper graph shows mean  $\pm$  SD of slow fibre number in the indicated somites from ten  
1082 independent embryos of each genotype. Lower graph shows fraction of wild type fibres  
1083 remaining in mutant. **B.** Trunk-specific residual slow muscle in *noto* mutant. **C.** 5-somite  
1084 stage (5ss) embryos from a *noto<sup>nl</sup>* heterozygote incross treated with cyA at 30% epiboly  
1085 stage, showing loss of adaxial *myod* mRNA in anterior presomitic mesoderm  
1086 (arrowheads), but retention in the most posterior pre-adaxial mesoderm (arrows) flanking  
1087 the chordoneural hinge (white outline). **D.** SU5402 (50  $\mu$ M) from tailbud stage ablates  
1088 residual pre-adaxial *myod* and *myf5* mRNAs in cyA-treated 8ss embryos (arrows).  
1089 Expression of paraxial *myod* in fast muscle precursors (asterisks) is not affected by cyA  
1090 but is decreased by SU5402. Bars: 50  $\mu$ m.

1091

1092 **Fig. 3. Dorsally-expressed Fgfs drive paraxial myogenesis**

1093 In situ mRNA hybridization for *myod* and *myf5* (A,C) or *aplnrb* (D) mRNAs at 80% epiboly  
1094 or *tbxta* (red) and *myod* (blue/brown) at tailbud stage (tb) (B). **A.** Reduction of *myod* and  
1095 *myf5* mRNAs in *dual* and *triple fgf* MO-injected wild type embryos. Arrowheads indicate  
1096 nascent adaxial cells. **B.** In contrast to 80% epiboly (compare Fig. S3C), at tailbud stage,  
1097 cyclopamine (cyA) treatment ablates anterior adaxial *myod* mRNA, but leaves pre-adaxial  
1098 expression intact (arrowheads). Injection of *fgf4+fgf8a* MOs ablate residual *myod* mRNA.  
1099 **C.** Fgf4 or Fgf6a mRNA injection up-regulates *myod* and *myf5* mRNAs around the  
1100 marginal zone. Note the widening of the unlabelled dorsal midline region (brackets).  
1101 Insets show the same embryos viewed from vegetal pole. **D.** Fgf4 mRNA injection down-  
1102 regulates *aplnrb* mRNA around the marginal zone (white dashes) but not anteriorly  
1103 (brackets) in the dorsalized embryo. Bars: 100  $\mu$ m.

1104

1105 **Fig. 4 Redundant Fgf/Tbx and Hh signals required for MRF induction**

1106 In situ mRNA hybridization for *tbxta* and *tbx16* in control untreated and SU5402-treated  
1107 from 30% embryos (A), for *myod*, *myf5*, *fgf8a*, *fgf3* and *fgf4*, in control, *tbxta* MO- and  
1108 *tbx16* MO-injected wild type (wt) embryos (B), and for *myod* and *myf5* in control, *tbxta* MO-  
1109 and *tbx16* MO-injected wt embryos treated with or without 0.1 mM cyA (C). **A.** In 10  $\mu$ M  
1110 SU5402-treated wt embryos, *tbxta* and *tbx16* transcripts are decreased (arrows) at 80%  
1111 epiboly, but almost normal at 6ss. Both transcripts are absent in 30  $\mu$ M SU5402-treated



1112 embryos at 6ss. **B.** Adaxial *myod* expression (black arrowheads) is completely ablated in  
1113 *tbxta* or *tbx16* morphants at 80% epiboly, and *myf5* expression is greatly decreased. *Fgf8a*  
1114 mRNA is ablated in posterior notochord of *tbxta* morphants (blue arrowheads), but  
1115 upregulated in *tbx16* morphants around the germ marginal zone at 80% and in posterior  
1116 notochord at 6ss (yellow arrowheads). Expression of *fgf3* and *fgf4* is absent in posterior  
1117 notochord of *tbxta* morphants, but enhanced in that location in *tbx16* morphants (green  
1118 arrowheads). **C.** At 6ss, pre-adaxial *myod* expression (black arrowheads) is lost in *tbxta*  
1119 morphant tailbud, and diminished in *tbx16* morphants. Injection of *tbx16* MO, but not *tbxta*  
1120 MO, reduces adaxial *myf5* mRNA to the level in paraxial mesoderm (orange arrowheads),  
1121 whereas *tbxta* MO but not *tbx16* MO up-regulates *myf5* mRNA in posterior tailbud  
1122 (asterisks). *Tbx16* MO abolishes pre-adaxial *myf5* mRNA in cyA-treated embryos (white  
1123 arrowheads). Adaxial *myf5* and *myod* transcripts recover in *tbxta* morphants, but are  
1124 ablated by cyA-treatment (red arrowheads). CyA-treatment of *tbx16* morphants ablates  
1125 adaxial *myod* expression throughout the axis (cyan arrowheads), leaving only residual  
1126 paraxial *myod* and *myf5* expression (mauve arrowheads). Bars: 100  $\mu$ m.

1127

### 1128 **Fig. 5. Tbx16 is necessary and sufficient for MRF induction**

1129 *In situ* mRNA hybridization for the indicated mRNAs of control uninjected and *fgf4* (A,B) or  
1130 *tbx16* (C,D) mRNA-injected embryos at 80% epiboly stage in *tbx16*<sup>+/-</sup> incross (A) and wild  
1131 type (B-D). Dorsal views. Insets ventral views. **A.** *Myf5* and *myod* mRNAs flank the  
1132 dorsal midline in siblings (sib), but are absent or greatly diminished in *tbx16*<sup>-/-</sup> mutants.  
1133 *Fgf4* widened notochord (bars) and induced ectopic *myod* and *myf5* mRNA around the  
1134 germ marginal zone of siblings, but did not rescue expression in *tbx16*<sup>-/-</sup> embryos. **B.**  
1135 *Tbxta* mRNA reveals widened notochord (bars) in wild type embryos injected with *fgf4*  
1136 mRNA. Both *tbxta* and *tbx16* mRNAs show clumping in the germ ring after overexpression  
1137 of *fgf4*. **C,D.** *In situ* mRNA hybridization at 80% epiboly stage for *myf5* and *myod* mRNAs  
1138 in wild type control or *tbx16* mRNA-injected embryos treated with SU5402 at 10  $\mu$ M (C) or  
1139 60  $\mu$ M (D). *Myf5* and *myod* mRNAs are ectopically induced in posterior mesoderm by  
1140 Tbx16 expression, decreased by administration of 10  $\mu$ M SU5402 in wild type, and  
1141 rescued in SU5402-treated embryos by overexpression of *tbx16*. High dose SU5402  
1142 prevents MRF expression, even after *tbx16* mRNA injection. Bars: 100  $\mu$ m.

1143

1144

1145 **Fig. 6. *Myf5* is a direct transcriptional target of *Tbx16***

1146 **A,B.** Chromatin immunoprecipitation followed by sequencing (ChIP-seq) on wild-type (wt)  
1147 embryos at 75-85% epiboly reveals endogenous *Tbx16* and *Tbx16a* binding events within  
1148 120 kb flanking the *myf5* transcriptional start site (TSS). RPM – ChIP-seq peak height in  
1149 reads per million reads. H3K4me3 indicates TSSs. H3K4me1 indicates putative enhancers.  
1150 H3K27ac indicates active enhancers. Known transcripts with exons (black) and introns  
1151 (arrowheads) are indicated. Multiz Alignments & Conservation from UCSC Genome  
1152 Browser (Haeussler et al., 2019) are shown beneath. Purple boxes indicate validated  
1153 *Tbx16* binding sites. Blue box indicates region expanded in panel B. Cyan boxes indicate  
1154 other *Tbx* sites mentioned in text. **C.** ChIP-qPCR validation of *Tbx16* peaks on *myf5*  
1155 distal element (5DE) and proximal element 2 (5PE1). Error bars indicate standard error of  
1156 the mean for biological triplicate experiments. **D.** Schematic showing how direct injection  
1157 into wt embryos of mRNA encoding the *Tbx16*-Glucocorticoid Response fusion protein  
1158 leads to target gene induction in the presence of protein synthesis inhibitor cycloheximide  
1159 (CHD) induced by nuclear translocation triggered by dexamethasone (DEX). CHD caused  
1160 ~5% delay in epiboly so when it was active. **E.** Wild-type embryos injected with *tbx16-GR*  
1161 mRNA treated with cycloheximide from germ ring stage and dexamethasone from shield  
1162 stage. Note that embryos treated with cycloheximide alone exhibit wild type *myf5*  
1163 expression at 75-80% epiboly, whereas embryos additionally treated with dexamethasone  
1164 exhibit ectopic *myf5* expression with strong (white arrowheads, comparable to wt pre-  
1165 adaxial level) and weak (black arrowheads, comparable to wt paraxial level) stain. Three  
1166 separate CDH+DEX treated embryos are shown. Numbers indicate the fraction of  
1167 embryos with the expression pattern(s) shown. Inset shows an unusual induction of *myf5*  
1168 in anterior regions that was not observed with *myod*. **F.** Injection of *tbx16* mRNA (200 pg)  
1169 into embryos from a *myf5*<sup>hu2022/+</sup> heterozygote incross led to ectopic up-regulation of *myod*  
1170 mRNA in the dorsal germ ring (arrows) irrespective of genotype. Numbers indicate fraction  
1171 of embryos showing ectopic mRNA/total analysed. Bars: 100 µm.

1172  
1173 **Fig. 7. *Tbx16a* is essential for *Fgf4*-driven induction of *myod* but not *myf5*.**

1174 Embryos from a *tbx16a*<sup>+/-</sup> incross injected with 150 pg *fgf4* mRNA or control. **A.** *Tbx16a*<sup>-/-</sup>  
1175 mutants lack *myod* mRNA (arrows) but retain *myf5* mRNA in presomitic mesoderm (white  
1176 dashes). *Fgf4* mRNA injection induced *myf5* and *myod* mRNAs throughout the posterior  
1177 mesoderm in siblings (white dashes), but failed to induce *myod* mRNA in mutants. **B.** *Fgf4*  
1178 suppressed *aplnrb* mRNA in posterior mesoderm above the germ ring (white dashes) in

1179 both *tbxta*<sup>-/-</sup> mutants and siblings. Individually genotyped embryos are shown in lateral  
1180 view, dorsal to right (A,B). **C.** Scoring of *myod* mRNA accumulation in response to *Fgf4*  
1181 mRNA injection into embryos from a *tbxta*<sup>+/-</sup> incross. Expanded: ventral expansion,  
1182 generally all around germ ring as in panel A. Adaxial/faint: Either wild type pattern or faint  
1183 version of it in small a small proportion of mutants, which was not significantly altered by  
1184 *Fgf4* mRNA. Left panel shows absolute number of embryos analysed from two  
1185 experiments to emphasise lack of induction in mutants (raw data in Table S3). Right panel  
1186 displays data as a percentage of the total to highlight reduced response in heterozygotes  
1187 compared to wild type ( $X^2$  test). **D.** Adaxial upregulation of *tbx16* mRNA is lost in *tbxta*<sup>-/-</sup>  
1188 mutant (arrowheads). *Fgf4* upregulates *tbx16* mRNA throughout ventral posterior  
1189 mesoderm (arrows) and causes mesodermal cell aggregation (asterisks). *Tbxta*<sup>-/-</sup> mutants  
1190 accumulate less *tbx16* mRNA than siblings and have less expression on the dorsal side  
1191 (brackets). Bars: 100  $\mu$ m.

1192

1193 **Fig. 8. Tbx16 is essential for Fgf4-driven upregulation of *tbx16* and suppression of**  
1194 ***tbxta*.** Embryos from a *tbx16*<sup>+/-</sup> incross injected with 150 pg *fgf4* mRNA or control. **A,B.**  
1195 By 24 hpf, *Fgf4*-injected embryos have disorganized heads and although lacking obvious  
1196 trunk or tail, some contain twitching muscle (A). In situ mRNA hybridization for *col1a2* for  
1197 dermomyotome/connective tissue and *myhz1* for skeletal muscle revealed muscle in *fgf4*-  
1198 injected sibs, but not in *tbx16*<sup>-/-</sup> mutants (B). Boxes are magnified to show the alternating  
1199 pattern of aggregated muscle and connective tissue in *Fgf4*-injected siblings, but the  
1200 reduced *col1a2* and absent *myhz1* mRNA in *Fgf4*-injected mutants. Note the aggregation  
1201 of posterior mesoderm cells into strands around the yolk. **C.** In situ mRNA hybridisation for  
1202 *tbx16* mRNA in embryos from a *tbx16*<sup>+/-</sup> incross at around 90% epiboly. Nonsense  
1203 mediate decay of the mutant transcript is apparent (arrow). *Fgf4* RNA injection increases  
1204 *tbx16* mRNA in paraxial mesoderm, widens dorsal axial notochord domain (asterisk) and  
1205 causes aggregation of paraxial cells in siblings, but suppresses residual *tbx16* transcript in  
1206 mutants. **D.** Immunodetection of *Tbxt* protein and *tbx16* mRNA in *Fgf4*-injected and  
1207 control embryos from a *tbx16*<sup>+/-</sup> incross. A *Fgf4*-injected *tbx16*<sup>-/-</sup> mutant (bottom) reveals  
1208 nuclear *Tbxt* protein in the entire posterior mesoderm. Residual *tbx16* mRNA in the  
1209 prechordal region (arrows) but absence in posterior mesoderm demonstrates the genotype.  
1210 **E.** Widespread up-regulation of *shha* mRNA reveals the notochord-like character of  
1211 posterior mesoderm in *Fgf4*-injected *tbx16*<sup>-/-</sup> mutant. Bars: 100  $\mu$ m.

1212

1213 **Table S1 Quantitation of data in Figures**

Figure panel	+	Assay	Treatment/genotype	Embryos with phenotype shown/Total (%)
1A		<i>myod</i> mRNA	con SU5402 con cyA	51/51 (100%) 40/40 (100%) 80/80 (100%) 60/60 (100%)
1A		<i>myf5</i> mRNA	con SU5402 con cyA	50/50 (100%) 36/36 (100%) 81/81 (100%) 68/68 (100%)
1A		<i>aplnrb</i> mRNA	con SU5402	30/30 (100%) 28/28 (100%)
1B		<i>myod</i> mRNA	cyA 6ss <i>smo</i> 6ss <i>smo</i> + cyA 6ss <i>smo</i> 15ss	83/83 (100%) 13/46 (28%) 14/54 (26%) 26/99 (26%)
1B		<i>ptc1</i> mRNA	<i>smo</i> 6ss cyA	11/67 (16%) 31/31 (100%)
2A		Slow MyHC	<i>shha</i> 24hpf	23/80 (29%)
2B		Slow MyHC	<i>noto</i> 24hpf	25/88 (28%)
2C		<i>myod</i> mRNA	sib <i>noto</i> sib + cyA <i>noto</i> + cyA	47/62 (76%) 15/62 (24%) 39/56 (70%) 17/56 (30%)
2D		<i>myod</i> mRNA	con cyA SU5402 SU5402 + cyA	67/67 (100%) 47/47 (100%) 116/116 (100%) 62/63 (98%)
2D		<i>myf5</i> mRNA	con cyA SU5402 SU5402+cyA	20/20 (100%) 15/15 (100%) 5/5 (100%) 13/13 (100%)
3A		<i>myod</i> mRNA	con <i>fgf6a</i> MO + <i>fgf8a</i> MO <i>fgf4</i> MO + <i>fgf8a</i> MO <i>fgf4</i> MO + <i>fgf6a</i> MO + <i>fgf8a</i> MO	35/40 (88%) + 8/19 (42%) 38/46 (83%) + 22/26 (85%) 25/36 (69%) 13/15 (87%)
3A		<i>myf5</i> mRNA	con <i>fgf6a</i> MO + <i>fgf8a</i> MO <i>fgf4</i> MO + <i>fgf8a</i> MO <i>fgf4</i> MO + <i>fgf6a</i> MO + <i>fgf8a</i> MO	38/40 (95%) + 20/20 (100%) 46/50 (92%) + 32/33 (97%) 11/38 (29%) 17/25 (68%)
3B		<i>myod</i> mRNA	con cyA <i>fgf4</i> MO + <i>fgf8a</i> MO <i>fgf4</i> MO + <i>fgf8a</i> MO + cyA	3/3 (100%) 5/5 (100%) 5/5 (100%) 4/5 (80%)
3C		<i>myod</i> mRNA	con ( <i>fgf4</i> ) + <i>_fgf4</i> mRNA con ( <i>fgf6a</i> )	129/129 (100%) 131/136 (96%) 20/20 (100%)

		+ <i>fgf6a</i> mRNA	20/20 (100%)
3C	<i>myf5</i> mRNA	con ( <i>fgf4</i> ) + <i>_fgf4</i> mRNA con ( <i>fgf6a</i> ) + <i>fgf6a</i> mRNA	30/30 (100%) 34/40 (85%) 20/20 (100%) 18/18 (100%)
3D	<i>aplnrb</i> mRNA	con + <i>fgf4</i> mRNA	32/32 (100%) 12/18 (67%)
4A	<i>tbxta</i> mRNA	con 80% low SU5402 80% con 6ss low SU5402 6ss high SU5402 6ss	21/21 (100%) 14/14 (100%) 52/52 (100%) 44/50 (88%) 32/32 (100%)
4A	<i>tbx16</i> mRNA	con 80% low SU5402 80% con 6ss low SU5402 6ss high SU5402 6ss	21/21 (100%) 15/15 (100%) 40/40 (100%) 41/41 (100%) 74/74 (100%)
4B	<i>myod</i> mRNA	con <i>tbxta</i> MO <i>tbx16</i> MO	28/28 (100%) 20/21 (95%) 20/22 (91%)
4B	<i>myf5</i> mRNA	con <i>tbxta</i> MO <i>tbx16</i> MO	30/30 (100%) 12/19 (63%) 19/20 (95%)
4B	<i>fgf8a</i> mRNA	con <i>tbxta</i> MO <i>tbx16</i> MO	30/30 (100%) 19/20 (95%) 18/20 (90%)
4B	<i>fgf3</i> mRNA	con <i>tbxta</i> MO <i>tbx16</i> MO	25/25 (100%) 17/17 (100%) 11/14 (79%)
4B	<i>fgf4</i> mRNA	con <i>tbxta</i> MO <i>tbx16</i> MO	28/28 (100%) 19/19 (100%) 12/14 (86%)
4B	<i>fgf8a</i> mRNA	con <i>tbxta</i> MO <i>tbx16</i> MO	30/30 (100%) 14/16 (88%) 10/12 (83%)
4C	<i>myod</i> mRNA	con <i>tbxta</i> MO <i>tbx16</i> MO cyA cyA + <i>tbxta</i> MO cyA + <i>tbx16</i> MO	31/31 (100%) 21/22 (95%) 24/24 (100%) 30/30 (100%) 23/23 (100%) 22/22 (100%)
4C	<i>myf5</i> mRNA	con <i>tbxta</i> MO <i>tbx16</i> MO cyA cyA + <i>tbxta</i> MO cyA + <i>tbx16</i> MO	40/40 (100%) 24/26 (92%) 25/25 (100%) 38/38 (100%) 19/20 (95%) 24/24 (100%)
5A	<i>myod</i> mRNA	sib <i>tbx16</i> <sup>-/-</sup> sib + <i>fgf4</i> mRNA <i>tbx16</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	82/122 (67%) 40/122 (33%) 95/128 (78%) 33/128 (26%)
5A	<i>myf5</i> mRNA	sib	95/120 (79%)

		<i>tbx16</i> <sup>-/-</sup> sib + <i>fgf4</i> mRNA <i>tbx16</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	25/120 (21%) 98/126 (78%) 28/126 (22%)
5B	<i>tbxta</i> mRNA	con <i>fgf4</i> mRNA	30/30 (100%) 20/21 (95%)
5B	<i>tbx16</i> mRNA	con <i>fgf4</i> mRNA	29/29 (100%) 61/61 (100%)
5C	<i>myod</i> mRNA	con + <i>tbx16</i> mRNA con + 10 $\mu$ M SU5402 + <i>tbx16</i> mRNA + 10 $\mu$ M SU5402	31/31 (100%) 7/42 (17%) 32/32 (100%) 7/35 (20%)
5C	<i>myf5</i> mRNA	con + <i>tbx16</i> mRNA con + 10 $\mu$ M SU5402 + <i>tbx16</i> mRNA + 10 $\mu$ M SU5402	24/24 (100%) 14/42 (33%) 29/29 (100%) 4/35 (11%)
5D	<i>myf5</i> mRNA	con + <i>tbx16</i> mRNA con + 60 $\mu$ M SU5402 + <i>tbx16</i> mRNA + 60 $\mu$ M SU5402	18/18 (100%) 2/23 (9%), 21/23 (91%) faint 8/8 (100%) 23/23 (100%)
5D	<i>myod</i> mRNA	con + <i>tbx16</i> mRNA con + 60 $\mu$ M SU5402 + <i>tbx16</i> mRNA + 60 $\mu$ M SU5402	15/15/ (100%) 2/24 (8%), 7/24 (29%) disrupted 15/15 (100%) 32/32 (100%)
6E	<i>myf5</i> mRNA	CHD alone CHD + DEX	75/75 (100%) 35/70 (50%)
6E	<i>myod</i> mRNA	CHD alone CHD + DEX	28/28 (100%) 11/21 (52%)
6F	<i>myod</i> mRNA	Control <i>myf5</i> het incross <i>myf5</i> <sup>+/+</sup> + <i>tbx16</i> mRNA <i>myf5</i> <sup>+/-</sup> + <i>tbx16</i> mRNA <i>myf5</i> <sup>-/-</sup> + <i>tbx16</i> mRNA	33/33 (100%) 6/6 (100%) 11/17 (65%) 2/4 (50%)
7A	<i>myod</i> mRNA	sib <i>tbxta</i> <sup>-/-</sup> sib + <i>fgf4</i> mRNA <i>tbxta</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	100/132 (76%) 32/132 (24%) 16/27 (59%) (for genotyping see 8/27 (29%) Table S4)
7A	<i>myf5</i> mRNA	sib <i>tbxta</i> <sup>-/-</sup> sib + <i>fgf4</i> mRNA <i>tbxta</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	108/138 (78%) 30/138 (22%) 50/74 (68%) 24/74 (32%)
7B	<i>aplnrb</i> mRNA	sib <i>tbxta</i> <sup>-/-</sup> sib + <i>fgf4</i> mRNA <i>tbxta</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	144/195 (74%) 51/195 (26%) 12/18 (67%) 6/18 (33%)
7D	<i>tbx16</i> mRNA	sib <i>tbxta</i> <sup>-/-</sup> sib + <i>fgf4</i> mRNA <i>tbxta</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	48/66 (73%) 18/66 (27%) 70/93 (75%) 23/93 (25%)

8A	Bright field	sib <i>tbx16</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	28/41 (68%) 13/41 (32%) 48
8B	<i>myhz1</i> mRNA	sib <i>tbx16</i> <sup>-/-</sup> sib + <i>fgf4</i> mRNA <i>tbx16</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	27/40 (68%) 13/40 (32%) 38/48 (79%) 10/48 (21%)
8C,D	<i>tbx16</i> mRNA+ Tbxta protein	sib <i>tbx16</i> <sup>-/-</sup> sib + <i>fgf4</i> mRNA <i>tbx16</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	33/47 (70%) 14/47 (30%) 40/57 (70%) 17/57 (30%)
8E		sib <i>tbxt16</i> <sup>-/-</sup> sib + <i>fgf4</i> mRNA <i>tbx16</i> <sup>-/-</sup> + <i>fgf4</i> mRNA	19/28 (68%) 9/28 (32%) 19/31 (61%) 12/31 (39%)
S1	<i>aplnr</i> mRNA	50%-10ss	approx. 25 embryos/stage
S2A	Slow MyHC	<i>smo</i> sib	27/40 (67.5%)
S2B	Slow MyHC	<i>smo</i> sib + <i>myod</i> mRNA	28/35 (80%)
S2C	Slow MyHC	<i>smo</i> <sup>-/-</sup>	13/40 (32.5%)
S2D	Slow MyHC	<i>smo</i> <sup>-/-</sup> + <i>myod</i> mRNA	7/35 (20%)
S2E	Slow MyHC	con	100/100 (100%)
S2F	Slow MyHC	<i>cyA</i>	73/73 (100%)
S2G	Slow MyHC	<i>cyA</i> + <i>myod</i> mRNA	20/32 (63%)
S2H	Slow MyHC + Prox1 + GFP	<i>cyA</i> + <i>myog</i> mRNA	26/46 (57%)
S2I	Slow MyHC + Prox1	24hpf	50/50 (100%)
S2J	Slow MyHC + Prox1	24hpf	26/43 (60%)
S3B	<i>myod</i> mRNA	con + <i>fgf3</i> MO + <i>fgf4</i> MO + <i>fgf6a</i> MO <i>fgf8a</i> <sup>-/-</sup>	39/47 58/65 46/70 52/69
S3B	<i>myf5</i> mRNA	con + <i>fgf3</i> MO + <i>fgf4</i> MO + <i>fgf6a</i> MO <i>fgf8a</i> <sup>-/-</sup>	51/51 (100%) 21/58 (36%) 19/58 (33%) 44/69 (64%)
S3C	<i>myf5</i> mRNA	sib + con MO <i>fgf8a</i> <sup>-/-</sup> + con MO sib + triple Fgf MO <i>fgf8a</i> <sup>-/-</sup> + triple Fgf MO	13/17 (76%) (2/2 genotyped sib) 4/17 (24%) (3/4 genotyped -/-) 19/31 (77%) (2/2 genotyped sib) 7/31(23%) (3/3 genotyped -/-)
S3D	<i>myod</i> mRNA	con + <i>fgf4</i> MO + <i>fgf6a</i> MO + <i>fgf4</i> MO + <i>fgf6a</i> MO	4/4 3/3 2/2 3/3

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1217 **Table S2 Sequences of morpholinos and primers**

<b>Morpholino</b>			
<b>Gene</b>	<b>Sequence</b> (start codon underlined)	<b>Quantity (ng)</b>	<b>Reference</b>
<i>fgf3</i>	5'- <u>CATT</u> GTGGCATGGCGGGATGTCGGC-3'	7.5	(Maroon et al., 2002)
<i>fgf4</i>	5'-GCAAGAGGGCTGACTGGACACT <u>CAT</u> -3'	2-6	
<i>fgf6a</i>	5'-TGAGGAACCTTTGCGCAGTGGCC <u>CAT</u> -3'	2-6	
<i>fgf8a</i>	5'-GAGTCT <u>CAT</u> GTTTATAGCCTCAGTA -3'	2	(Furthauer et al., 2001)
<i>tbx16</i>	5'-GCTTGAGGTCTCTGATAGCCTG <u>CAT</u> -3'	0.5	(Bisgrove et al., 2005)
<i>tbxta</i>	5'-GACTTGAGGCAGG <u>CAT</u> ATTTCCGAT -3' 5'-GCTGGTCGGGACTTGAGGCAGAC <u>CAT</u> -3'	0.25 2	(Bisgrove et al., 2005; Feldman and Stemple, 2001)
control	5'-CCTCCTACCTCAGTTACAATTTATA -3'	3-6	Gene Tools standard
<b>Primers</b> (start and stop codons underlined)			
<b>Gene</b>	<b>Forward</b>	<b>Reverse</b>	<b>Reference</b>
<i>fgf4</i>	5'-GAGCTCGAGCTC <u>CAT</u> GAGTGTCCAGTCGGCCCTTG-3'	5'-GTCGACGTCGACT <u>CAA</u> ATTCTAGGCAAG-3'	
5DE_ChIP-qPCR	5'-TTCCTCACCGTACCTTTTGC-3'	5'-CATTTCCTCCCAACAATACACC-3'	
5PE1_ChIP-qPCR	5'-GTGCAATTTTGGCTCAGCTT-3'	5'-AGATCGGGGAACTTCGCTAT-3'	
Negative region ( <i>rhod</i> )	5'-GACTCCACACAATCTGCAACAT-3'	5'-ACCACCTACGCTAAAGAAACCA-3'	Morley et al., 2009

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1219



1220 **Table S4. Tbx1a dosage controls response of *myod* to Fgf.**

Fgf4 mRNA (pg)	Genotype	Number genotyped [%]¶	<i>Myod</i> mRNA expression pattern in genotyped embryos (%)		
			Absent	Adaxial only	Expanded ventrally
0	-/-	4*	4 (100%)§	0 (0%)	0 (0%)
	+/-	3*	0 (0%)	3 (100%)	0 (0%)
	+/+	2*	0 (0%)	2 (100%)	0 (0%)
	Total	9	8 ((15%))∞	45 ((85%))	0 ((0%))
100	-/-	8 [30%]	7 (88%)	1 (12%)	0 (0%)
	+/-	14 [52%]	0 (0%)	11 (79%)	3 (21%)
	+/+	5 [19%]	0 (0%)	0 (0%)	5 (100%)
	Total	27	7 (26%)	12 (44%)	8 (30%)
0	-/-	5*	5 (100%)	0 (0%)	0 (0%)
	+/-	2*	0 (0%)	2 (100%)	0 (0%)
	+/+	3*	0 (0%)	3 (100%)	0 (0%)
	Total	10	9 ((30%))	21 ((70%))	0 ((0%))
100	-/-	1 [6%]	1 (100%)	0 (0%)	0 (0%)
	+/-	8 [50%]	0 (0%)	3 (37%)	5 (63%)
	+/+	7 [44%]	0 (0%)	1 (14%)	6 (86%)
	Total	16	1 (6%)	4 (25%)	11 (69%)
150	-/-	6 [25%]	5 (100%)	1 (17%)	0 (0%)
	+/-	9 [38%]	0 (0%)	6 (67%)	3 (33%)
	+/+	9 [38%]	0 (0%)	2 (22%)	7 (78%)
	Total	24	5 (21%)	9 (38%)	10 (42%)
225	-/-	15 [24%]	12 (80%)	3 (20%)	0 (0%)
	+/-	26 [42%]	0 (0%)	11 (42%)	15 (58%)
	+/+	21 [34%]	0 (0%)	4 (19%)	17 (81%)
	Total	62	12 (19%)	18 (29%)	32 (52%)
<b>Summary</b>					
0	-/-	9*	9 (100%)	0 (0%)	0 (0%)
	+/-	5*	0 (0%)	5 (100%)	0 (0%)
	+/+	5*	0 (0%)	5 (100%)	0 (0%)
	Total	19	17 ((20%))	66 ((80%))	0 ((0%))
100-225	-/-	30 [23%]	25 (88%)	5 (12%)	0 (0%)
	+/-	57 [44%]	0 (0%)	31 (54%)	26 (46%)
	+/+	42 [33%]	0 (0%)	7 (17%)	35 (83%)
	Total	129	25 (19%)	43 (33%)	61 (47%)

1221 \* Only a subset of control embryos were genotyped (to ensure reproducibility).

1222 § Percentages in curved brackets represent fraction of embryos of indicated genotype showing  
1223 listed *myod* expression pattern.

1224 ¶ Percentages in square brackets represent fraction of embryos in sample with each genotype.

1225 ∞ Percentages in double brackets represent fraction of embryos in sample showing listed *myod*  
1226 expression pattern.

1227 **Fig. S1. Expression of *aplnrb* mRNA during zebrafish axis formation.**

1228 Wholmount in situ mRNA hybridization of apelin receptor b (*aplnrb*) mRNA in zebrafish embryos  
1229 at the indicated stages, shown in animal (An), lateral (La), dorsal (Do, animal to top), ventral (Ve,  
1230 animal to top) and posterior (Po, dorsal to top) views. Ant = anterior. Note significant expression in  
1231 early germ ring (arrows), future cranial mesoderm (large and small brackets highlight comparable  
1232 regions of expression) and adaxial cells (arrowheads). Expression is lacking in paraxial mesoderm  
1233 (white dashes) that expresses *myf5* and later *myod* mRNAs (see Fig. 1C).

1234

1235

1236 **Fig. S2. MRF over-expression rescues trunk slow myogenesis.**

1237 Confocal stacks showing immunodetection of slow fibres with Slow MyHC in *smo* mutant (identified  
1238 by lack of tail circulation), *smo* sibling, un-injected control or cyA-treated embryos injected with  
1239 *myod* or *myog* RNA. All embryos orientated anterior to left dorsal up showing 2-3 trunk somites (A-  
1240 H) or entire trunk and tail (I,J). **A,B.** *Myod* RNA-injected *smo* siblings have slow muscle with  
1241 disrupted somite morphology. **C,D.** Rare slow fibres present in the trunk region of *smo* mutants  
1242 (arrow) are more common after *myod* RNA injection. **E,F.** CyA-treatment prevents slow fibre  
1243 formation. Presence of maternal Smo protein may account for the greater number of residual slow  
1244 fibres in *smo* mutant compared to cyA-treated embryos. **G,H.** *Myod* or *myog* RNA injection  
1245 rescues slow fibre formation in cyA-treated embryos. Inset in H shows co-expression of slow  
1246 MyHC, Prox1 and GFP in a cyA-treated embryo after injection of *myog-IRES-GFP* RNA. **I,J.** *Myog*  
1247 RNA rescues slow fibres in trunk but not tail. Insets show co-expression of Prox1 and slow MyHC  
1248 in short confocal stacks. **K.** Slow fibres were counted at 24 hpf in each somite of seven control  
1249 *smo* mutants and seven *smo* mutants injected at 1 cell stage with *myod* RNA. **L.** Slow fibres were  
1250 counted at 24 hpf in each somite of ten control uninjected and ten embryos injected at 1 cell stage  
1251 with *myog* RNA that were each subsequently treated with cyA from 30% epiboly. Bars: 50  $\mu$ m.

1252

1253

1254 **Fig. S3. Dorsal fgf expression and requirement for adaxial myogenesis.**

1255 In situ mRNA hybridization for fgfs in wild type embryos at 80% epiboly, tailbud (tb) and 6ss (A), for  
1256 *myod* and *myf5* in control and fgf MO-injected and *fgf8a*<sup>-/-</sup> embryos at 80% (B,C) and for *tbxta* (red)  
1257 and *myod* (blue/brown) (D). **A.** *fgf8a*, *fgf4*, *fgf6a* and *fgf3* transcripts appear successively in wild  
1258 type embryos in the dorsal midline (arrows) and CNH (arrowheads). **B.** *Myod* and *myf5* mRNAs in  
1259 *fgf3* MO, *fgf4* MO and *fgf6a* MO wild type embryos and in sequence-genotyped *fgf8a*<sup>-/-</sup> embryos at  
1260 80% epiboly (upper rows, con and single MOs from a representative experiment). Note that  
1261 siblings of the *fgf8a* mutants had similar MRF expression. Arrowheads indicate nascent adaxial  
1262 cells. **C.** *Myf5* mRNA in sibling embryos from an incross of heterozygous *fgf8a*<sup>+/-</sup> fish injected with  
1263 6 ng control MO or 2 ng each of *fgf3*, *fgf4* and *fgf6a* MO. Note the successively stronger reduction  
1264 in signal as more fgf function is removed. **D.** Rows showing replicate fgf MO-injected embryos  
1265 had reduced accumulation of *myod* mRNA in pre-adaxial cells of compared to control (con)  
1266 (arrowheads). Note widening of notochord in *fgf6a* morphants. Bars: 100 µm.

1267

1268 **Fig. S4. *AplnrB* mRNA changes in Tbx mutants.**

1269 In situ mRNA hybridization for *aplnrB* in wild type sibling and *tbx16* mutant and *tbxta* mutant  
1270 embryos at 80% epiboly. Single embryos are shown from dorsal, left lateral and ventral views.  
1271 Labelling (brackets) is in a band of anterior mesoderm. Note the unlabelled region in wild type and  
1272 *tbxta* mutant that is missing in *tbx16* mutant (white dashes). Adaxial *aplnrB* mRNA up-regulation  
1273 (arrowheads) is lacking in mutants.

1274

1275 **Fig. S5. ChIP-seq analysis of *myod* locus.**

1276 ChIP-seq on wt embryos at 75-85% epiboly indicates endogenous Tbx16 and Tbx16  
1277 binding events within 75 kb flanking *myod* TSS. H3K4me3 marks TSSs; H3K4me1 marks  
1278 enhancers; H3K27ac indicates active enhancers; RPM – ChIP-seq peaks height in reads  
1279 per million reads. Multiz Alignments & Conservation from UCSC Genome Browser  
1280 (Haeussler et al., 2019) are shown beneath. Purple boxes indicate significant Tbx binding  
1281 for Tbx16 and Tbx16 (DDE1) and Tbx16 alone (DDE3). Cyan boxes indicate of Tbx sites  
1282 mentioned in text. Significant H3K4me1 marks are present at both DDE1 and DDE3, while  
1283 only DDE3 has a significant H3K27ac mark.

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