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Spatiotemporal coordination of FGF and Shh signalling underlies the specification of myoblasts in the Zebrafish embryo

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SUMMARY

Somitic cells give rise to a variety of cell types in response to Hh, BMP and FGF signaling. Cell position within the developing Zebrafish somite is highly dynamic: how, when, and where these signals specify cell fate is largely unknown. Combining four-dimensional imaging with pathway perturbations, we characterize the spatiotemporal specification and localization of somitic cells. Muscle formation is guided by highly orchestrated waves of cell specification. We find that FGF directly and indirectly controls the differentiation of fast and slow-twitch muscle lineages respectively. FGF signaling imposes tight temporal control on Shh induction of slow muscles by regulating the time at which fast-twitch progenitors displace slow-twitch progenitors from contacting the Shh-secreting notochord. Further, we find a reciprocal regulation of fast and slow muscle differentiation, morphogenesis and migration. In conclusion, robust cell fate determination in the developing somite requires precise spatiotemporal coordination between distinct cell lineages and signaling pathways.

KEY WORDS

Zebrafish, Myogenesis, Adaxial cells, Sonic hedgehog, FGF signaling, Temporal regulation, Somite polarity, Somite rotation

ABBREVIATIONS

BMP: Bone Morphgenetic Protein
FGF: Fibroblast Growth Factor
Shh: Sonic Hedgehog Protein
SDF: Stromal cell-derived factor
CycA: cyclopamine
DM: Dorsomorphin
PSM: Presomitic Mesoderm
MP: Muscle Pioneer
SSF: Superficial Slow Muscle Fiber
ECL cells: External cell layer cells
ABC: anterior border cell
S-1, S0, S1,…: Somite stage -1, 0, 1
AP: Anterior-posterior
DV: Dorsal-ventral
ML: Medial-lateral
Mins: Minutes
Hpf: Hours post fertilization
FISH: Fluorescent *in situ* hybridization

INTRODUCTION

Cell fate decisions in metazoa are determined by an assemblage of receptor stimulations in response to a limited number of extracellular signals (Cooper and Hausman, 2007). During development, numerous cell types and diverse patterns are induced through combinations of inputs from these signals. Extensive studies over the past two decades have revealed the crucial importance of precise spatio-temporal control of signaling pathway activation (Housden and Perrimon, 2014; Ingham and Placzek, 2006) in imparting cells with unique positional information (Kicheva et al., 2007; Perrimon et al., 2012;

62 Wolpert, 1969). However, positional information must often be conveyed to cells in the context of
63 dynamic rearrangements. For example, during the transition of the neural plate to the neural tube,
64 extensive cell migration, intercalation and proliferation take place concurrently with Shh gradient
65 formation and interpretation (Xiong et al., 2013). There is currently a dearth of understanding of how
66 cells integrate biochemical and biomechanical inputs whilst also undergoing morphological and
67 positional changes. To gain deeper insight, here we analyze the process of skeletal muscle
68 differentiation in the zebrafish somite, a tissue that undergoes rapid cell morphogenesis and cell
69 migrations.

70 Previous studies have implicated inputs from three different signaling systems in the specification of
71 muscle fiber identity: Sonic Hedgehog (Shh) along the medial-lateral (ML) axis (Blagden et al 1997;
72 Wolff et al., 2003); Bone Morphogenetic Protein (BMP) along the dorsal-ventral (DV) axis (Maurya et
73 al., 2011; Nguyen-Chi et al., 2012); and Fibroblast Growth Factor (FGF) along the anterior posterior
74 (AP) axis (Nguyen-Chi et al., 2012). Two major skeletal muscle lineages are specified in response to
75 these signals, giving rise to the slow-twitch (henceforth slow) and fast-twitch (henceforth fast) muscle
76 fibers (Devoto et al., 1996) (Figure 1A). These fiber-types differ in their physiological and metabolic
77 properties, with very different contraction velocity, mitochondrial number and motor-neuron innervation
78 (Schiaffino and Reggiani, 2011). The progenitors of the muscle fiber types originate from distinct
79 locations within the somite (Devoto et al 1996; Stickney et al., 2000): the earlier differentiating slow-
80 muscles arise from an epithelial-like cell group (termed adaxial cells) within the presomitic mesoderm
81 (PSM), located medially in the somite close to the notochord (Devoto et al., 1996; Hirsinger et al., 2004)
82 (Figure 1B). These cells differentiate into elongated slow muscle fibers soon after somite segmentation
83 (Figure 1C) (Daggett et al., 2007); subsequently, the majority migrate radially to form a monolayer of
84 fibers on the surface of the myotome (the so-called superficial slow fibers (SSFs)) leaving behind a
85 small number of medially located fibers, termed muscle pioneers (MPs) (Felsenfeld et al., 1991), located
86 at the dorsal-ventral midline in close apposition to the notochord (Figure 1D) (Devoto et al 1996; Henry
87 and Amacher, 2004).

88 At a similar time to slow muscle differentiation, the more lateral somitic cells differentiate into fast muscle
89 fibers and external cell layer (ECL) cells (Figure 1A and 1D). The ECL cells are equivalent to the
90 dermomyotome of amniotes and provide a pool of progenitors for post-embryonic muscle growth and
91 repair (Amthor et al., 1999; Goulding et al., 1994). The ECL cells are located lateral to the SSFs by the
92 end of primary myogenesis (Figure 1D) (Hollway et al., 2007; Stellabotte et al., 2007). In addition to
93 skeletal muscles and muscle progenitors, somitic cells also give rise to a variety of non-muscle
94 progenitors (Figure 1A and 1D) including fin mesenchyme cells, dorsal aorta, vasculature and apical
95 ectodermal ridge (Lee et al., 2013; Masselink et al., 2016; Nguyen et al., 2014; Shimada et al., 2013).
96 However, it remains largely unknown precisely from where the various progenitors arise and how these
97 different cell fates are specified.
98
99 The notochord is an important signaling center for the induction of slow muscle cell types, in particular
100 by acting as a source of Hedgehog family proteins (Blagden et al., 1997). The differentiation of MPs is
101 Hh dependent (Currie and Ingham, 1996), as is the specification of the entire slow muscle lineage
102 (Blagden et al., 1997; Lewis et al., 1999). Modulation of Shh signaling strength and duration suggests
103 MPs require both higher levels and longer duration of Shh exposure compared to the SSFs for their
104 specification (Wolff et al., 2003). A combination of Shh-mediated activation and BMP-mediated
105 inhibition determines MP cell fate amongst the adaxial cells, in part by controlling the transcription of
106 the homeobox gene *engrailed2A* (*eng2A*) (Maurya et al., 2011). The extracellular matrix between the
107 notochord and somite also plays a role in MP differentiation, by modulating BMP signaling (Dolez et al.,
108 2011).

109 FGF appears to participate in the further differentiation of both the slow (MPs and SSFs) and fast (fast
110 muscle fibers and ECL cells) twitch lineages (Groves, 2005; Nguyen-Chi et al., 2012). Compared with
111 the direct roles of Shh and BMP signaling in regulating *eng*-family expression, the role of FGF signaling
112 in MP differentiation is less well defined. Nguyen-Chen and colleagues have presented evidence that
113 modulation of the FGF signaling pathway in adaxial cells restricts MP cell fate to the anterior-most cells
114 in each somite (Nguyen-Chi et al., 2012). However, earlier studies had indicated that the FGF pathway
115 is not directly activated within adaxial cells (Groves, 2005; Hamade et al., 2006). Hence, there remains
116 uncertainty as to whether FGF signaling acts directly - in concert with Shh and BMP signaling - to
117 specify MP cell fate. In contrast, there is good evidence that FGF pathway is activated among fast
118 muscle progenitors and directly controls their differentiation (Groves, 2005). Since the migration and

119 further differentiation of slow muscles is closely associated with fast muscle differentiation, it seems
120 possible that FGF might influence MP specification indirectly through its regulation of fast fiber
121 differentiation.

122 Here, we combine live imaging with targeted pathway perturbations using drug treatments and genetic
123 manipulation, to characterize systemically the spatiotemporal specification and localization of various
124 types of somitic cells. We adopt a multi-dimensional whole-somite imaging approach, which enables
125 simultaneous monitoring of cell morphogenesis and migration, together with the expression of key
126 markers of cell identity. We use this to create a cell fate map for the whole somitic cell population. Our
127 data reveal that cell fate specification within the newly formed somite is highly polarized both temporally
128 and spatially. Distinct domains within newly formed somites robustly give rise to MPs, SSFs, ECL cells,
129 fast muscles, and non-muscle progenitors. During somite patterning, orchestrated waves of muscle
130 formation occur in both the slow and fast muscle lineages. These waves occur along distinct axes of
131 the somite (AP axis for fast muscle fibers and DV axis for slow muscle fibers), and help to ensure that
132 different cell types locate to their specific destination within it. In particular, we have revisited the role of
133 FGF signaling in the patterning of both slow and fast muscle lineages. Our results indicate that FGF
134 directly patterns the fast muscle progenitors along the AP axis; by contrast, rather than directly
135 regulating *eng2A* expression as previously implied (Nguyen-Chi et al., 2012), FGF signaling influences
136 MP cell fate by modulating adaxial cell migration from the notochord, the source of Shh. Further analysis
137 suggests the migration of SSFs requires the myogenesis of their neighboring fast muscle progenitors.
138 Thus, we conclude that FGF acts indirectly to determine the precise temporal window of exposure of
139 slow muscle progenitors to Shh and BMP signaling, through its direct effect on fast muscle
140 differentiation.

141 ## RESULTS

142

143 **Differential Hedgehog activity along the DV axis in the PSM endows a wave of slow muscle**
144 **morphogenesis**

145 We used confocal and light-sheet microscopy to collect movies of muscle fiber formation, starting from
146 somite stage -2 (S-2) at PSM through to the end of primary myogenesis at around 10 hours after somite
147 segmentation. We imaged at sufficient spatial and temporal resolution to enable tracking of every
148 somitic cell in each somite (see Methods). We also recorded cell shape and, where appropriate,
149 recorded expression of cell specific transgenes such as *Tg(eng2a:eGFP)*, a marker of MPs (Maurya et
150 al., 2011) or *Tg(prdm1a:GFP*), a marker of all slow-muscle cells (Elworthy et al., 2008). We used these
151 tracks to curate maps of muscle fiber location throughout myogenesis (Figure 1E and Video S1) and
152 also the spatio-temporal variation in reporter expression (Figure 1F). These maps form the basis of the
153 results presented below. Our approach is highly robust, as it utilizes cell tracking on the full adaxial cell
154 and lateral somitic cell population across contiguous somites, based both on a cell fate marker as well
155 as final cell morphology. We refer to the developing muscle segments from stage S1 to S8 as somites,
156 whilst the more mature tissue after S8 are referred to as muscle segments.
157
158 Adaxial cells intercalate with each other and elongate to span the whole somite soon after segmentation.
159 Using single cell tracking, we identified a wave of muscle morphogenesis among adaxial cells within
160 each somite, emanating outward from the DV midline (Figure 2A and Video S2). We quantified the
161 timing for each slow muscle progenitor to elongate fully such that it spanned the whole somite. We
162 found most adaxial cells along the DV midline fully elongated 40-60mins after segmentation of their
163 corresponding somite (Figure 2A(iii-iv) and Figure 2B). In contrast, adaxial cells on the dorsal and
164 ventral edges of the PSM displayed a large variation in the timing of complete fiber elongation, ranging
165 from 50mins to 150mins after segmentation (Figure 2A(v-viii) and Figure 2B).

166 We mapped all the slow muscles from four contiguous somites back to their initial location within the
167 PSM (Figure 2C-C''). Consistent with previous understanding of the role of Shh signaling in slow muscle
168 specification, almost all the slow muscle progenitors are in direct contact with the notochord in the PSM,
169 whilst a subset of them on the dorsal and ventral sides displays only very limited contact of their basal
170 surface with the notochord (Figure 2C). Careful examination of the absolute timing of muscle elongation
171 throughout four contiguous somites revealed a temporal gradient of elongation along the AP body axis
172 (Figure 2C'). Among cells at similar AP position, we noticed large temporal variations along the DV axis,
173 particularly toward the dorsal and ventral edges of the somite (Figure 2C'). The timing of slow fiber
174 myogenesis is hence dependent on both the AP and DV position of the adaxial cells. However, within

175 each somite, the temporal variation appears predominantly dependent on DV position (Figure 2C'' and
176 Figure S1A).

177 One explanation for the temporal variation along the DV axis is differential Shh activity within the adaxial
178 cell population. Using expression of the Shh target gene *ptch2* as a reporter of transcriptional responses
179 to Shh signaling (Figure S1B), we observe differential responses to Shh within the adaxial cell
180 population along the DV axis (Figure S1C). Midline adaxial cells display stable and relatively high levels
181 of *ptch2* expression, whilst near the margins of the notochord there is large cell-to-cell variation in *ptch2*
182 expression (Figure S1B and S1C). This variability may be due to restricted physical contact with the
183 notochord of such cells. To test this possibility further, we utilized low concentrations of the Shh pathway
184 inhibitor cyclopamine (CycA) to inhibit partially the response to Shh signaling. Elongation of adaxial
185 cells was delayed by 1 hour or 2 hours of CycA treatment (Figure S1D). Taken together, our data show
186 that a wave of slow muscle morphogenesis occurs from the midline to the dorsal and ventral sides of
187 each somite, mediated at least in part by differential Shh activity along the DV axis.

188 **MPs originate from midline adaxial cells without AP bias**
189
190 We identified MPs using the Tg(*eng2a:eGFP*) transgenic line and based on their distinctive shape and
191 location within the somite, and then tracked them back to their initial location within the PSM at S0 or
192 S-1. We found that adaxial cells initially located at either anterior and posterior positions within the
193 somite can take on a MP cell fate (Figure 2D-E, and Video S3). In order to locate the spatial origin of
194 MP cells, we constructed the MP cell fate map from 52 somites (for somite numbers from 8 to 25,
195 $n_{Embryos}$=16) (Figure 2F). Our data indicate that MPs originate from the DV midline but display no overall
196 AP bias in their provenance. To estimate the variability in the origin of MPs within somites, we quantified
197 the AP bias in each single somite (Figure 2G). Notably, 30% (16/52) of somites analyzed showed at
198 least a 0.5-fold difference in AP bias, with 15% (8/52) of somites displaying at least a 1-fold difference
199 between the anterior and posterior portions of the somite. Despite this significant variability in MP origin
200 between somites, there is, however, on average no AP bias in MP cell fate determination; this contrasts
201 with the conclusions of a previous study that reported an AP bias in MP specification (Nguyen-Chi et
202 al., 2012).
203
204 The MP fate map can be explained through the migration pattern induced by the muscle morphogenesis
205 wave along the DV axis. Anterior and posterior adaxial cells near the DV midline elongate and
206 intercalate with each other first, which also extrudes the dorsal and ventral adaxial cells further away
207 from the notochord (Figure 2E). Subsequently, the cells at the midline experience sustained Shh-
208 mediated activation and BMP-mediated inhibition, whereas notochord-derived signals are diminished
209 for the more dorsal and ventral adaxial cells (Figure S1E-G). In effect, sequential slow muscle
210 morphogenesis induces feedback into adaxial cell fate by clearly demarcating between cells that
211 continuously contact the notochord (both MPs and SSFs) and cells that migrate away from the
212 notochord (always SSFs) (Figure 1E).
213
214 **FGF signaling participates in further differentiation of both slow and fast muscle lineages**
215
216 *Fgf8a*, a key ligand of FGF signaling in zebrafish body axis patterning (Dorey and Amaya, 2010), is
217 expressed in the anterior border cells of immature somites (Groves, 2005). Perturbation of FGF
218 signaling has been shown to alter both fast and slow muscle cell fate (Groves, 2005; Nguyen-Chi et al.,
219 2012). However, it remains unclear how different types of muscle progenitors interpret and respond to
220 the FGF ligand. Using fluorescent *in situ* hybridization (FISH) combined with immunostaining of
221 Tg(*prdm1a:GFP*) embryos, we determined the response of distinct cell populations to the FGF ligand.
222 Consistent with previous observations, we find *fgf8a* to be expressed in a stripe of the anterior-most
223 cells in each somite (Figure S2A). The downstream activator of FGF signaling, *pea3*, displays
224 expression across the anterior region of each somite (Figure 3A), whilst another downstream activator,
225 *erm*, is expressed ubiquitously in lateral regions of each somite (Raible and Brand, 2001; Roehl and
226 Nüsslein-Volhard, 2001) (Figure 3B). These distinct patterns of *erm* and *pea3* expression are
227 suggestive of differential activation by FGF signaling. However, the expression of both *pea3* and *erm*
228 is barely detectable within the slow muscle lineage in the anterior PSM or in immature somites (Figure
229 3A'-3A'', 3B'-3B'' and S2B). By contrast, *sprouty4* (*spry4*), which encodes an intracellular inhibitor of
230 tyrosine kinases that antagonises FGF signaling (Fürthauer et al., 2001), is expressed within the slow
231 muscle lineage from stage S2 (Figure 3C-C'') but displays neither AP nor DV bias among slow muscle
232 cells (Figure 3C''' and Figure S2C). In all, the expression patterns of these FGF downstream activators

233 and inhibitor suggest that the FGF signaling pathway is differentially activated in slow and fast muscle
234 lineages.
235
236 Consistent with previous work, the initial separation between slow and fast muscle lineages is
237 independent of FGF signaling, as the number of slow muscle remains largely unperturbed under FGF
238 inhibition (Figure S2D). However, the further differentiation of both slow and fast muscle lineages is
239 subject to FGF perturbations (Groves, 2005; Nguyen-Chi et al., 2012; Reifers et al., 1998). To explore
240 the basis of this effect further, FGF signaling was modulated by: (i) a pharmacological inhibitor SU5402;
241 (ii) heat shock of Tg(*hsp70l:dn-fgfr1-eGFP*) embryos, which carry a heat shock inducible cDNA
242 encoding a dominant negative form of the FGF receptor 1 (*hs:dn-fgfr1*); and (iii) heat shock of
243 Tg(*hsp70l:ca-fgfr1*), a heat shock inducible construct encoding a constitutively active form of the FGF
244 receptor 1 (*hs:ca-fgfr1*). In the first two perturbations, in which FGF signaling is inhibited, ectopic MPs
245 (assayed by Tg(*eng2a:eGFP*) expression) were observed (Figure 3D-F and Figure 3H). In contrast, the
246 induction of constitutive FGF receptor activity resulted in significantly decreased numbers of MPs in
247 most muscle segments (Figure 3G-H), and a complete absence in some (3/40) (Figure S2E). We also
248 note that ectopic MPs were induced in the muscle segments that segmented soon after drug treatment,
249 and these muscle segments still had a stereotypic size (Figure S3A-B). This suggests that the induction
250 of ectopic MPs under FGF inhibition is not due to abnormally enlarged somites caused by the
251 perturbation (Sawada et al., 2001).
252
253 By the end of primary myogenesis, the ECL cells of the fast muscle lineage reside at the lateral surface
254 of the myotome and remain Pax7 positive (Figure 3I). In wild type embryos, there are around 30-40
255 ECL cells per muscle segment. Inhibition of the FGF pathway results in induction of an ectopic cell
256 population located laterally to the SSFs after their migration (Groves, 2005) (Figure 3J-K). This ectopic
257 lateral population displays a similar localization to the ECL cells in wild type embryos, as well as Pax7
258 expression. In contrast, over-activation of FGF signaling results in the loss of ECL cells (Figure 3L and
259 3M), and the SSFs migrate more laterally than in wild type embryos. Thus, FGF signaling determines
260 the further differentiation of slow and fast muscle lineages within similar timeframes.
261
262 **The direct and indirect roles of FGF signaling in the further differentiation of fast and slow**
263 **lineages respectively**
264
265 Though we do not observe *pea3* or *erm* activity in slow muscle fibers, this does not preclude the
266 possibility that these cells may respond to FGF signaling. To test this further, we utilized the UAS:GAL4
267 binary mis-expression system with the promoter of the *slow myosin heavy chain 1* (*smyhc1*) gene
268 (Elworthy et al., 2008), to drive expression of *dn-fgfr1* and *ca-fgfr1* exclusively in adaxial cells and slow
269 muscle fibers (Figure 4A-A’). The *dn-fgfr1* and *ca-fgfr1* were designed as previously described (Lee,
270 2005; Neilson and Friesel, 1996) and their activity verified by injecting the encoded mRNA into embryos
271 at the one cell stage (Figure S3C-D). Microinjection of the corresponding UAS plasmids into
272 Tg(*smyhc1:gal4*) embryos induced mosaic FGF perturbations in the slow muscle fibers identifiable
273 through mCherry expression (Figure 4B-C). Interestingly, both dn-fgfr1-p2a-mCherry positive and ca-
274 fgfr1-p2a-mCherry positive slow muscle cells display a similar fraction of MPs compared with that of
275 control, which only express mCherry (Figure 4D and Figure S3E). Therefore, neither selective inhibition
276 nor over-activation of FGF signaling in slow muscles had a significant effect on MP differentiation,
277 suggesting FGF signaling determines MP cell fate indirectly.
278
279 To investigate further the role of FGF signaling in fast muscle specification, we induced mosaic
280 expression of *dn-fgfr1* or *ca-fgfr1* using the heat shock promoter *hsp70l* (Figure 4E-F). Heat shock was
281 performed at the 16-somite stage to induce mosaic FGF inhibition or over-activation regardless of cell
282 type. We calculated the percentage of positive ECL cells among the total population of mCherry positive
283 cells in fast muscle lineages in muscle segments 16-22 at 30hpf - when primary myogenesis is
284 completed in the corresponding muscle segments. Cells positive for dn-fgfr1-mCherry displayed a
285 significantly larger fraction of ECL cells compared with the control, which only express mCherry under
286 *hsp70l* (Figure 4G and Figure S3F). In contrast, almost all the lateral somitic cells with cafgfr1-p2a-
287 mCherry expression committed to the fast muscle fate. Consistent with the previous UAS:GAL4 binary
288 mis-expression system, the cell fate decision between MPs and SSFs is not significantly changed at a
289 single cell level by either the mosaic expression of dn-fgfr1-mCherry or ca-fgfr1-p2a-mCherry (Figure
290 S3G) under the *hsp70l* promoter. We observed a reduction in the number of MPs in a subset of muscle
291 segments where only fast muscle progenitors are over-activated with ca-fgfr1-p2a-mCherry (1 or 2 MPs,
292 4 out of 32 segments), whilst all the wild type muscle segments analyzed contained 3 or more MPs (55

293 out of 55 segments) (Figure 4F). However, there is no significant difference in the average number of
294 MPs per muscle segment between them, possibly because only a small portion of fast muscles are
295 activated under the mosaic expression of ca-fgfr1-p2a-mCherry (Figure S3H). Taken together, these
296 results suggest FGF pathway activity determines the further differentiation of fast and slow muscle
297 lineages directly and indirectly respectively.
298
299 **A posterior to anterior wave of fast muscle elongation leads to somite rotation**
300
301 We next analyzed the ontogeny of fast muscle fibers using our four-dimensional dataset. The
302 development of fast muscle cells begins in the posterior of each somite around 180-220mins after its
303 formation. These posterior fast muscle progenitors elongate anteriorly and extend between slow
304 muscles in reaching the anterior somite border (Figure 5A). Concomitant with this initial phase of fast
305 muscle elongation, the SSFs are gradually displaced by the elongating fast muscle fibers and move
306 away from the notochord (Figure 5B-E). By following every cell in the lateral region of the somite, we
307 determined that fast fiber formation occurs sequentially as a wave along the AP axis, starting from the
308 posterior to anterior (Figure 5F-G). In addition to the posterior to anterior wave of fast muscle elongation,
309 fast muscle morphogenesis occurs from the medial to lateral sides of the somite (Figure 5B). Thus, the
310 most posterior cells elongate first and occupy the most medial position of myotome after their elongation
311 (Figure 5C). Cells initially located centrally within the somite elongate later and are positioned more
312 laterally within the myotome (Figure 5D). Finally, cells initially located in the anterior migrate eventually
313 migrate to the lateral surface of myotome and differentiate into dermomyotome (Figure 5E). This
314 sequential elongation of fast muscle progenitors induces an apparent somite rotation through 90º , as
315 previously reported (Hollway et al., 2007; Stellabotte et al., 2007).
316
317 **FGF signaling determines cell fate directly in the anterior of each somite**
318
319 To characterize fully the somite polarity in generating fast muscle fibers, we tracked backwards every
320 fast muscle progenitor starting from full elongation to its location in stage S2. As shown in Figure 5F,
321 the whole somite displays heterogeneous cell behaviors in the course of fast fiber differentiation along
322 the AP, DV and ML (Figure 5F(ii-iii)) axes. We next characterized the role of FGF signaling in specifying
323 cell fate along the AP axis. We quantified the timing of elongation of medial fast muscle progenitors
324 (Figure 5G) along the DV midline (rectangular region in Figure 5F(ii)). Inhibition of FGF activity
325 significantly delays muscle elongation (Figure 5G and H') compared to wildtype embryos (Figure 5G
326 and H) and induces ectopic ECL cells from the anterior half somite (Figure 5I and I'). In contrast, over-
327 activation of FGF activity leads to premature muscle elongation in the middle somite (Figure 5G, 5H'')
328 and ectopic fast muscle cells at the expense of ECL cells at anterior half somite (Figure 5I and I''). Thus,
329 the anterior border cells (ABCs) commit into dermomyotome as previously reported, whilst the rest of
330 cells in the anterior half somite correspond to the lateral fast muscle fibers, which have previously been
331 reported as sensitive to FGF inhibition (Groves, 2005; Hollway et al., 2007; Stellabotte et al., 2007).
332 Cell fate in the posterior somite is relatively independent of FGF activity, though a slight delay in
333 elongation was observed under FGF inhibition (Figure 5G).
334
335 **Tempo-spatial expression profiles of *eng2a* reveals the importance of lateral migration in MP**
336 **differentiation**
337
338 We used the Tg(*eng2a:eGFP*) line as a live reporter for the transcriptional activity of *eng*, enabling cell
339 fate determination of MPs to be studied at single cell resolution. Eng2a:eGFP expression in wild type
340 embryos was detected 100-150mins after the segmentation of the corresponding somite from the PSM
341 (Figure 6A-A'). Premature Eng2a:eGFP expression was observed in embryos exposed to dorsomorphin
342 (DM), an inhibitor of BMP and VEGF signalling (Figure 6B-B'). This observation is consistent with the
343 previous demonstration that BMP signaling acts as an upstream inhibitor of *eng* in slow muscle cells
344 (Maurya et al., 2011). However, no premature expression of Eng2a:eGFP was detected under FGF
345 inhibition, even when ectopic MPs were induced (Figure 6C-C'). On the contrary, an extended time
346 window of MP induction was observed, ranging from 100- 180mins after somite segmentation. Taken
347 together, these data suggest that FGF signaling is not directly involved in driving *eng* expression in slow
348 muscle fibers. Instead, the extended time window for MP induction might be a result of delayed
349 migration of slow muscle cells in response to FGF inhibition.
350
351 A portion of SSFs display Eng2a:eGFP expression (denoted as Eng2a:eGFP+ SSFs) (Nguyen-Chi et
352 al., 2012). To test the fidelity of *eng* expression in the Eng2a:eGFP+ SSFs, we stained Tg(*eng2a:eGFP*)

353 embryos with anti-Eng antibody 4D9, (Figure S4A-A'). The Eng2a:eGFP+ SSFs showed stronger 4D9
354 intensity than SSFs without Eng2a:eGFP expression, but consistently lower intensity levels than in MPs
355 (Figure S4B). Of the 126 Eng2a:eGFP positive slow muscle fibers tracked, 20 had a SSF cell fate, with
356 the rest becoming MPs. The 20 Eng2a:eGFP+ SSFs migrated to the lateral surface of the myotome,
357 and resembled the rest of the SSFs in terms of cell morphology and migration (Figure 6D and Figure
358 S4C). Further, the intensity of Eng2a:eGFP in these cells became weaker compared with that of MPs
359 after they migrated away from the notochord. In contrast, the MPs, which remain in contact with the
360 notochord, displayed increasing GFP intensity. Perturbations to FGF signaling resulted in changes to
361 both MPs and Eng2a:eGFP+ SSFs populations, though with opposite trends. As shown above, FGF
362 inhibition results in increased numbers of MPs. However, under these conditions we also observed a
363 smaller fraction of Eng2a:eGFP+ SSFs compared to unperturbed embryos (Figure 6E and Figure 6G-
364 G'). Conversely, over-activation of FGF signaling led to an increase in the number of Eng2a:eGFP+
365 SSFs (Figure 6F-G, Figure S4D and Video S4), with more Eng2a:eGFP+ SSFs than MPs (2.1±0.9
366 compared with 1.7±0.5, $n_{Somites}$=28, p-value=0.05). Therefore, *eng* expression alone is insufficient to
367 determine MP cell fate; FGF signaling is also required to segregate properly between SSFs and MPs.
368 As shown in Figure 5A, the lateral migration of slow muscles coincides with the elongation of fast muscle
369 progenitors. Thus, FGF signaling likely determines the timing of SSF migration by modulating the
370 morphogenesis of fast muscle progenitors.
371
372 **FGF signaling controls the timing, velocity, and range of lateral migration**
373
374 We used our cell tracks to explore the role of FGF signaling on muscle fiber migration (Figure 6H-J and
375 Video S5). To quantify the timing of lateral migration of SSFs, the initial breaking of the slow muscle
376 monolayer integrity was used to denote the start of migration, (Figure 5A(v-vi) and Figure 6H(i)). In wild
377 type embryos, SSFs started to migrate around 200mins after somite segmentation (Figure 6H and
378 Figure 6K). Under FGF signaling pathway inhibition (SU5402 (Figure 6I) or *hs:dn-fgfr1* (Figure S5A),
379 the start of lateral migration was significantly delayed (Figure 6K). This observation indicates that FGF
380 signaling is necessary for the proper lateral migration of SSFs. However, another possibility is that the
381 ectopic MPs induced by FGF inhibition obstruct the lateral migration of the remaining SSFs. To test this,
382 we quantified the initiation time of lateral migration in DM treated embryos and found it to resemble that
383 in WT embryos, despite the presence of ectopic MPs (Figure 6K). Likewise, over-activation of FGF
384 signaling did not change the initiation of lateral migration (Figure 6K).
385
386 To measure the changes in position of fast muscle fibers and SSFs during migration, we quantified the
387 size of the area lateral to the migrating SSFs from the transverse somite (muscle segments) aspect. To
388 account for variability in somite size, we normalized each measurement by dividing it by the initial size
389 of the lateral area before migration. As fast muscle cells exchange position with SSFs, the size of the
390 area lateral to the migrating SSFs gradually decreases (Figure 6L). In WT embryos, this area began to
391 decrease around 180mins after somite segmentation and stabilized at about 50% of its initial size about
392 240mins later. Inhibition or over-activation of FGF signaling significantly changed the timing and velocity
393 of lateral migration of slow muscle cells. Under SU5402 treatment, the lateral area decreased more
394 slowly and had a larger final size (around 30% larger than that of wild type embryos, p-value<0.001),
395 suggesting that both the velocity and range of SSF migration is compromised by FGF inhibition. The
396 larger lateral area is populated by ectopic ECL cells under FGF inhibition (Figure 3J-K). In contrast,
397 over-activation of FGF activity speeds up the lateral migration of SSFs compared to unperturbed
398 embryos (Figure 6L). Taken together, these data indicate that FGF signaling influences the timing,
399 velocity and range of SSF migration.
400
401 **Lateral migration of SSFs depends on fast muscle myogenesis**
402 The above results show that FGF signaling determines the timing and velocity of slow muscle migration
403 and their final position within the myotome by modulating the process of fast myogenesis and the
404 segregation between fast muscles and ECL cells. We confirmed that SSFs selectively inhibited for FGF
405 pathway activity displayed no obvious defects in their lateral migration (Figure S5B). Therefore, the
406 lateral migration of SSFs appears to require participation of the neighboring fast muscle progenitors.
407 To test this conclusion further, we took advantage of the observation that fast muscle fiber specification
408 is disrupted by perturbation of Ripply1 function. Together with Tbx6 and Mesp-b, Ripply1 regulates
409 somite boundary formation and fast muscle myogenesis (van Eeden et al., 1996; Windner et al., 2015).
410 Injection of a morpholino oligonucleotide targeting the *ripply1* mRNA, resulted in the complete absence
411 of fast muscle myogenesis and the stalling of adaxial migration such that all the slow muscle fibers
412 remained at the medial surface of each muscle segment (Figure 7A-B) with the concomitant induction

of ectopic MPs (Figure 7A'-B'). In contrast, the migration of SSFs remains largely unperturbed in *tbx6*$^{-/-}$ *(fused-somite)* mutants, in which somite boundaries, but not cell fates, are disrupted (Figure 7C-C'). In both of these experiments, our cell tracking suggests that the posterior to anterior wave of muscle elongation within each somite is lost (Figure 7D). The fast muscle progenitors in *tbx6*$^{-/-}$ mutants sequentially elongate from the anterior to posterior along the AP body. In contrast, there is no fast muscle progenitor elongation in the Ripply1 morphant (Figure 7D). This observation further supports our hypothesis that fast muscle myogenesis is required for migration of SSFs and robust MP differentiation.

SSF migration is necessary for complete fast muscle myogenesis

We next addressed whether SSFs play a role in fast fiber myogenesis. The formation of fast muscle fibers is significantly delayed in *smo*$^{-/-}$ (*smoothened*) mutants, which lack Hh pathway activity and consequently, all slow muscle fibers (Figure 7E) (Barresi et al., 2000). We noticed that AP polarized sequential fast muscle elongation still occurs in *smo*$^{-/-}$ mutants, suggesting the AP polarity of fast muscle progenitors within each somite is independent of Shh signaling and the population of slow muscle cells. We next analyzed *prdm1a*$^{-/-}$ *(*U boot) mutants, in which the development of slow muscle fibers is aborted before the onset of their lateral migration (Roy et al., 2001). Fast muscle elongation is significantly delayed in the *prdm1a*$^{-/-}$ mutant (around 1-3 hours of delay), depending on the AP position within the somite compared with the wild type embryo (Figure 7E). These observations indicate that the migration of slow muscles promotes fast muscle myogenesis reciprocally, consistent with the finding that transplanted wild type slow muscles can rescue fast fiber myogenesis in *smo*$^{-/-}$ mutants (Henry and Amacher, 2004).

From examination of our live movies, we noticed that fast muscle progenitors undergo cell-cell fusion between migrating slow muscle fibers (Figure 7F(iii-v)). After the cell-cell fusion, the multinucleated fast muscle fibers further expand and eventually fill the space vacated by the migrating SSFs (Figure 7F). We hence hypothesized that the migrating SSFs play an important role in shaping fast muscle fibers. To test this idea, we compared the patterning of fast muscle fibers between *prdm1a*$^{-/-}$ and wild type embryos (Figure 7G-H). The fast muscle fibers in *prdm1a*$^{-/-}$ mutants are not only smaller (Figure 7I), but also display much larger cell-to-cell variations (Figure 7I'). Therefore, the migration of SSFs appears to play important roles in controlling the timing of fast fiber myogenesis and in size control of fast muscle fibers.

DISCUSSION

By utilizing a multi-dimensional whole-somite imaging approach, we have monitored cell morphogenesis, cell migration, and relevant spatio-temporal gene expression profiles throughout the differentiation of cells within the developing somite. The formation of the myotome and dermomyotome are underpinned by a series of highly organized and directional cell morphogenesis and cell migration events. These highly orchestrated cellular behaviors ensure robust cell fate determination and somite compartmentalization. The sequential wave of slow muscle morphogenesis determines the cell fate of adaxial cells by positioning them at different DV locations. The midline adaxial cells remain attached to the notochord, thereby continuing to receive the highest levels of Shh input and eventually become MPs. The sequential elongation of fast muscle progenitors along the AP axis within each somite rotates almost all the cells through 90º, transforming the AP polarity of the somite into a ML polarity, and positioning ECL cells to the lateral surface of the somite. This tightly coordinated series of cell movements and rearrangements ensures spatial segregation of different cell fates along the DV axis (MPs, SSFs, and non-muscle progenitors) and the AP axis (ECL cells and fast muscle fibers). Another interesting point is that our results suggest that commitment to the MP fate depends upon a finely balanced competition between migration and signaling activation (e.g. of Eng). This concept may well be applicable to other developmental processes in ensuring the proper partitioning of cell fate. For example, in the self-renewal of germline stem cells in the *Drosophila* ovary, different levels of E-cadherin can stimulate their competition for niche occupancy, thus eventually determining distinct cell fates (Jin et al., 2008; Ting, 2013).

An apparent rotation of the somites has previously been observed based on single cell labelling and time-lapse imaging of somitogenesis in the coronal plane (Hollway et al., 2007; Stellabotte et al., 2007). Two models for the mechanism of the somite rearrangement have been proposed. (1) The whole-somite rotation is induced by the directional and coherent movement of most or all of the somitic cells

473 under control by the Sdf family of secreted cytokines (Hollway et al., 2007). (2) The rearrangement of
474 somitic cells is due to the active lateral migration of slow muscles and/or the active morphogenesis of
475 the posterior fast muscle progenitors (Stellabotte et al., 2007). In this study, we systematically explored
476 cell morphogenesis and cell migration by reconstructing the developing somite in 4D. Consistent with
477 Stellabotte *et al.*, we find that the earliest elongating fast muscles originate from the posterior-most
478 region of each somite and occupy the most medial position of the myotome (Stellabotte et al., 2007).
479 The ABCs eventually move to the lateral surface of the myotome after the primary myogenesis. Our 4D
480 cell tracking data further reveals a polarization of the somite along its AP axis in terms of the timing of
481 fast muscle elongation, which leads to the buildup of the myotome sequentially from the posterior fast
482 muscle progenitors to the anterior. This induces a rotation of most somitic cells through around 90º
483 (Figure S6A). However, the apparent somite rotation starts soon after somite segmentation, a time
484 earlier than the elongation of fast muscle progenitors and the lateral migration of slow muscles,
485 consistent with the report of Hollway et al., 2007. This observation can be explained by the dynamic
486 cell shape changes of slow muscles and fast muscle progenitors prior to the slow muscle migration.
487 The slow muscles remain comparatively flat along the medial surface of the somite at stages S1 and
488 S2 (Figure S6B-B'). Soon after stage S2, the posterior fast muscle progenitors move toward the medial
489 direction and start to display a sawtooth pattern along the posterior somite border (Figure S6C)
490 (McMillen et al., 2016). Almost simultaneously, the slow muscles extend laterally in the anterior half
491 somite (Figure S6C'). Thus, the early cell shape changes of slow muscles and the movement of lateral
492 cells appear to initiate a whole somite rearrangement prior to slow muscle migration. This distinctive
493 sawtooth shape of the posterior fast muscle progenitors is thought to depend on Cadherin-2 (McMillen
494 et al., 2016).
495
496 One way to distinguish between the two models is to map the relative movement of neighboring somitic
497 cells throughout the rearrangement (Stellabotte and Devoto, 2007). Whole-somite rearrangement
498 should largely preserve the cellular neighbor relationships, whilst dynamic cell rearrangements should
499 significantly change the relative positions of the somitic cells. In this study, we quantified the neighbor
500 changes of lateral somitic cells during somite rotation by tracking neighboring cells (Figure S6D-F).
501 Interestingly, cells located in the anterior-lateral region collectively moved towards the posterior
502 direction, with few neighbour changes throughout somite rearrangement (Figure S6D). In contrast,
503 dynamic cell rearrangements were observed in the middle and posterior regions of the somite shortly
504 before their elongation (Figure S6E-F). During muscle elongation, the posterior somitic cells appear to
505 elongate actively and move in an anterior direction (Figure S6F). Thus, cell motility varies both across
506 the somite and stages. The apparent rotation of the somite is due to dynamic cell rearrangement and
507 active morphogenesis of the fast muscle progenitors and the coherent migration of the dermomyotome
508 cells. The somite appears to rotate largely through the rearrangement cells with most of the cells rotated
509 through varying angles in a less cohesive manner (Figure S6A).
510
511 Partial or total loss of slow muscles affects the timing of fast muscle elongation. Yet, the temporal wave
512 of fast muscle elongation can still be found within the *smo*$^{-/-}$ and *prdm1a*$^{-/-}$ mutants, indicating the somite
513 can still 'rotate' even in the absence of slow muscles (Figure 7E). SDF signaling has been implicated in
514 dermomyotome formation and possibly the somite rotation (Hollway et al., 2007). However, it remains
515 largely unclear whether the Sdf ligand directly induces the directional movement of dermomyotome
516 progenitors or fast muscle progenitors as a cytokine. Another possibility is that SDF signaling is involved
517 in the cell fate segregation of dermomyotome and fast muscles, given the fact that knockdown of SDF1a
518 or CXCR4b inhibit early myogenesis in Zebrafish (Chong et al., 2007). Further study is required to
519 compare and distinguish the two scenarios. Above all, we believe the apparent somite rotation is
520 underpinned by the sequential cell shape changes and muscle elongation from the posterior cells to
521 anterior, potentially under the control of the somite AP polarity genes including *ripply1* and *mesp-b*
522 (Sawada et al., 2000; Windner et al., 2015) (Figure 7D).
523
524 Here, we have focused predominantly on myogenic cell fates of skeletal muscles. However, distinct
525 origins of non-muscle progenitors have been revealed by our single cell tracks and further tested with
526 photo-conversion of the photo-convertible protein Kaede (Figure S7). There appear to be three domains
527 of non-muscle progenitors located at the dorsal margin, lateral ventral margin, and medial ventral
528 margin of each somite. Due to the time limitations of the live imaging, we were unable to identify the
529 future cell fate of these groups of cells, though we have been able to follow their distinct migratory
530 routes away from the somite. It will be interesting to track these cells from early somite stages through
531 to their final location within the developing embryo. Light-sheet microscopy should enable such long-
532 term imaging with sufficient spatial resolution to perform such analyses.

533
534 In this study, we have revealed the framework for somite patterning of the posterior trunk (somite 9-15)
535 and tail (somite 16-30) somites. However, the anterior trunk somites (somite 1-8) differ from their
536 posterior trunk and tail counterparts both in their embryonic origin and the molecular players underlying
537 their segmentation (Holley, 2006; Row et al., 2016; Yin and Solnica-Krezel, 2007). In contrast to the
538 tailbud-derived posterior trunk and tail somites, the anterior trunk somites are generated from the
539 mesoderm during gastrulation. In addition, complementary genetic networks have been observed, with
540 Integrin-α5 and Notch dominating the boundary formation of the anterior and posterior trunk somites
541 respectively (Jülich et al., 2005). In contrast, the establishment of somite AP (rostro-caudal R-C) polarity
542 appears independent of somite position. The somite AP polarity genes *mesp-b* and *ripply1* are
543 expressed in the rostral and the caudal regions of somites respectively from somite 1 to somite 15
544 (Lackner et al., 2013; Sawada et al., 2000; Windner et al., 2015; Yabe et al., 2016). Thus, the sequential
545 waves of fast muscle myogenesis are likely to occur regardless of the somite position. The DV polarity
546 within the adaxial cell population is likely established in both of the trunk and tail somites, since the
547 notochord provides essential stimuli to the somites in these regions. Though the general framework for
548 patterning skeletal muscles is expected to be largely similar in different somites, the induction of the
549 myogenic and non-muscle progenitors of the limbs and fins is reliant on the position of an individual
550 somite (Haines, 2004; Masselink et al., 2016). Further study is required to compare the differences
551 between the trunk and tail somites in somitic cell differentiation and migration, especially for the
552 progenitors of limbs and fins that underlay the body plan.
553
554 Cell fate decisions in response to Shh signaling have been shown to depend not just upon how strong
555 the Shh input is, but also for how long the signal is received (Balaskas et al., 2012; Dessaud et al.,
556 2007; Ribes and Briscoe, 2009). However, such a mechanism implies tight temporal – and not just
557 spatial – regulation of morphogen signaling. In this study, we have shown that carefully timed cell
558 migration can effectively act as a timer to “switch off” Shh signaling in SSFs through spatial
559 rearrangements with fast muscle fibers. FGF effectively controls slow muscle cell fate by (indirectly)
560 regulating the duration of Shh pathway activation and BMP pathway inhibition in slow muscle cells
561 (Figure 7J-J’). In effect, FGF signaling influences slow muscle cell fate non-autonomously, through
562 controlling the timing, velocity and range of the migration of SSFs. In systems with multiple
563 morphogenetic inputs, each morphogen may not always be acting to provide spatial information, but
564 may also contribute temporal information that plays a role in the interpretation of other morphogenetic
565 inputs. This insight opens up new avenues for understanding the spatio-temporal regulation of
566 morphogenesis in complex three-dimensional tissues.
567
568 In this study, we have systematically explored the interactions between cells of the slow and fast muscle
569 lineages. Previous work (Henry and Amacher 2004) characterized the role of slow muscle migration in
570 driving fast muscle myogenesis. Our results significantly extend these conclusions by providing
571 evidence that the interactions between fast and slow muscle lineages are indeed reciprocal to each
572 other in regulating differentiation, morphogenesis and migration. Our results also broaden our
573 understanding of the diverse roles of FGF signaling in AP patterning of the somite. Lateral somitic cells
574 display a varying dependency upon FGF signaling along the AP axis in determining their behaviors and
575 cell fates. Importantly, we are able to demonstrate how the meticulous tuning of FGF activity is key to
576 the proper segregation between dermomyotome (Hollway et al., 2007; Stellabotte et al., 2007) and
577 lateral fast muscle fibers (Groves, 2005), which occurs in the anterior half somite. In contrast, no AP
578 bias was found in slow muscle lineages in the muscle elongation and cell fate determination.
579
580 There are several open questions that arise from our study. Even though we have determined that fast
581 muscle myogenesis is closely coupled with slow muscle migration and MP differentiation, the molecular
582 mechanisms underlying the interaction between the two muscle lineages remains largely unknown.
583 Reciprocal expression waves of M-Cadherin (M-Cad) and N-Cadherin (N-Cad) have previously been
584 implicated in the lateral migration of slow muscles (Cortés et al., 2003). The change in the adhesive
585 environment during fast muscle myogenesis may help to drive migration of SSFs. It will be interesting
586 to image such changes in living embryos using reporters such as the *TgBAC(cdh2:cdh2-sfGFP-*
587 *TagRFP)* (Revenu et al., 2014). However, perturbation of M- or N-Cad expression only induces limited
588 defects on the lateral migration of SSFs (Cortés et al., 2003; Ono et al., 2015). This suggests that other
589 mechanisms might be involved into the lateral migration of SSFs. It is also not clear if (and if so, how)
590 the notochord restricts the migration of MPs. Notably, *itga5*, a molecule implicated in the cell-matrix
591 junction, is expressed exclusively in MPs after lateral migration (Thisse et al., 2001). Further study is

needed to reveal the possible participation of cell-matrix junctions between the myotome-notochord interface.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, and five movies and can be found online with this article.

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AUTHOR CONTRIBUTIONS

PWI, TES and JY conceived and designed the study. JY performed experiments with support from RL and YO. JY performed image analysis and cell tracking with assistance from TES. JY, PWI and TES interpreted the data and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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792

793 **Figure Legends**
794
795 **Figure 1: The differentiation and migration of somitic cells.**
796 (A) Schematic diagram of the differentiation of somitic cells and the further differentiation of slow and
797 fast muscle lineages. MPs: Muscle Pioneers. SSFs: Superficial Slow Muscle Fiber. ECL cells: external
798 cell layer cells. (B-D). Schematic representation of the localization of various cell types at somite stage
799 S0 (B), S3 (C) and the muscle segments around 10 hours after somite segmentation after primary
800 myogenesis (D) at transverse view, using same color coding for cell fate as in (A). (E): 3D representation
801 of the slow muscle migration and differentiation throughout primary myogenesis. Red and blue denote
802 cells with MP and SSF cell fates respectively. (F): The temporal-spatial expression profile of
803 Eng2a:eGFP in (E), normalized to the maximum Eng2a:eGFP expression in the last time point.
804
805 **Figure 2: Morphogenesis of adaxial cells and the determination of MPs from DV midline**
806 (A)Time-lapse of the muscle morphogenesis of adaxial cells from segmentation (i) to 140mins after
807 segmentation (viii). Anterior to the left and dorsal to the top in all parasagittal views unless otherwise
808 stated. Timing refers to the relative time after the formation of corresponding somite in all figures unless
809 otherwise stated. White brackets label fully elongated adaxial cells. White dash lines denote to somite
810 boundaries unless otherwise stated. Images are taken at somite 18-20. (B) The timing of elongation of
811 each individual adaxial cell at different positions relative to DV midline (n_{Cells}=135, from total of 8 somites
812 taken from 8 embryos), ***p < 0.001, Student's t test). (C-C'') Mapping of all slow muscles from four
813 contiguous somites back to the PSM with colors denoting distinct cell fates (C), timing of elongation
814 relative to the segmentation of somite S0 (C') or relative to the segmentation of each somite (C''). Black
815 dash lines label the position of notochord. White asterisks in (C) label the adaxial cells lying on the
816 dorsal or ventral margin of the notochord. (D) Time-lapse of MP differentiation with the live MP cell fate
817 marker eng2a:GFP from PSM (i) to 290mins later (ix). Images are taken at somite 19-20. (E) 2D
818 representation of the adaxial cell rearrangement during slow muscle myogenesis. The varying colors in
819 each track denote the corresponding time relative to the somite segmentation. Black circles and
820 squares denote to the initial positions of future SSFs and MPs respectively. (F) Probability map of MP
821 initial position constructed from adaxial cell positions in S0 ($n_{Somites}$=52, $n_{Embryos}$=16). Left, right, top and
822 bottom refer to anterior, posterior, dorsal and ventral side of adaxial cell population. (G) Distribution of
823 AP-Bias of the quantified 52 somite. (i) The AP-Bias in each somite is defined by
824 $\log(\sum area(A)/\sum area(P))$. $\sum area(A)$ and $\sum area(P)$ denote the area taken by the future MPs anteriorly and
825 posteriorly in the somite respectively.
826
827 **Figure 3: FGF signaling participates in the further differentiation of both slow and fast muscle**
828 **lineages**
829 (A-C) Fluorescent *in situ* of FGF downstream activators *pea3* (A-A'') and *erm* (B-B'') and inhibitor *spry4*
830 (C-C''). Images are taken at the lateral somitic cells (A-C) and the slow muscle fibers (A'-C') at 18-
831 somite stage. Constructed transverse images are taken at somite S3 and are displayed in (A''-C'') with
832 lateral to the top and dorsal to the left. Slow muscles are labeled with Prdm1a:GFP and white short
833 arrows. (C''') *Spry4* expression level per cell along the DV axis. The expression of *spry4* is measured
834 cell by cell by calculating the average intensity within the nucleus (n_{Cells}=153, from total of 9 somites
835 taken from 5 embryos, ^{NS}P >0.05, Student's t test). (D-G): Perturbations of FGF signaling change the
836 number of MPs with MPs identified by Eng2a:eGFP expression. Wild type embryo (D), SU5402
837 treatment at 60μM (E), heat shock of hsp70l:dn-fgfr1-eGFP (F) and heat shock of hsp70l:ca-fgfr1 (G)
838 analyzed, all imaged at 28hpf at muscle segments 16-20, with quantification of MP number shown in
839 (H), where $n_{Segments}$=55, 60, 40, 40 (from 11, 12, 10, 10 embryos) for conditions (D-G) respectively. (I-
840 L) Perturbations of FGF signaling change the number of ECL cells, with ECL cells identified by Pax7
841 antibody staining. White asterisks label ECL cells with moderate Pax7 intensity. The much brighter cells
842 labelled with white short arrows are neural crest cells. Wild type embryo (I, I'), SU5402 treatment at
843 60μM (J, J'), heat shock of hsp70l:dn-fgfr1 (K, K') and heat shock of hsp70l:ca-fgfr1 (L, L') analyzed at
844 28hpf at muscle segments 16-20. Transverse views shown in (I-L) with lateral to the top and dorsal to
845 the left. (M) Quantification of ECL cell number, where $n_{Segments}$=16, 21, 15, 19 (from 6 embryos in each
846 condition) for conditions (I-L) respectively. ***p < 0.001, Student's t test.
847
848 **Figure 4: The direct and indirect roles of FGF signaling in the further differentiation of fast and**
849 **slow lineages respectively**
850 (A-D) Mosaic FGF perturbations in slow muscle cells driven by smyhc1:gal4;UAS:dn-fgfr1-p2a-mCherry
851 (A) or smyhc1:gal4;UAS:ca-fgfr1-p2a-mCherry (A') respectively. (B-C) Mosaic FGF inhibition (B) or
852 over-activation (C) in the slow muscle fibers is identifiable through mCherry expression. Images are

853 taken at 28hpf at muscle segments 13-16 and projected along ML axis. (B'-C') Transverse views of (B-
854 C) made at the sites of dash lines with lateral to the left and dorsal to the top. (D) Fraction of mCherry
855 positive MPs among mCherry positive slow muscles in control group (60/296), FGF inhibited group
856 (52/262) and FGF over-activated group (45/214). (E-G) Mosaic FGF perturbations regardless of cell
857 types with the expression of dn-fgfr1-mCherry (E) or ca-fgfr1-p2a-mCherrry (F) driven by heat shock
858 promoter hsp70l. The perturbed cells are identifiable through membrane localized dn-fgfr1-mCherry (E)
859 or uniformly distributed mCherry respectively (F) and labelled with white short arrows. (E'-F')
860 Transverse views of (E-F) made at the sites of dash lines with lateral to the left and dorsal to the top.
861 (G) Fraction of mCherry positive ECL cells among the total population of mCherry positive cells in fast
862 muscle lineage (both fast muscle fibers and ECL cells) in control group (104/495), FGF inhibited group
863 (373/777) and FGF over-activated group (41/696). Heat shock was performed at 16-somite stage. The
864 quantification of cell fates was performed at muscle segments 16-22 at 30hpf.
865
866 **Figure 5: AP polarity of fast muscle myogenesis and the roles of FGF signaling in the AP**
867 **patterning**
868 (A) Time-lapse of the earliest fast muscle elongation and the displacement of slow muscles. Yellow,
869 white, blue and magenta labels the contours of elongating fast muscle progenitors. Slow muscles are
870 labeled with Prdm1a:GFP. (B-E) Time-lapse of the cell rearrangement and differentiation of lateral
871 somitic cells throughout the primary myogenesis at coronal view (B) with lateral to the top and anterior
872 to the left and parasagittal optical views (C-E). Yellow, white and blue circles label cells from posterior
873 (C), central (D) and anterior (E) part of somite S2. In C-E, the z-plane selected corresponds to the
874 position of the center of each cell being tracked (corresponding z-position is clear in B). White arrows
875 label the elongating fast muscle progenitors. (F) Maps of the timing of fast muscle elongation
876 constructed at somite stage S2 (i) at parasagittal planes 20 µm (ii) or 36 (iii) µm away from the notochord.
877 Cells labeled with gray color did not elongate throughout the primary myogenesis. (G) The timing of
878 elongation of fast muscle progenitors quantified from the rectangle region of 5F(ii) along the AP axis in
879 wild type embryo (blue, n_{Cells}=57 from total of 7 somites taken from 6 embryos), embryos under heat
880 shock of hsp70l:dn-fgfr1-eGFP (green, n_{Cells}=35 from total of 6 somites taken from 5 embryos) or heat
881 shock of hsp70l:ca-fgfr1 (red, n_{Cells}=45 from total of 6 somites taken from 5 embryos). The ellipse
882 represents the minimum-volume covering of data points in each group. (H-I) Maps of timing of fast
883 muscle elongation (H-H'') and cell fates (I-I'') at somite stage S2 in in wild type embryo (H and I),
884 embryos under heat shock of hsp70l:dn-fgfr1-eGFP (H' and I') or heat shock of hsp70l:ca-fgfr1 (H'' and
885 I''). Images above are taken at somite 16-18.
886
887 **Figure 6: FGF signaling determines MP cell fate by regulating slow muscle migration**
888 (A-C) Time lapses of Eng2a:eGFP expression during MP differentiation in wild type embryos (A),
889 embryos under treatment of dorsomorphin (DM) at 50µM (B) or SU5402 at 60µM (C). White short
890 arrows denote the onset of Eng2a:eGFP expression that can be identified. (A'-C') The corresponding
891 temporal expression profile of Eng2a:eGFP for individual MP under same conditions of (A-C). All the
892 MPs in each group are sorted along the Y-axis according to the timing of initial Eng2a:eGFP expression.
893 The color map represent differential intensity of Eng2a:eGFP for each cell. White and red lines label
894 the start and end of the time window for the onset of Eng2a:eGFP expression. (A') (n_{Cells}=22 from total
895 of 6 somites taken from 5 embryos); (B') (n_{Cells}=28 from total of 6 somites taken from 4 embryos);
896 (C')(n_{Cells}=34 from total of 7 somites taken from 5 embryos). (D-F) Time lapses of Eng2a:eGFP
897 expression in MPs and Eng2a:eGFP+ SSFs in wild type embryos (D), embryos under treatment of
898 SU5402 at 60µM (E) and heat shock of hsp70l:ca-fgfr1 (F). White short arrows label Eng2a:eGFP+
899 SSFs. (G-G') The number of MPs and Eng2a:eGFP+ SSFs (G') per muscle segments in conditions of
900 (D-F) ($n_{Segments}$=31, 22, 28, $n_{Embryos}$=7, 7, 5, ***$p < 0.001$, Student's t test). (H-J): Time lapses of slow
901 muscle migration in wild type embryos (H), embryos under treatment of SU5402 at 60µM (I) or heat
902 shock of hsp70l:ca-fgfr1 (J). Slow muscles are identified by the expression of Prdm1a:GFP. (H'-J') The
903 reconstructed transverse view of (H-J) with medial to the left and dorsal to the top. White arrows denote
904 the breaking of the slow muscle monolayer by fast muscle elongation. Dash lines label the area at the
905 lateral side of slow muscles. White asterisk in (I'(III)) denotes the ectopic ECL cells under SU5402
906 treatment. (K) Timing of the initiation of lateral migration in wild type embryos ($n_{Somites}$=15 from 5
907 embryos), embryos under dorsomorphin (DM) treatment at 50µM ($n_{Somites}$=12 from 5 embryos)), heat
908 shock of hsp70l:ca-fgfr1 ($n_{Somites}$=12 from 5 embryos), SU5402 treatment at 60µM ($n_{Somites}$=15 from 5
909 embryos) and heat shock of hsp70l:dn-fgfr1-eGFP ($n_{Somites}$=15 from 5 embryos) (***$p < 0.001$, $^{NS}P > 0.05$,
910 Student's t test). (L) The relative size of lateral area (labelled with dash lines in Figure 6H-J) throughout
911 the lateral migration of SSFs. Blue, green and red denote the profiles under conditions of (H-J) (total of

912 5 somites from 5 separate embryos in each condition). ***p < 0.001, Student's t test. Images taken at
913 muscle segments 16-18.
914
915 **Figure 7: Reciprocal interactions of fast and slow muscle fibers guides their differentiation**
916 (A-C) The lateral migration of SSFs (A-C) and MPs specification (A'-C') in wild type embryos (A),
917 Ripply1 morphants (B) and *tbx6*$^{-/-}$ mutants (C). Slow muscles are either labeled with Prdm1a:GFP (A,
918 B) or Smyhc1:lyn_eGFP (C). MPs are either labeled with Eng2a:eGFP (A', B') or 4D9 antibody (C').
919 Images are taken at muscle segments 12-16 at 30hpf. (D) The timing of elongation of individual fast
920 muscle progenitors along the DV midline from a region corresponding to four adjacent somites. Shaded
921 regions represent individual somites in wild type embryos. Blue, orange and violet dots denote cells
922 from WT embryos ($n_{Embryos}$=5), Ripply1 morphants ($n_{Embryos}$=5) and *tbx6*$^{-/-}$ mutants ($n_{Embryos}$=5)
923 respectively. Cells with ECL cell fate are placed at the top of the panel. (E) AP dependence of the timing
924 of elongation relative to somite segmentation of individual fast muscle progenitors along the DV midline
925 in wild type embryos (n_{Cells}=57 from total of 7 somites taken from 6 embryos), *prdm1a*$^{-/-}$ (n_{Cells}=70 from
926 total of 6 somites taken from 6 different embryos) and *smo*$^{-/-}$ mutants (n_{Cells}=42, from total of 4 somites
927 taken from 4 different embryos). The ellipse represents the minimum-volume covering of data points in
928 each group. (F) Time-lapse of the patterning of fast muscle fibers in WT embryos at muscle segments
929 16-18. The fusing fast muscle progenitors are labeled with blue and yellow asterisks. White short arrows
930 label the dissolving membrane between the fusing cells. The intercalated slow and fast muscles are
931 labeled with long white arrows. (G-H) Parasagittal optical view of wild type embryos and *prdm1a*$^{-/-}$
932 mutant labelled with membrane marker lyn-td tomato at muscle segments 12-15. Transverse views of
933 (G-H) are displayed in (G'-H') with dorsal to the left and lateral to the top and manually segmented in
934 (G''-H''). (I) Distribution of the transverse size of fast muscle fibers in wild type embryo (n_{Cells}=1246 from
935 total of 15 muscle segments taken from 8 embryos) and *prdm1a*$^{-/-}$ mutant (n_{Cells}=1126 from total of 12
936 muscle segments taken from 6 embryos) (***p < 0.001, Student's t test). (I') Probability density of (I).
937 (J) Schematic presentation of how FGF signaling regulates MPs differentiation in wild type embryos by
938 precisely controlling the timing of SSFs migration and hence their exposure to Shh and BMP signaling.
939 (J') In the absence of FGF signaling, ectopic MPs are induced not only at the expense of *eng*+ SSFs,
940 but also in an extended time window due to the delay of lateral migration.
941
942

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Timothy E Saunders (dbsste@nus.edu.sg).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Adult fish were maintained on a 14h light/10h dark cycle at 28°C in the Agri-Food and Veterinary Authority (AVA) of Singapore certificated Institute of Molecular and Cell Biology (IMCB) Zebrafish Facility (Biopolis, Singapore). Wild type and transgenic zebrafish embryos were obtained by crossing male and female adults aged 3-18 months and raised at 28 °C. Embryos were staged by hours post-fertilization (hpf) at 28 °C and/or morphological features (Kimmel et al., 1995). All experiments with zebrafish embryos were approved by the Singapore National Advisory on Laboratory Animal Research and performed under the A*STAR Biological Resource Centre IACUC project #090434.

Previously described transgenic Zebrafish lines were used as follows: Tg(*PACprdm1:GFP*)^{i106} (Elworthy et al., 2008) and *Tg(-10en2a:EGFP)*^{i233} (Maurya et al., 2011) for the labelling of slow muscles and muscle pioneers respectively; Tg(*hsp70:ca-fgfr1*)^{pd3} (Marques et al., 2008), Tg (*hsp70:dn-Fgfr1-EGFP*)^{pd1} (Lee, 2005), Tg(*smyhc1:GAL4-VP16*)^{i316} for the global and selective perturbations of FGF activity; and *smo*$^{b641/b641}$(Varga et al., 2001), *ubo/prdm1a*$^{tp39/tp39}$(van Eeden et al 1996; Baxendale et al., 2004) and *tbx6*$^{ti1/ti1}$(Nikaido et al., 2002) for the generation of homozygous *smo, prdm1a* and *tbx6* mutants respectively.

Embryos selected for experiments were typically less than 24hpf, a stage at which sex cannot be readily determined and is unlikely to influence the biological processes under study.

METHOD DETAILS

Assembly of DNA constructs and RNA for live imaging

For transcription of RNA for time-lapse imaging, we assembled pcs2/SP6-lyn-tdTomato, pcs2/SP6-lyn-eGFP, pcs2/SP6-lyn-kaede and pcs2/SP6-h2b-EGFP. Plasmids were linearized with NotI before transcription of capped RNA using an mMessage-mMachine kit (Ambion). We assembled pTol2 (UAS:ca-fgfr1-p2a-mcherry), pTol2(UAS:dn-fgfr1-p2a-mcherry), pTol2(UAS:mcherry), pTol2 (hsp70l:ca-fgfr1-p2a-mcherry), pTol2 (hsp70l:dn-fgfr1-mcherry) and pTol2 (hsp70l:mcherry) for mosaic FGF perturbations using Gibson Assembly (NEB). The *dn-fgfr1* and *ca-fgfr1* of pTol2(UAS:dn-fgfr1-p2a-mcherry) and pTol2 (UAS:ca-fgfr1-p2a-mcherry) were designed as previously described (Lee, 2005; Neilson and Friesel, 1996) and tested by injecting the encoded mRNA into embryos at the one cell stage (Figure S4C-D). The *ca-fgfr1* and *dn-fgfr1* of pTol2 (hsp70l:ca-fgfr1-p2a-mcherry) and pTol2 (hsp70l:dn-fgfr1-mcherry) were amplified from Tg(*hsp70:ca-fgfr1*)^{pd3} (Marques et al., 2008) and Tg (*hsp70:dn-Fgfr1-EGFP*)^{pd1} (Lee, 2005).

Injections and morpholino knockdown

30ng/ml of mRNA encoding lyn-tdTomato, lyn-eGFP, lyn-kaede or h2b-GFP were injected into embryos at the one cell stage. 30ng/ml of DNA encoding UAS:dn-fgfr1-h2a-mcherry, UAS:dn-fgfr1-h2a-mcherry or UAS:mcherry were injected into embryos from crosses between Tg(*smyhc1:GAL4-VP16*) and Tg(*PACprdm1:GFP*)^{i106}. 30ng/ml of DNA encoding hsp70l:dn-fgfr1-mcherry, hsp70l:ca-fgfr1-p2a-mcherry or hsp70l:mcherry were injected into embryos from Tg(*smyhc1:GAL4-VP16*). Morpholino (MO) oligonucleotides were purchased from Gene Tools (Philomath, OR) using previously reported sequences (Windner et al., 2015). Morpholinos were dissolved in water to a stock solution of 1mM. The stock solution of the *Ripply1* MO was diluted in injection solution to a final concentration of 200µM and 1–2nL injected into 1–2 cell stage embryos.

Drug treatments and heat shock inductions

SU5402 (Sigma) was added to embryo medium at 14-somite stage 2h before live imaging at a concentration of 60µM. The same concentration of SU5402 was applied in the agarose-embedding solution throughout live imaging. For immunostaining purpose, SU5402 was added to embryo medium

996 4-somite stage. Dorsomorphin (Sigma) was added to agarose-embedding solution and embryo medium
997 at 14-somite stage 2h before live imaging at a concentration of 50μM. Cyclopamine (Abcam) was added
998 either 1 or 2h before live imaging at a concentration of 30μM. The dose of drug treatment was based
999 on previous reports (Dolez et al., 2011; Groves, 2005; Nguyen-Chi et al., 2012; Wolff et al., 2003). Heat
1000 shock of Tg(*hsp70:ca-fgfr1*)^{pd3}, Tg(*hsp70:dn-fgfr1-EGFP*)^{pd1} or transient transgenic embryos with
1001 hsp70l promoter was performed at 16-somite stage at 38°C for 50min. Embryos were immediately
1002 transferred for live imaging after heat shock.
1003
1004 **Time-lapse live imaging**
1005 Zeiss Lightsheet Z.1 and Zeiss LSM 700 were used for the live imaging of zebrafish embryos. For live
1006 imaging of anterior somites, embryos were mounted into FEP tube with 1% low melting agarose in
1007 embryo medium at 8-10 somites stage (around 13hpf). Well-mounted embryos were transferred to
1008 Zeiss Lightsheet Z.1 and imaged using a 20x water immersion objective. For live imaging of posterior
1009 somites, embryos were mounted into microwell dishes tube with 0.3% low melting agarose in embryo
1010 medium at 18-20 somites stage (around 19hpf). Well-mounted embryos were transferred to Zeiss LSM
1011 700 and imaged using a 40x oil-immersion objective. During live imaging, the environmental
1012 temperature was maintained at 26℃.
1013
1014 **Fluorescent *In situ* hybridization and antibody staining**
1015 Fluorescent *in situ* hybridization (FISH) followed the online protocol:
1016 https://zfin.org/ZFIN/Methods/ThisseProtocol.html. SIGMAFAST™ Fast Red TR (Sigma) was utilized
1017 for generating red fluorescence with alkaline phosphatase. Rabbit Anti-GFP antibody (TP401) was used
1018 to reveal eGFP in FISH. For RNA probe generation, regions of the coding sequences of *fgf8a, pea3,*
1019 *erm, ptch2* were amplified by PCR using the sequences in the key resources table. Antibody staining
1020 was performed at the following dilutions: mAb 4D9 (anti-engrailed; DHSB) at 1:50-1:200; mAb pax7
1021 (anti-pax7; DHSB) at 1:100-1:200; Specimens were imaged using a 40x oil immersion objective on a
1022 Zeiss LSM 700 confocal microscope.
1023

1024 QUANTIFICATION AND STATISTICAL ANALYSIS

1025 Images were analyzed using ImageJ software or homemade MATLAB code. Cell tracking, cell fate
1026 mapping, timing measurement and mapping were performed manually with high reliability.
1027 Quantification of the size of the lateral region of a somite or individual muscle fibers was based on
1028 automatic image analysis with homemade MATLAB code combined with manual corrections. All p-
1029 values unless otherwise stated are calculated using a two-tailed t-test, performed in MATLAB.
1030 Minimum-volume covering of data points (Figure 5G and 7E) was performed in MATLAB. The
1031 expression levels of *ptch2*, *pea3*, *erm* and *sprouty4* were obtained through FISH and imaged by
1032 confocal microscopy. The expression profile of *pea3* was measured directly along the medial-lateral
1033 axis and then normalized according to the maximum intensity of each profile. The expression level of
1034 *spry4* and *ptch2* was measured cell by cell by calculating the average intensity within each nucleus.
1035 The *spry4* expression displayed very large cell-to-cell variations even within the same somite so no
1036 normalization was performed on it. The *ptch2* expression level was normalized according to the mean
1037 expression level among the midline adaxial cells.
1038

1039 DATA AND SOFTWARE AVAILABILITY

1040 All images and data are available upon request.

1041
1042

1043 **Supplemental Video Legends**
1044
1045 **Video S1: The specification of MPs and lateral migration of SSFs, related to Figure 1E**
1046 3D representation of the cell rearrangement of adaxial cells during slow muscle morphogenesis and
1047 the lateral migration of SSFs. Red and blue denote to MPs and SSFs respectively.
1048
1049 **Video S2: Wave of slow muscle elongation emanating outward from the DV midline, related to**
1050 **Figure 2A**
1051 Time-lapse of the slow muscle morphogenesis of adaxial cells from somite segmentation to 400mins
1052 after somite segmentation. H2a:eGFP and Lyn-td tomato label cell histone and cell membrane
1053 respectively.
1054
1055 **Video S3: MPs originate from midline adaxial cells, related to Figure 2D**
1056 Time-lapse of MPs specification from segmentation to 290mins later. MPs are identified by the
1057 expression of Eng2a:eGFP.
1058
1059 **Video S4: Ectopic Eng2a:eGFP positive SSFs are induced under heat shock of hs:ca-fgfr1,**
1060 **related to Figure 6F**
1061 Left and right panels are the parasagittal optical view and reconstructed transverse view of the
1062 developing muscle segments.
1063
1064 **Video S5: Perturbations of FGF change the timing, velocity, and range of lateral migration of**
1065 **SSFs, related to Figure 6H-L**
1066 Comparison of the lateral migration of SSFs of wild type embryos (top), embryos under treatment of
1067 60μM SU5402 (middle) and embryos under heat shock of hs:ca-fgfr1 (bottom). Left and right panels
1068 are the parasagittal optical view and reconstructed transverse view of the developing muscle segments.
1069 Slow muscles are labeled with Prdm1a:GFP.
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