1 **Zic1 advances epaxial myotome morphogenesis to cover the neural tube via Wnt11r**

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3 Authors: Ann Kathrin Heilig[1,2,3], Ryohei Nakamura[1], Atsuko Shimada[1], Yuka Hashimoto[1],

4 Yuta Nakamura[1], Joachim Wittbrodt[2], Hiroyuki Takeda[1*], Toru Kawanishi[1*]

5 1: Department of Biological Sciences, Graduate School of Science, University of Tokyo,

6 Tokyo, Japan

7 2: Centre for Organismal Studies, Heidelberg University, Heidelberg, Germany

8 3: Heidelberg Biosciences International Graduate School, Heidelberg, Germany

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10 *Corresponding authors: htakeda@bs.s.u-tokyo.ac.jp (H.T.), toru.kawanishi@bs.s.u-

11 tokyo.ac.jp (T.K.)

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13 **Abstract**

14 The dorsal axial muscles, or epaxial muscles, are a fundamental structure covering the spinal

15 cord and vertebrae, as well as mobilizing the vertebrate trunk. To date, mechanisms

16 underlying the morphogenetic process shaping the epaxial myotome are largely unknown. To

17 address this, we used the medaka *zic1/zic4*-enhancer mutant *Double anal fin* (*Da*), which

18 exhibits ventralized dorsal trunk structures resulting in impaired epaxial myotome

19 morphology and incomplete coverage over the neural tube. In wild type, dorsal

20 dermomyotome (DM) cells, progenitors of myotomal cells, reduce their proliferative activity

21 after somitogenesis and subsequently form unique large protrusions extending dorsally,

22 potentially guiding the epaxial myotome dorsally. In *Da*, by contrast, DM cells maintain the

23 high proliferative activity and form mainly small protrusions. By combining RNA- and ChIP-

24 sequencing analyses, we revealed direct targets of Zic1 which are specifically expressed in

25 dorsal somites and involved in various aspects of development, such as cell migration,

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26   extracellular matrix organization and cell-cell communication. Among these, we identified

27   *wnt11r* as a crucial factor regulating both cell proliferation and protrusive activity of DM

28   cells. We propose that the dorsal movement of the epaxial myotome is guided by DM cells

29   and that Zic1 empowers this activity via Wnt11r to achieve the neural tube coverage.

30

31   **Introduction**

32   Active locomotion, which is powered by skeletal muscles in vertebrates, is critical for

33   animals to survive. Vertebrate skeletal muscles consist of axial muscles (head, trunk and tail

34   muscles) and appendicular muscles (limb muscles). Axial muscles first arose in the chordates

35   to stabilize and enable side-to-side movement of the body axis. In jawed vertebrates, the

36   subdivision of axial muscles into epaxial (dorsal) and hypaxial (ventral) muscles led to an

37   increased range of movement: dorsoventral undulation in fish and lateral movements in

38   terrestrial vertebrates (Glass and Goodrich, 1960; Romer and Parsons, 1986; Fetcho, 1987;

39   Clifton, 2002; Sefton and Kardon, 2019). Among these muscles, epaxial muscles are

40   characterized by their unique anatomical structure which extends dorsally and surrounds the

41   vertebrae. This morphology also ensures mechanical support and protection of the vertebrae

42   and the spinal cord inside. While we have a detailed understanding of how the myotome,

43   precursors of epaxial and hypaxial muscles, differentiates from the somites (Kalcheim and C,

44   1999; Gros, Scaal and Marcelle, 2004; Hollway and Currie, 2005), we only begin to

45   understand the cellular and molecular mechanisms of the subsequent morphogenetic

46   processes generating the epaxial muscles. Previous studies in rats and mice suggested that

47   myocytes of the epaxial myotome do not actively migrate dorsally but are guided by external

48   forces (Deries, Schweitzer and Duxson, 2010; Deries *et al.*, 2012). However, what exerts

49   such forces to drive extension of epaxial myotome is still unclear.

50   Fish have been extensively utilized to study myotome development thanks to the

51   transparency of their bodies throughout embryonic development (Nguyen *et al.*, 2017;

52   Ganassi *et al.*, 2018). Additionally, their epaxial trunk muscles have a simple structure

53   consisting of only one anatomical unit (reviewed in (Sefton and Kardon, 2019)). Like other

54   vertebrates, fish myotomes, on either side of the neural tube, extend dorsally after somite

55   differentiation and eventually cover the neural tube by the end of embryonic development

56   (Figure 1A). The spontaneous medaka (*Oryzias latipes*) mutant *Double anal fin* (*Da*) displays

57   a particular epaxial myotome morphology, in which the dorsal ends of the left and right

58   epaxial myotome fail to extend sufficiently and thus do not cover the neural tube at the end of

59   embryonic development. Previous studies demonstrated that the dorsal trunk region of the *Da*

60   mutant is transformed into the ventral one, including not only the myotome but also the body

61   shape, skeletal elements, pigmentation and fin morphology (Figure 1B, D) (Ishikawa, 1990;

62   Ohtsuka *et al.*, 2004). Given the unique morphological features, the medaka *Da* mutant is an

63   excellent model to study the morphogenesis of epaxial myotome. Genetic analysis of the *Da*

64   mutant revealed that this phenotype is due to a dramatic reduction of the expression of the

65   transcription factors *zic1* and *zic4* in the dorsal somites (Figure 1C, E), and identified

66   *zic1*/*zic4* as master regulators of trunk dorsalization (Ohtsuka *et al.*, 2004; Kawanishi *et al.*,

67   2013). The down-regulation of *zic1/zic4* specifically in the dorsal somites is caused by the

68   insertion of a large transposon, disrupting the dorsal somite enhancer of *zic1/zic4* (Moriyama

69   *et al.*, 2012; Inoue *et al.*, 2017). While the function and downstream targets of Zic1 and Zic4

70   have been studied in the nervous system (Aruga and Millen, 2018), the molecular mechanism

71   of how these Zic genes control dorsal trunk morphogenesis has not been investigated so far.

72   Here we describe the morphogenetic process of the formation of the epaxial myotome

73   of the back, which we termed "dorsal somite extension" (Figure 1A). By *in vivo* time-lapse

74   imaging we uncovered its cellular dynamics; during dorsal somite extension, dorsal

75   dermomyotome (DM) cells reduce their proliferative activity and subsequently form unique

76   large protrusions extending dorsally, potentially guiding the epaxial myotome dorsally. In the

77   *Da* mutant, by contrast, DM cells keep their high proliferative activity and mainly form small

78   protrusions. Mechanistically, we identify a Zic1 downstream-target gene, *wnt11r*, as a crucial

79   factor for dorsal somite extension. We demonstrate that Wnt11r regulates cellular behavior of

80   dorsal DM cells by promoting protrusion formation and negatively regulating proliferation.
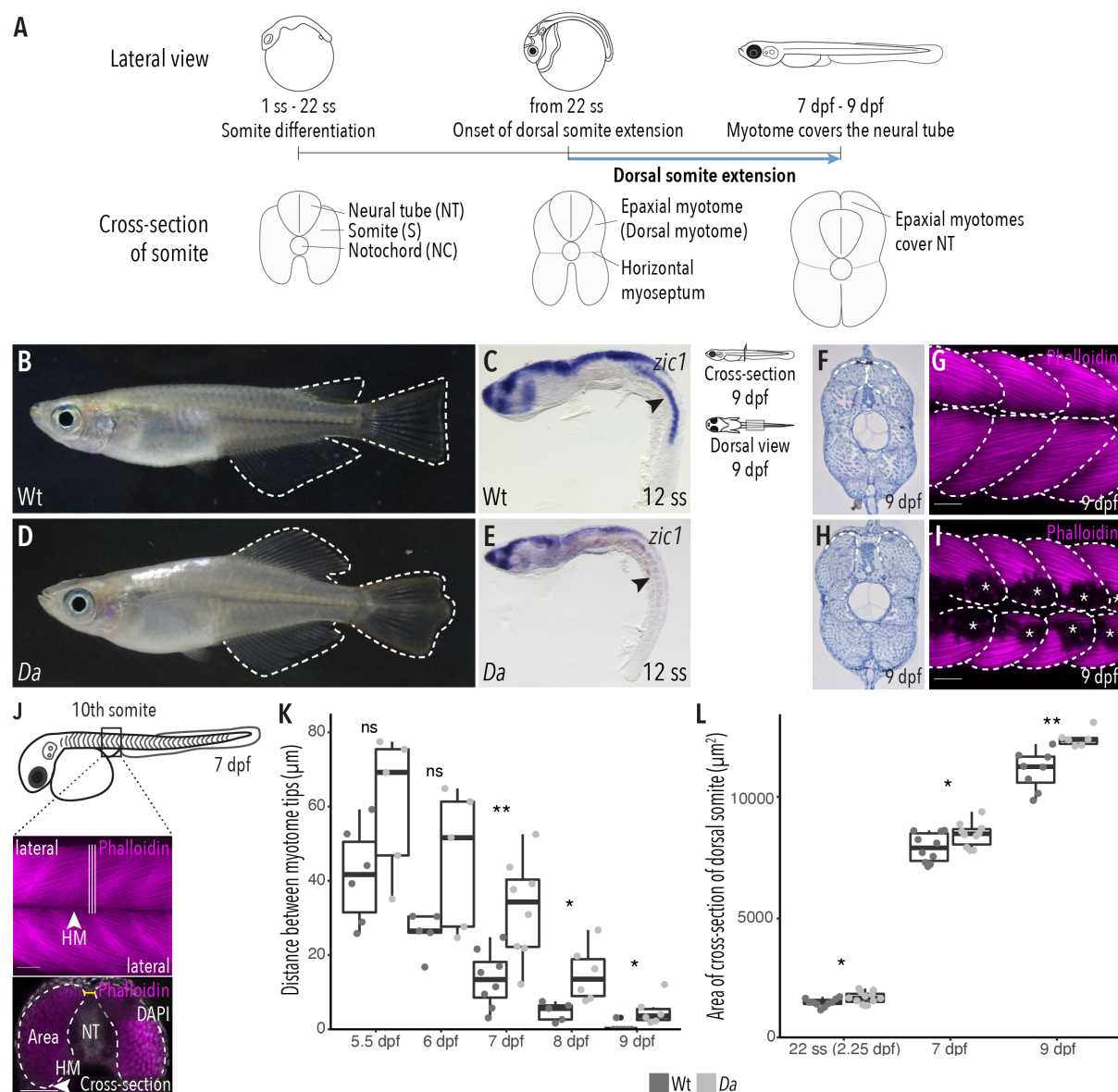
81

82   **Results**

83   Our previous study showed that *zic1* and *zic4* expression starts at embryonic stages and

84   persists throughout life (Kawanishi *et al.*, 2013). Phenotypic analysis of homozygous adult

85   *Da* mutants implies long-term participation of Zic-downstream genes in the formation of

86   dorsal musculatures, which eventually affects the external appearance of the fish adult trunk.

87   Here, we examined the initial phase of this long-term dorsalization process. In the following

88   of the study, we will focus on *zic1*, since *zic1* and *zic4* are expressed in an identical fashion

89   with overlapping functions in trunk dorsalization of medaka, and *zic4* is expressed slightly

90   weaker than *zic1* (Moriyama *et al.*, 2012; Kawanishi *et al.*, 2013).

91

92   **The dorsal myotome of the *Da* mutant fails to cover the neural tube**

93   In wild type (Wt) medaka, the dorsal ends of the myotomes first came in contact at 7 days

94   post fertilization (dpf, stage 37) and formed the tight, thick myotome layer covering the

95   neural tube at the end of embryonic development (9 dpf, stage 39) (Figure 1F-G, Figure 1 -

96   figure supplement 1A-F). In the ventralized *Da* mutant, however, the dorsal ends of the

97   myotomes did not extend sufficiently and failed to cover the neural tube at the end of

98   embryonic development (Figure 1H-I, Figure 1 - figure supplement 1G-L). The ends of the

4

99   ventralized dorsal myotome in the mutant displayed a round shape (not pointed as found in

100   Wt) which resembled the morphology of the ventral myotome.

101   We wondered if there are other morphological differences between Wt dorsal

102   myotome and the ventralized dorsal myotome of the *Da* mutant. Indeed, the cross-sectional

103   area in the *Da* mutant was significantly larger compared to Wt (Figure 1L). Possible

104   explanations for a larger cross-sectional area in the *Da* mutant could be a larger myofiber

105   diameter or a higher number of myofibers which make up the myotome. We measured the

106   diameter of dorsal myotome muscle fibers in Wt and *Da* mutant embryos but could not

107   observe a difference, suggesting that dorsal myotome of the *Da* mutant has a higher number

108   of myotomal cells (Figure 1 - figure supplement 1M).

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Figure 1: The epaxial myotome of the *Da* mutant fails to cover the neural tube at the end of embryonic development.

(A) Schematic representation of dorsal somite extension which results in the full coverage of the neural tube by the epaxial myotomes at the end of embryonic development.

(B) Lateral view of adult Wt medaka. Dorsal, caudal and anal fins are outlined.

(C) Lateral view of whole-mount *in situ* hybridization against *zic1* in a 12 ss (1.7 dpf, stage 23) Wt embryo. *Zic1* expression can be observed in the brain, neural tissues and the dorsal somites (arrowhead).

119   (D) Lateral view of adult *Da* mutant. Dorsal, anal and caudal fins are outlined. The dorsal

120   trunk region resembles the ventral trunk region.

121   (E) Lateral view of whole-mount *in situ* hybridization against *zic1* of a 12 ss *Da* embryo. *Zic1*

122   expression can be observed in the brain and the neural tissues, but is drastically decreased in

123   the dorsal somites (arrowhead).

124   (F, H) Cross-sections of tail regions of hematoxylin stained 9 dpf embryos. Dorsal ends of

125   myotomes are outlined. (F) In Wt, the left and the right myotome come in close contact at the

126   top of the neural tube and form a gapless muscle layer. (H) In the *Da* mutant, the left and

127   right myotome fail to come in contact at the top of the neural tube.

128   (G, I) Dorsal view of whole-mount Phalloidin (magenta) immunostaining labeling the

129   myotome of (G) Wt and (I) *Da* embryos. Epaxial myotome is outlined, asterisks label

130   melanophores.

131   (J) Schematic representation of measurements to analyze the distance between the left and the

132   right dorsal tip of the myotome (yellow) and the cross-sectional area of the dorsal myotome.

133   For each measurement, three consecutive optical cross sections of the 10th somite were

134   analyzed and averaged.

135   (K) Analysis of the distance between the left and right tip of the dorsal myotome 5.5 dpf – 9

136   dpf (n = 6 Wt embryos 5.5 dpf (stage 35), n = 5 *Da* embryos 5.5 dpf, n = 5 Wt embryos 6 dpf

137   (stage 36), n = 5 *Da* embryos 6 dpf, n = 8 Wt embryos 7 dpf, n = 8 *Da* embryos 7 dpf, n = 5

138   Wt embryos 8 dpf (stage 38), n = 6 *Da* embryos 8 dpf, n = 7 Wt embryos 9 dpf, n = 6 *Da*

139   embryos 9 dpf, median, first and third quartiles, $^{ns}P_{5.5\ dpf} = 0.097$, $^{ns}P_{6\ dpf} = 0.075$, $**P_{7\ dpf} =$

140   0.0047, $*P_{8\ dpf} = 0.019$ $*P_{9\ dpf} = 0.034$).

141   (L) Analysis of cross-sectional area of the dorsal somites at 22 ss (2.25 dpf, stage 26; n = 10

142   somites of 5 Wt embryos, n = 12 somites of 6 *Da* embryos), 7 dpf (n = 10 somites of 5 Wt

143   embryos, n = 10 somites of 5 *Da* embryos) and 9 dpf (n = 8 somites of 4 Wt embryos, n = 6

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144 somites of 3 *Da* embryos) (median, first and third quartiles, $*P_{22\ ss} = 0.038$, $*P_{7\ dpf} = 0.044$,

145 $**P_{9\ dpf} = 0.0019$).

146 Anterior = left, HM = horizontal myoseptum, NT = neural tube, scale bar = 50 μm.
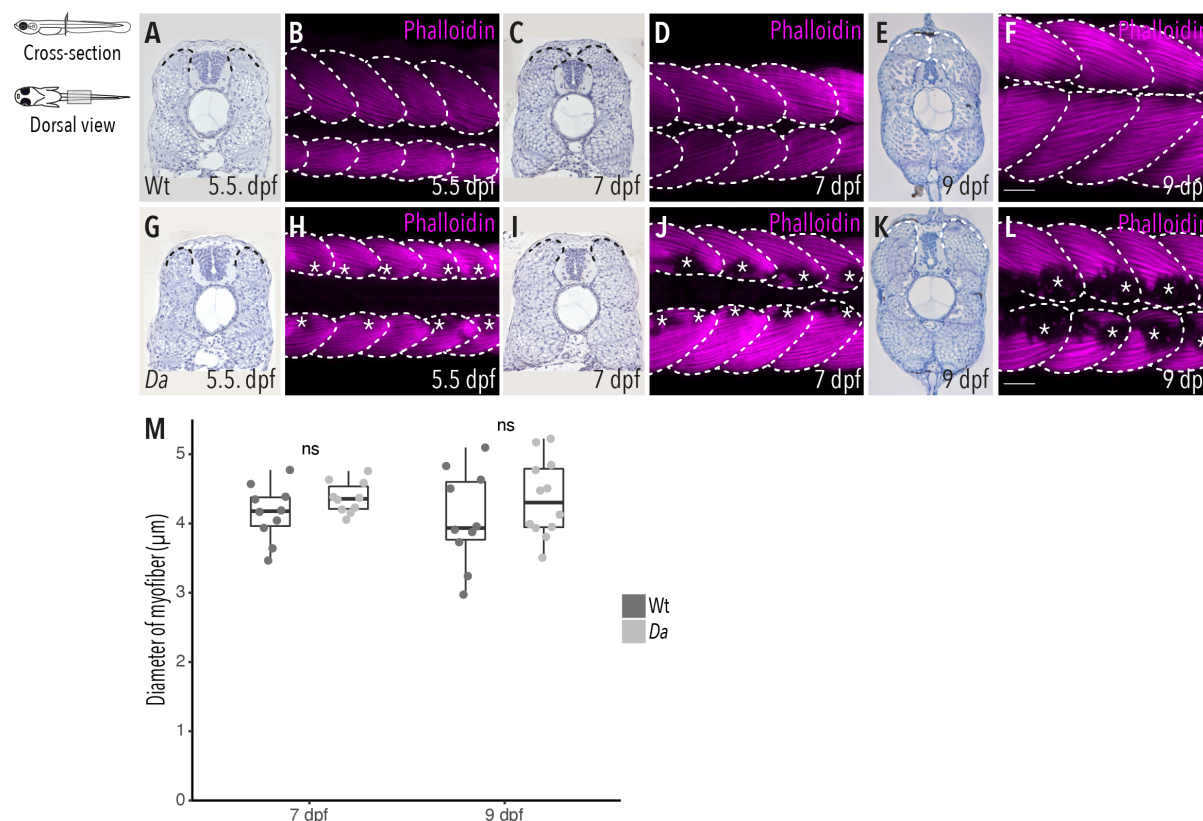
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149 Figure 1 - figure supplement 1: The ventralized epaxial myotome of the *Da* mutant fails to

150 extend sufficiently to cover the neural tube at the end of embryonic development.

151 (A, C, E,) Hematoxylin staining of cross-sections from Wt and (G, I, K) *Da* mutant embryos

152 and dorsal view of whole-mount Phalloidin (magenta) immunostaining to label the myotome

153 of Wt (B, D, F) and *Da* mutant embryos (H, J, L).

154 (M) Analysis of diameter of myofibers in dorsal myotome of 7 dpf embryos (n = 10

155 myofibers from 10 somites of 5 Wt embryos, n = 10 myofibers from 10 somites of 5 *Da*

156 embryos) and 9 dpf embryos (n = 10 myofibers from 10 somites of 5 Wt embryos, n = 12

157 myofibers from 12 somites of 6 *Da* embryos) (median, first and third quartiles, $^{ns}P_{7\,dpf} = 0.15$,

158 $^{ns}P_{9\,dpf} = 0.31$).

159 Anterior = left, scale bar = 50 μm.

160

161 **Proliferative activity of the dorsal DM cells is enhanced in the *Da* mutant**

162 In fish, as in other vertebrates, the DM gives rise to muscle precursor cells which ultimately

163 differentiate into myofibers. In medaka the DM is a one cell-thick, Pax3/7-positive cell layer

164 encompassing the myotome (Figure 2A-B'') (Hollway *et al.*, 2007; Abe *et al.*, 2019). A high

165 proliferative activity of the dorsal DM could explain a larger cross-sectional area of the

166 dorsal myotome of the *Da* mutant. To test this, we performed immunohistochemistry against

167 the mitotic marker phosphor-histone H3 (PH3) and the DM marker Pax3/7 on Wt and *Da*

168 embryos (Figure 2C-E). In both Wt and *Da* embryos, PH3-positive cells were randomly

169 distributed in the dorsal DM without obvious bias (Figure 2C-D). At the 12-somite stage (12

170 ss, 1.7 dpf stage 23), when *zic1* expression in the somites becomes restricted to the dorsal

171 region (Kawanishi *et al.*, 2013), the number of PH3-positive DM cells per dorsal somite was

172 not significantly different between Wt and *Da*. Remarkably, from 16 ss (1.8 dpf, stage 24)

173 onwards, the number of PH3-positive cells became reduced in the Wt, whereas in *Da*, no

174 such reduction was observed (Figure 2E). At 35 ss (3.4 dpf, stage 30), PH3-positive cells

175 increased both in the Wt and *Da*, but the mutant DM cells were more proliferative (Figure

176 2E). The number of PH3-positive cells in the ventral DM was not significantly different in

177 Wt and *Da* embryos at 22 ss (2.75 dpf, stage 26) (Figure 2—figure supplement 1A). These

178 results suggest that *zic1* reduces proliferative activity of the DM, which becomes evident

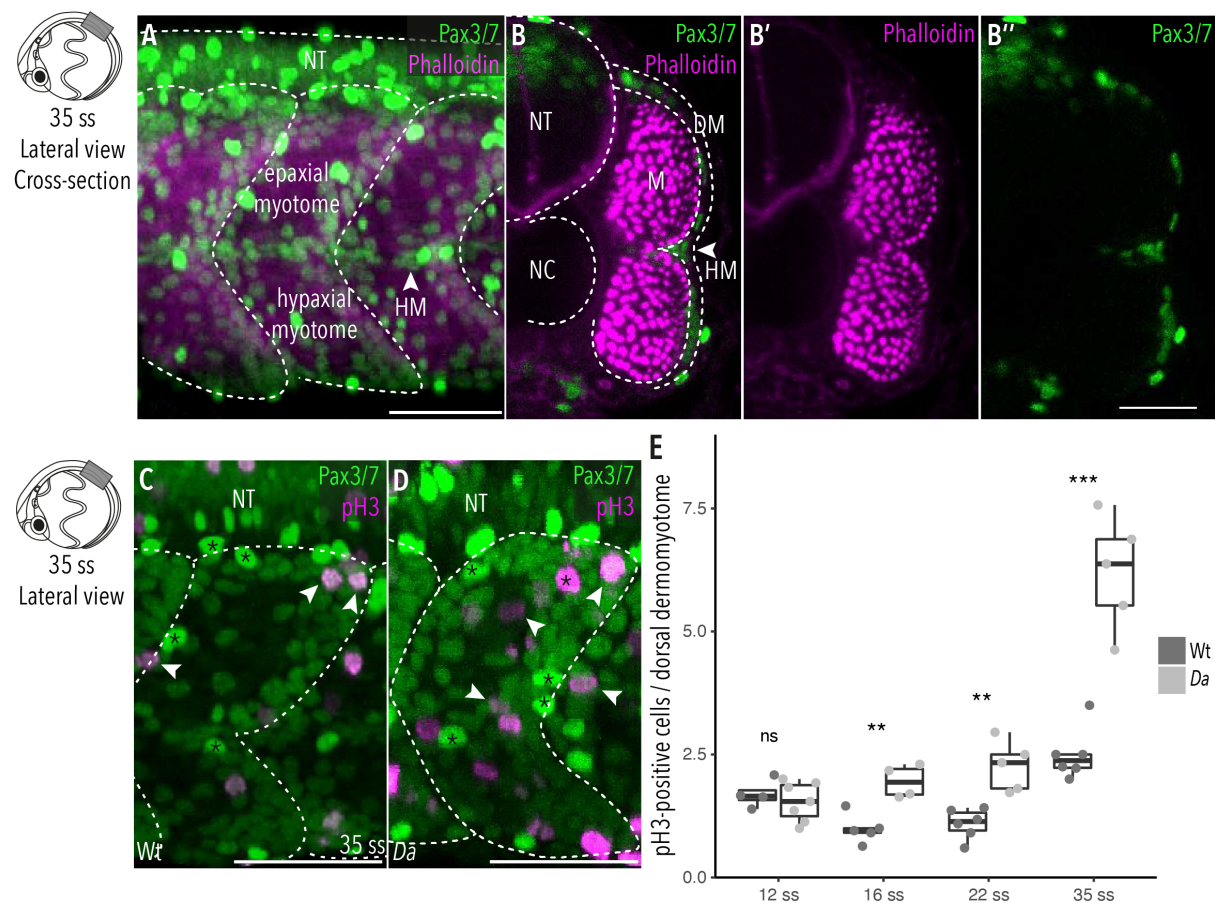179 following the confinement of its expression to the dorsal somite region.

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Figure 2: Wt dorsal DM cells show lower proliferative activity after the confinement of *zic1* expression to the dorsal somite.

(A) Lateral view of 35 ss (3.4 dpf, stage 30) embryo, 10th somite is positioned in the center. Pax3/7 (green) labels DM cells and Phalloidin (magenta) labels myotome. The horizontal myoseptum (HM) separates the myotome into epaxial myotome (dorsal) and hypaxial myotome (ventral).

(B–B'') Optical cross sections, myotome is labelled by Phalloidin (magenta) and encompassed by a one-cell thick layer of DM labelled with Pax3/7 (green).

(C-D) Lateral view of (C) Wt and (D) *Da* 35 ss embryos. DM is labelled with Pax3/7 (green), mitotic active cells are PH3-positive (magenta, arrowheads (exemplary)). Asterisks exemplary mark neural crest cells which are highly Pax3/7-positive.

(E) Quantification of PH3-positive cells in Wt and *Da* at 12 ss (n = 46.5 somites from 4 Wt embryos, n = 95 somites from 7 *Da* embryos), 16 ss (n = 54.5 somites from 5 Wt embryos, n

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195   = 42.5 somites from 4 *Da* embryos), 22 ss (n = 66 somites from 6 Wt embryos, n = 40.5

196   somites from 5 *Da* embryos,) and 35 ss (n = 49 somites from 6 Wt embryos, n = 47.5 somites

197   from 5 *Da* embryos,) (median, first and third quartiles, $^{ns}P_{12\ ss}$ = 0.48, **$P_{16\ ss}$ = 0.0038, **P_{22}

198     $_{ss}$ = 0.0035, ***$P_{35\ ss}$ = 0.0008).

199   Anterior = left, dorsal = up, DM = dermomyotome, HM = horizontal myoseptum, M =

200   myotome, NT = neural tube, NC = notochord, scale bar = 50 μm.

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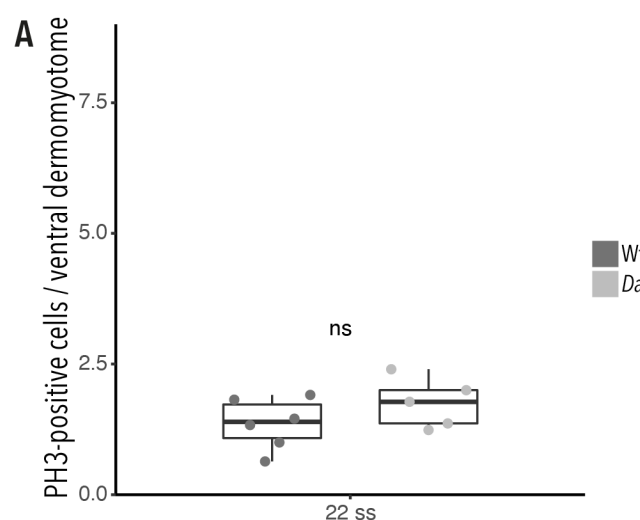
203   Figure 2 - figure supplement 1: Difference in proliferative activity is not observed between

204   Wt and *Da* ventral DM.

205   (A) Quantification of PH3-positive cells in the ventral DM of 22 ss embryos (n = 66 somites

206   from 6 Wt embryos, n = 51 somites from 5 *Da* embryos) (median, first and third quartiles, ^{ns}P

207   = 0.2).

208

209   **Wt dorsal DM cells form numerous large, motile protrusions at the onset of dorsal**

210   **somite extension**

211   The epaxial myotome, on either side of the neural tube, extends dorsally to cover the neural

212   tube by the end of embryonic development. To examine the behavior of *zic1*-positive cells

213   underlying this dorsal somite extension, we performed *in vivo* time-lapse imaging of dorsal

214   somites using the transgenic line Tg(*zic1:GFP; zic4:DsRed*), which expresses GFP under the

215   control of the *zic1* promoter and enhancers to visualize the dorsal somitic cells (Kawanishi *et*

216   *al.*, 2013) (hereafter called Tg(*zic1:GFP*) since the DsRed fluorescence was negligible in the

217   following analyses). Intriguingly, around 22 ss and onwards, cells at the tip of the dorsal

218   somites started to form numerous large protrusions extending dorsally towards the top of the

219   neural tube (Figure 3A, Video 1). We defined the beginning of protrusion formation as the

220   onset of dorsal somite extension. Close-up views of the time-lapse images (Figure 3A, Video

221   1) showed that these protrusions were motile and dynamically formed new branches at their

222   dorsal tips (Figure 3J). Immunohistochemistry revealed that the protrusion-forming cells

223   belong to the DM (Figure 3 - figure supplement1 A-A'').

224   To characterize the protrusions, we classified them according to their length into

225   small (< 8 μm, Figure 3C arrowheads) and large (≥ 8 μm, Figure 3C arrow) protrusions

226   (Figure 3 - figure supplement 1B-C). Based on their shape, we reasoned that the small

227   protrusions correspond to lamellipodia (Figure 3C, arrowheads), while the large protrusions

228   appeared more complex. To investigate the nature of large protrusions, we injected Actin-

229   Chromobody GFP mRNA to visualize the actin skeleton. The large protrusions were found to

230   exhibit a complex architecture consisting of a lamellipodia-like core structure (Figure 3D,

231   bracket) with additional multiple bundles of filopodia (protrusions with linear arranged actin

232   filaments) branching out from their dorsal tips (Figure 3D, arrows, summarized in Figure

233   3E).

234   Interestingly, time-lapse *in vivo* imaging of Tg(*zic1:GFP*);*Da* showed that in the *Da*

235   background, protrusions started to form later (Figure 3B, Video 2) and the number of large

236   protrusions and protrusions in total was significantly lower than in Wt (Figure 3I-I''). In

237   addition, protrusions were transient and mostly failed to form new branches at their dorsal

238   tips (Figure 3K). While no difference in the actin skeleton of small protrusions could be

239   observed, filament bundles branching out from large protrusions of *Da* DM cells contained

240   fewer and shorter filopodia compared to Wt (Figure 3G, arrowheads, summarized in Figure

241   3H). These results indicate that the protrusive activity, especially the ability to form large

242   protrusions, is significantly reduced in the *Da* mutant. The large protrusions of the dorsal DM

243   cells continuously appeared at later stages of dorsal somite extension, too (Figure 3 - figure

244   supplement 2).

245   Taken together, the unique large protrusions of the dorsal DM might be involved in

246   guiding the epaxial myotome dorsally and *zic1* might promote this function.
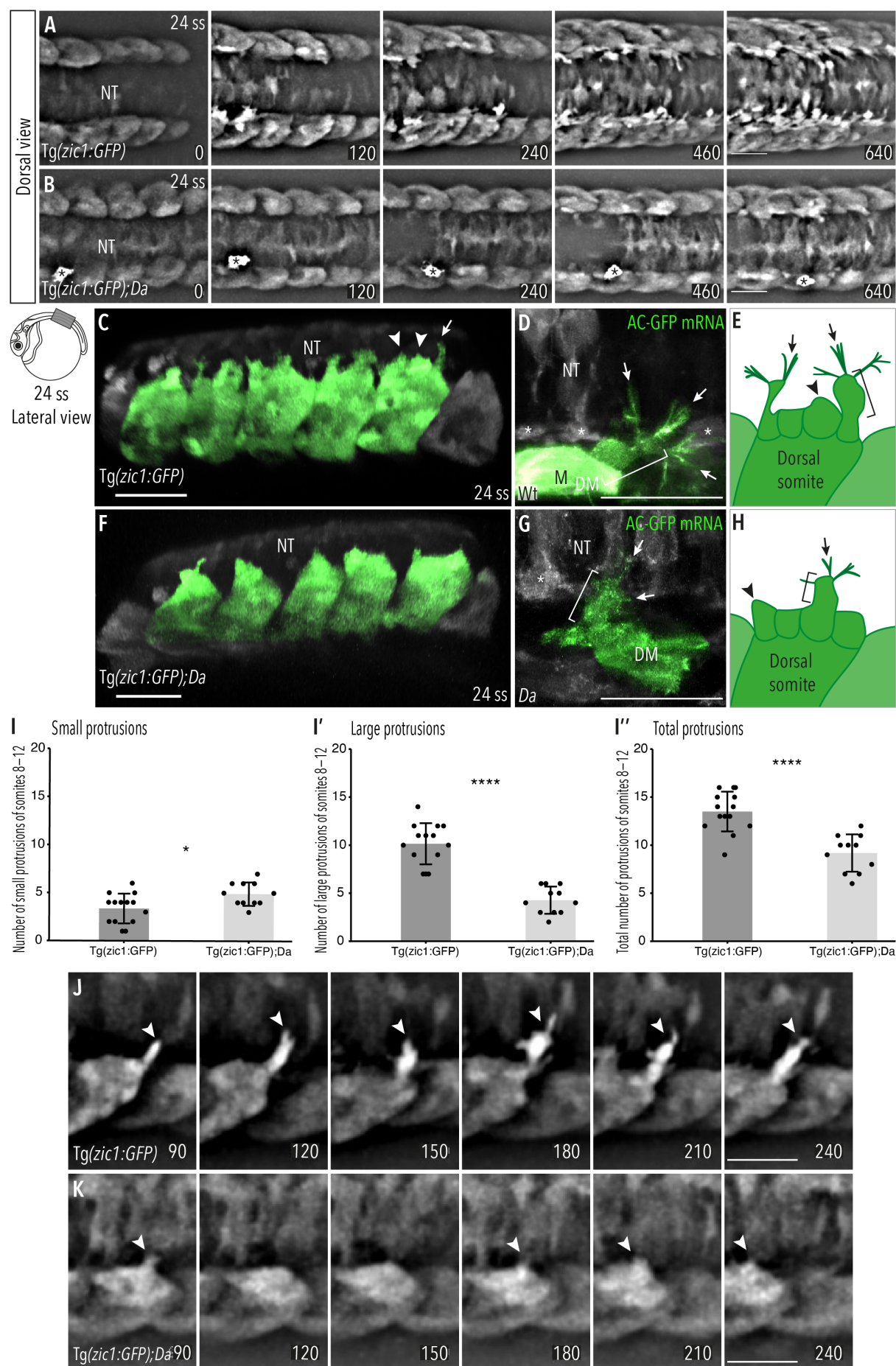
248   Figure 3: Wt dorsal DM cells form numerous large, motile protrusions at the onset of somite

249   extension.

250   (A-B) Dorsal view of time-lapse *in vivo* imaging of onset of dorsal somite extension at 24 ss

251   (2.4 dpf, stage 27) of (A) Tg(*zic1:GFP*) and (B) Tg(*zic1:GFP);Da* embryos. 15th somite is

252   positioned in the center, z-stacks were imaged every 10 min, time is displayed in min.

253   Asterisks indicate migrating melanophore. Scale bar = 50 μm.

254   (C, F) Lateral view of 3D-reconstructions of *in vivo* imaging of (C) Tg(*zic1:GFP*) and

255   (F)Tg(*zic1:GFP*);*Da*, dorsal somites are colored green. Arrowheads indicate small

256   protrusions, arrow indicates large protrusion. Scale bar = 50 μm.

257   (D, G) Dorsal view of 3D-reconstruction of *in vivo* imaging of large protrusion in (D) Wt and

258   (G) *Da*. Embryos were injected with Actin-Chromobody GFP (AC-GFP) mRNA, large

259   protrusions are colored green. Brackets indicated lamellipodia-like structure, arrows indicate

260   filopodia branching out from dorsal tips of lamellipodia-like core. Asterisks indicate

261   sclerotome cells, scale bar = 25 μm.

262   (E, H) Summary of dorsal DM cell protrusions in (E) Wt and (H) *Da* embryos. Arrowheads

263   indicate small protrusions, brackets indicate lamellipodia-like core structure of large

264   protrusions, arrows indicate filopodia bundles branching off from dorsal tips of large

265   protrusions.

266   (I-I'') Quantification of protrusions from Tg(*zic1:GFP*) (n = 14 embryos) and

267   Tg(*zic1:GFP*);*Da* (n = 11 embryos). Protrusions of the 8th-12th somite were counted (mean ±

268   SD, $*P_{small\ protrusions} = 0.01$, $****P_{large\ protrusions} = 3.3e\text{-}08$, $****P_{total\ protrusions} = 2.1e\text{-}05$).

269   (J-K) Lateral view of protrusions extracted from time-lapse imaging of (A-B). Arrowheads

270   indicate tip of protrusions, time is displayed in min. (J) Protrusion observed in Tg(*zic1:GFP*).

271   (K) Protrusion observed in Tg(*zic1:GFP*);*Da*. Scale bar = 25 μm.

272   Anterior = left, DM = dermomyotome, M = myotome, NT = neural tube.

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273

274   Video 1: Onset of dorsal somite extension in Tg(*zic1:GFP*).

275   Dorsal view of time-lapse *in vivo* imaging of 24 ss Tg(*zic1:GFP*) embryo. 15th somite is

276   positioned in the center, z-stacks were imaged every 10 min, time is displayed in min.

277   Anterior = left, scale bar = 50 μm.

278

279   Video 2: Onset of dorsal somite extension in Tg(*zic1:GFP*);*Da*.

280   Dorsal view of time-lapse *in vivo* imaging of 24 ss Tg(*zic1:GFP*);*Da* embryo. 15th somite is

281   positioned in center, z-stacks were imaged every 10 min, time is displayed in min. Bright cell

282   at the bottom migrating to the right is a melanophore. Anterior = left, scale bar = 50 μm.

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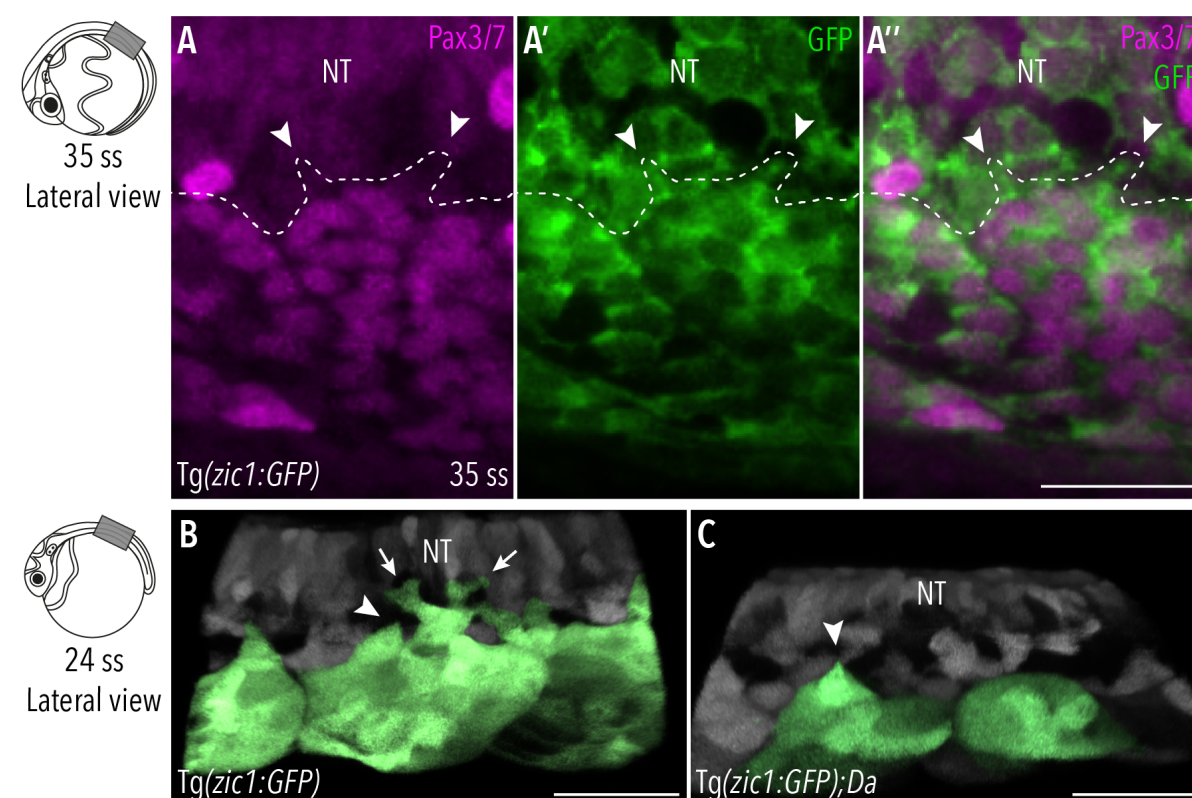
285   Figure 3 - figure supplement 1: Protrusion forming cells at the tips of the dorsal somites are

286   DM cells.

16

287   (A-A'') Dorsal view of immunostaining performed on Tg(*zic1:GFP*) embryos at 35 ss. GPF

288   labels Zic1-positive dorsal somite cells (green), Pax3/7 labels DM cells (magenta).

289   Arrowheads indicate protrusions, scale bar = 50 μm.

290   (B-C) Lateral view of 3D-reconstractions of dorsal somites (green) from (B) Tg(*zic1:GFP*)

291   and (C) Tg(*zic1:GFP*);*Da* embryos at 24 ss. 10th somite is positioned in center, small and

292   large protrusions are exemplary labeled by arrowheads and arrows respectively. Scale bar =

293   25 μm.

294   Anterior = left, dorsal = up, NT = neural tube.

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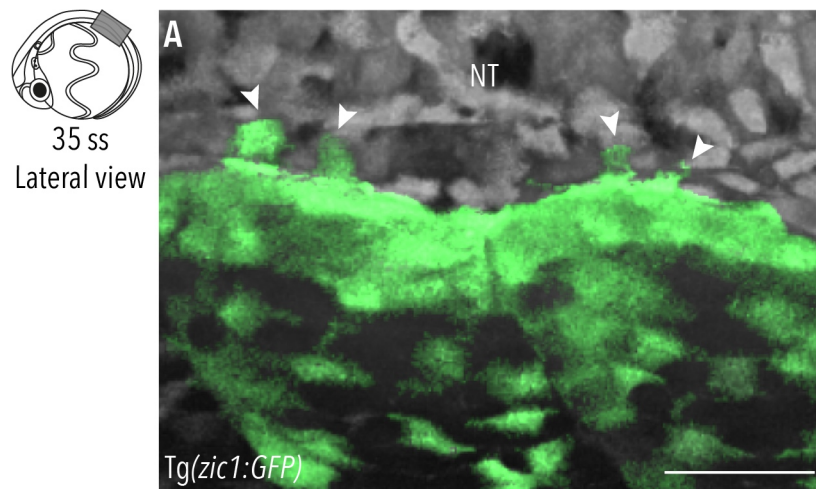
297   Figure 3 - figure supplement 2: Dorsal DM cells form protrusions throughout dorsal somite

298   extension.

299   (A) Lateral view of 3D-reconstractions of dorsal somites (green) from Tg(*zic1:GFP*) at 35 ss

300   5th somite is positioned in center, large protrusions are indicated with arrowheads.

301   Anterior = left, dorsal = up, NT = neural tube, scale bar = 50 μm

302

303   **DM cells delaminate and accumulate between opposing somites during the late phase of**

304   **dorsal somite extension**

305   We continued to trace the behavior of the DM tip cells until the somites reach the top of the

306   neural tube. Indeed, *in vivo* imaging of Tg(*zic1:GFP*) revealed that dorsal DM cells

307   continued to form protrusions, and additionally, some of them delaminated to become Zic1-

308   positive mesenchymal cells accumulating in the space between the dorsal ends of the left and

309   the right somites (Figure 4A, star-shaped cells, arrowheads) from 4.5 dpf (stage 33) onwards.

310   This is consistent with previous observations of strongly *zic1* expressing mesenchymal cells

311   in Wt at late embryonic stages (Ohtsuka *et al.*, 2004). As dorsal somite extension proceeded,

312   the number of these mesenchymal cells increased, filling the space between the two

313   myotomes (Figure 4A-D, arrowheads, Video 3-4, arrowheads indicate representative

314   mesenchymal cells). These mesenchymal cells formed protrusions towards neighboring

315   mesenchymal cells and DM cells at the tip of somites, creating a dense cellular network

316   between the dorsal ends of the somites. Mosaic cell-labeling demonstrated that the

317   mesenchymal cells originated from the DM (Figure 4 - figure supplement 1A-F, arrowheads).

318   Interestingly, while mesenchymal cells dynamically formed protrusions, they showed no

319   extensive migratory behavior and were rather stationary (Video 3-4, Figure 4 – figure

320   supplement 2). This could suggest that these protrusions fulfill a non-migratory function.

321   When the opposing somites came in contact with each other at 8 dpf (stage 38, one day

322   before hatching), the mesenchymal cells tended to attach to the nearest DM cells at the tip of

323   the somite, bridging the gap between the left and right DM cells (Figure 4K-K'''').

324   In *Da* mutants, mesenchymal DM cells were also detected in the space between the two

325   myotomes, but the timing of their appearance was delayed, i.e. between 5 dpf (stage 34) and

326   5.5 dpf (stage 35) (Figure 4E-H) (4.5 dpf in Wt). Additionally, *Da* mesenchymal cells

327   exhibited a rounder morphology and formed significantly fewer protrusions compared to Wt

328   (Figure 4I-J, Figure 4 - figure supplement 1G-L).

329   Collectively, dorsal DM cells and the mesenchymal cells derived from them seem to

330   actively participate in the entire process of dorsal somite extension, from its onset to neural

331   tube coverage at the end.
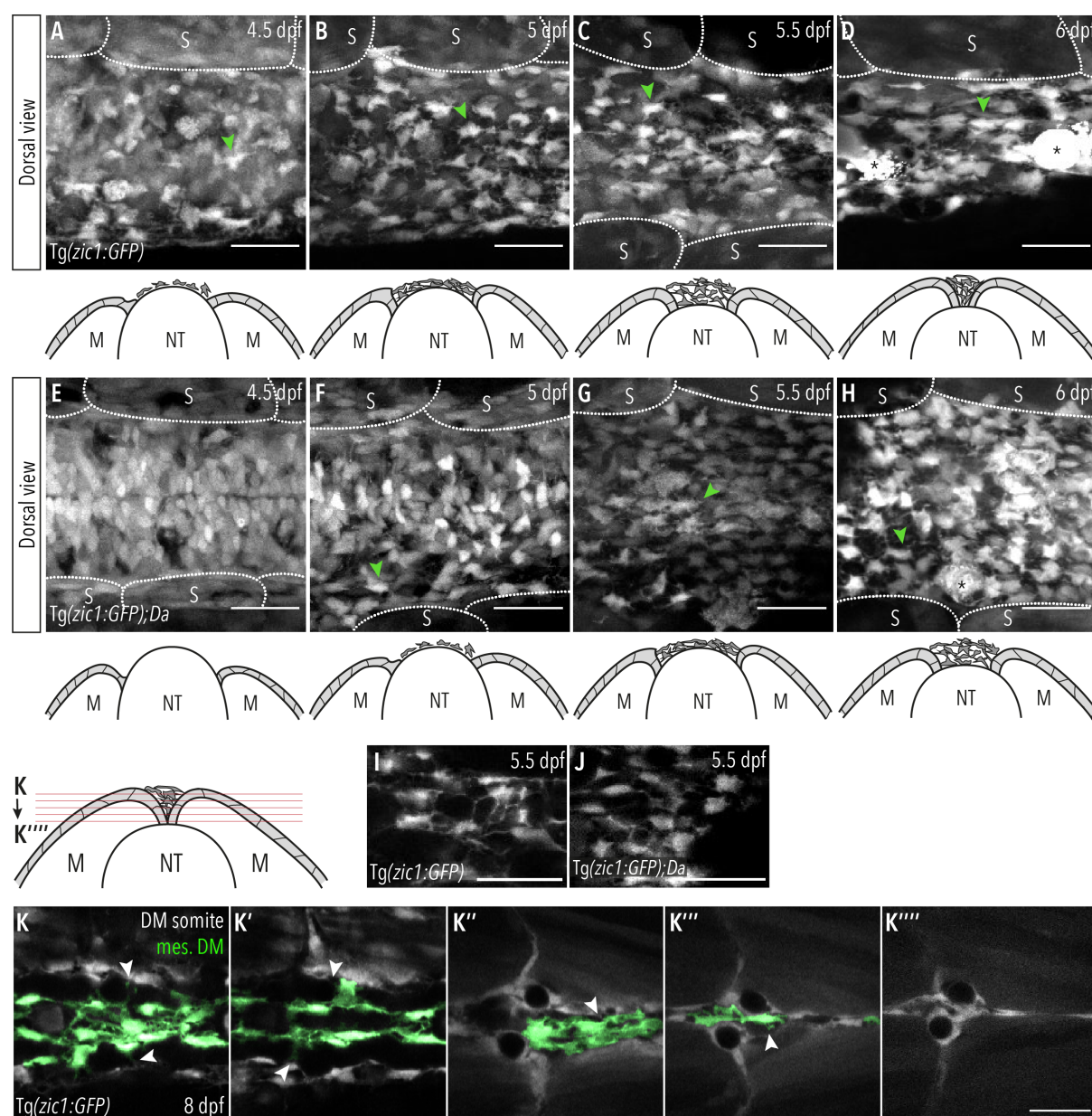
332

333

334   Figure 4: DM cells delaminate from the dorsal somite at the end of dorsal somite extension

335   and accumulate between somites.

336   (A-H) Dorsal view of maximum projection of Tg(*zic1:GFP*) at (A) 4.5 dpf, (B) 5 dpf, (C) 5.5

337   dpf and (D) 6 dpf and Tg(*zic1:GFP*);*Da* at (E) 4.5 dpf, (F) 5 dpf, (G) 5.5 dpf and (H) 6 dpf.

338   10th somite positioned in center, arrowheads point to exemplary mesenchymal DM cells,

339   asterisks mark melanophores. Corresponding schematic representation of cross-section of the

340   trunk beneath each dorsal view.

341   (I-J) Maximum projection of mesenchymal DM cells of Tg(*zic1:GFP*) (I) and

342   Tg(*zic1:GFP*);*Da* (J).

343   (K-K''''') Consecutive z-planes of dorsal view of 10th somite of Tg(*zic1:GFP*) embryo.

344   Mesenchymal DM cells are colored green, arrowheads indicate protrusions formed between

345   somitic DM cell and mesenchymal DM cell.

346   Anterior = left, M = myotome, NT = neural tube, S = somite, scale bar = 25μm.

347

348   Video 3: Mesenchymal DM cells during dorsal somite extension at 4.5 dpf.

349   Dorsal view of time-lapse *in vivo* imaging of 4.5 dpf Tg(*zic1:GFP*) embryo. 10th somite

350   positioned in center, z-stacks were imaged every 10 min, time is displayed in min.

351   Arrowhead indicates representative mesenchymal DM cell. Anterior = left, scale bar = 50

352   μm.

353

354   Video 4: Mesenchymal DM cells during dorsal somite extension at 5.5 dpf.

355   Dorsal view of time-lapse *in vivo* imaging of 5.5 dpf Tg(*zic1:GFP*) embryo. 10th somite is

356   positioned in center, z-stacks were imaged every 10 min, time is displayed in min.

357   Arrowhead indicates representative mesenchymal DM cell. Anterior = left, scale bar = 50
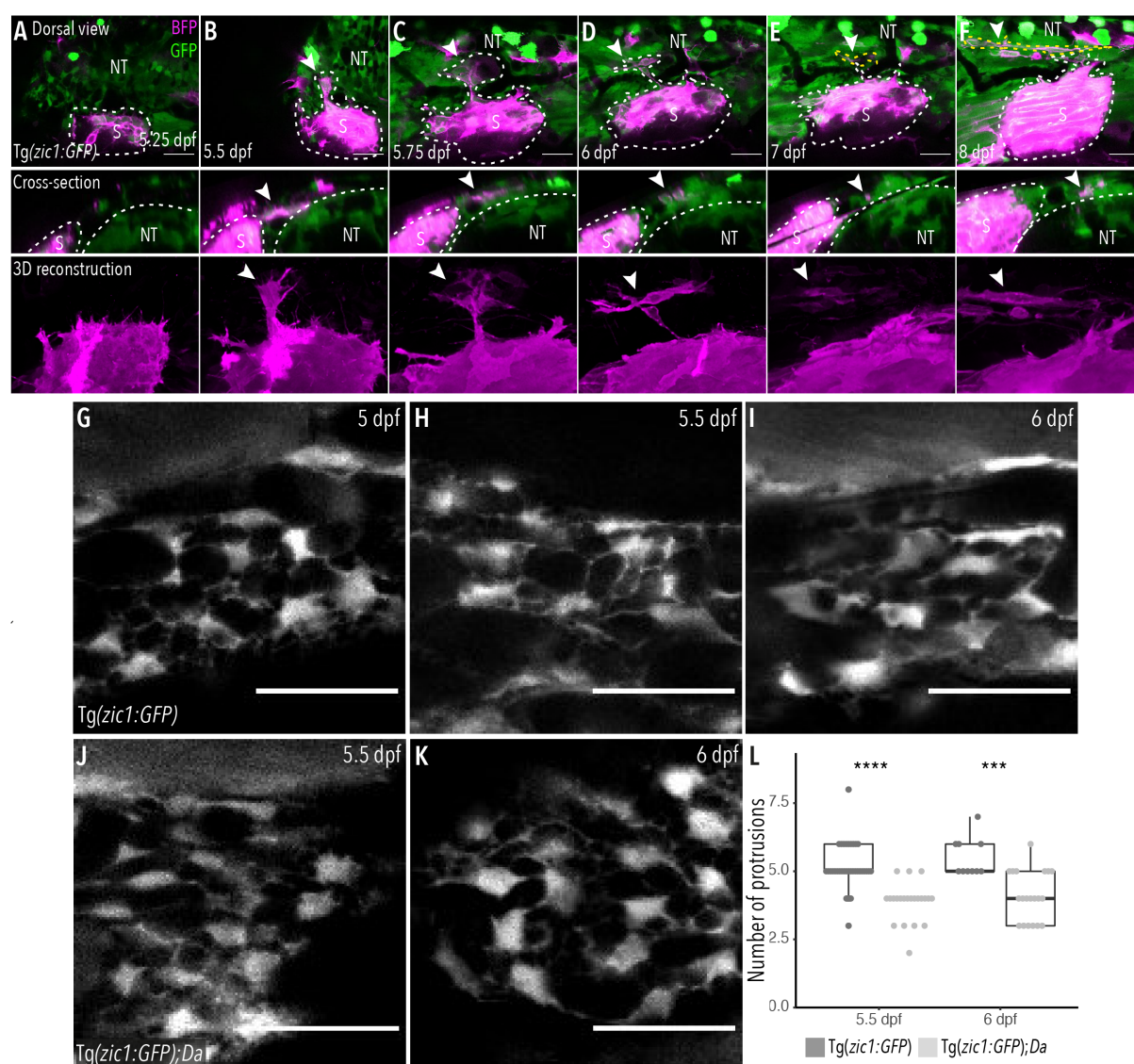
358   μm.

359

360

361

Figure 4 - figure supplement 1: Mesenchymal DM cells originate from dorsal somites.

(A-F) Lineage tracing experiment to clarify the origin of mesenchymal DM cells. Dorsal somite cells express BFP (magenta). (B-F) Arrowheads indicate DM cell originating from labelled somite which (B-C) extends dorsally and (D) delaminates, becoming a mesenchymal DM cell. Somites are outlined in white, mesenchymal DM cell is outlined in yellow (E, F).

(G-K) Maximum projections of mesenchymal DM cells of Tg(*zic1:GFP*) at (G) 5 dpf, (H) 5.5 dpf and (I) 6 dpf and Tg(*zic1:GFP*);*Da* at (J) 5.5 dpf and (K) 6 dpf.

(L) Quantification of protrusions of mesenchymal DM cells at 5.5 dpf (n = 40 cells from Tg(*zic1:GFP*), n = 22 cells from Tg(*zic1:GFP*);*Da*) and 6 dpf (n = 10 cells from

372   Tg(*zic1:GFP*), n = 20 cells from Tg(*zic1:GFP*);*Da*)(median, first and third quartiles,

373   ****$P_{5.5\ dpf}$ = 4.4e-07, ***$P_{6\ dpf}$ = 0.00012,).

374   Anterior = left, NT = neural tube, scale bar = 25μm.
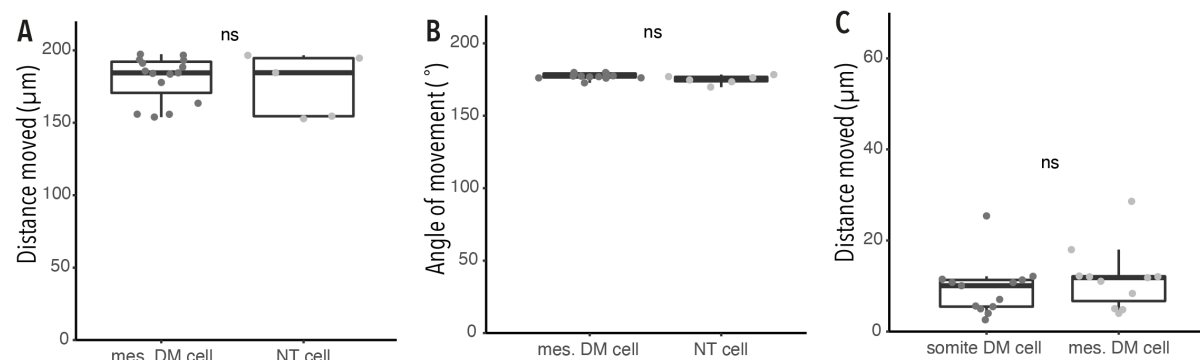
375

377   Figure 4 - figure supplement 2: Mesenchymal DM cells do not migrate significantly.

378   (A) Analysis of distance moved relative to imaging field by mesenchymal DM cells (mes.

379   DM cell, n = 15 DM cells) and cells of the neural tube (NT cell, n = 5 NT cells) during 13.3 h

380   *in vivo* imaging of 4.5 dpf Tg(*zic1:GFP*) embryo (video 3) (median, first and third quartiles,

381   ^{ns}P = 0.73).

382   (B) Analysis of angle moved relative to imaging field by mesenchymal DM cells (n = 15 DM

383   cells) and cells from the neural tube (n = 5 NT cells) during 13.3 h *in vivo* imaging of 4.5 dpf

384   Tg(*zic1:GFP*) embryo (video 3) (median, first and third quartiles, ^{ns}P = 0.1).

385   (C) Analysis of distance moved relative to imaging field by DM cells from the tip of the

386   dorsal somite (somite DM cell, n = 11 somite DM cells) and mesenchymal DM cells (mes.

387   DM cells, n = 13 mesenchymal DM cells) during 15 h *in vivo* imaging of 5.5 dpf

388   Tg(*zic1:GFP*) embryo (video 4) (median, first and third quartiles, ^{ns}P = 0.4).

389

390   **Zic1 regulates the expression of dorsal-specific genes during somite differentiation**

391   We then addressed the molecular machinery controlling the dorsal somite extension

392   investigated above. Since somite extension is impaired in the *zic1*-enhancer mutant *Da*

22

393   (Moriyama *et al.*, 2012; Kawanishi *et al.*, 2013), we reasoned that downstream genes of Zic1

394   are regulators of this process.

395   First, we identified genes which are specifically expressed in the dorsal somites. For

396   this, we dissected somites of the transgenic line Tg(*zic1:GFP*) and FACS sorted them into

397   cells from the dorsal (GFP+) and ventral (GFP-) somites, including the DM, and performed

398   RNA-seq and ATAC-seq on both cell populations (Figure 5A, Figure 5 - figure supplement

399   1A). The RNA-seq identified 1,418 differentially expressed genes. Among them 694 genes

400   showed higher expression in the dorsal somites (termed hereafter dorsal-high genes), and 724

401   genes showed higher expression in the ventral somites (termed hereafter dorsal-low genes)

402   (Figure 5C). We confirmed that *zic1* and *zic4* were found among the dorsal-high genes

403   (Figure 5C, Supplementary table 1).

404   Next, we identified potential direct Zic1-target genes by investigating Zic1 binding

405   sites. Since there were no suitable antibodies available to perform ChIP-seq against medaka

406   Zic1, we created a transgenic line expressing a Myc tagged *zic1* in the *Da* background under

407   the control of *zic1* promoter and enhancers (Tg(*zic1:zic1-Myc;zic4:DsRed*));*Da,* called

408   *Tg(zic1:zic1-Myc);Da* hereafter). This transgene *(zic1:zic1-Myc)* efficiently rescued the

409   ventralized phenotype (dorsal and anal fin shape, pigmentation pattern, and body shape) of

410   the *Da* mutant (Figure 5 - figure supplement 1B-C), verifying the full functionality of the

411   tagged Zic1 protein. Somites of the transgenic line *Tg(zic1:zic1-Myc);Da* were dissected and

412   subjected to ChIP-seq using antibodies against Myc (Figure 5B). Since somites contain a low

413   number of cells, we applied ChIPmentation (Schmidl *et al.*, 2015) to identify genome-wide

414   Zic1 binding sites. From two biological replicates, 5,247 reliable ChIP peaks were identified,

415   and we confirmed that the enriched DNA motif among these peaks was showing high

416   similarity with previously identified binding motifs of ZIC family proteins (Figure 5 - figure

417   supplement 2A-B). Then, we associated each Zic1 peak to the nearest gene within 50 kb and

23

418   identified 3,232 genes as Zic1 target genes. By comparing the ChIPmentation with RNA-seq

419   data, we found that Zic1 target genes are overrepresented in dorsal-high and dorsal-low

420   genes. While 47% of the dorsal-high genes and 27% of the dorsal-low genes were found to be

421   Zic1 target genes, only 12% of genes which showed no differential expression in dorsal or

422   ventral somites were potential Zic1 downstream target genes (Figure 5D, Supplementary

423   table 1). This suggests that Zic1 can function as transcriptional activator and repressor of

424   versatile genes, but the former role seems to be dominant.

425

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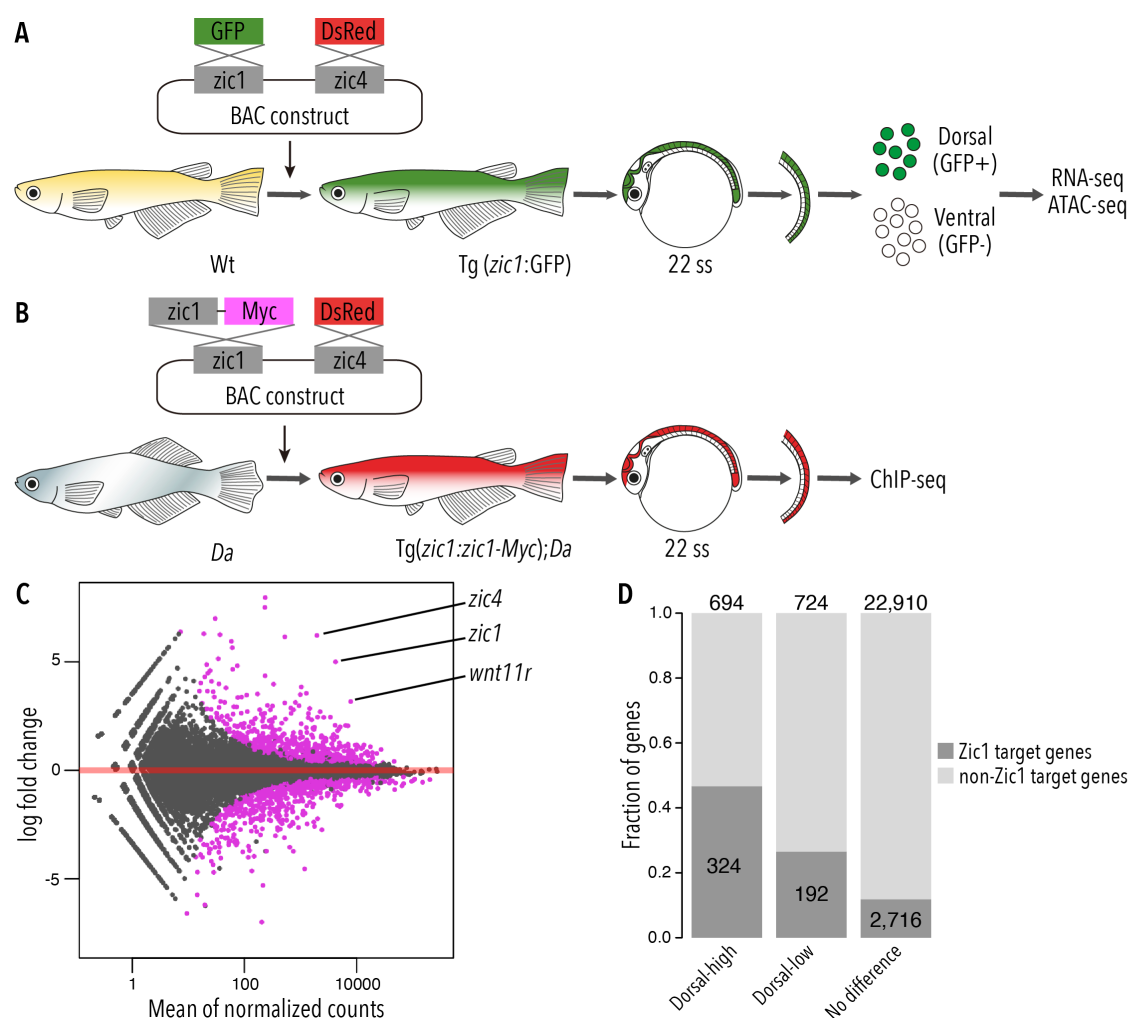
427   Figure 5: Zic1 regulates dorsal-specific expression of genes in the somites.

428   (A) Schematic representation of preparation of dorsal and ventral somite cells for RNA-seq

429   and ATAC-seq.

24

430   (B) Schematic representation of generating the transgenic line Tg(zic1:*zic1-Myc*);*Da.*

431   Somites expressing *zic1-Myc* are subjects to ChIPmentation against Myc.

432   (C) Analysis of RNA-seq revealed that 694 genes are expressed specifically in the dorsal

433   somites (among these genes are *zic1* and *zic4*) and 724 genes are expressed specifically in

434   ventral somites. Magenta indicates differentially expressed genes (adjusted p-value<0.01).

435   (D) The ChIPmentation against Zic1 revealed that 324 of dorsal-high genes, 192 dorsal-low

436   genes and 2,716 non differential expressed genes in the somites are potential Zic1 target
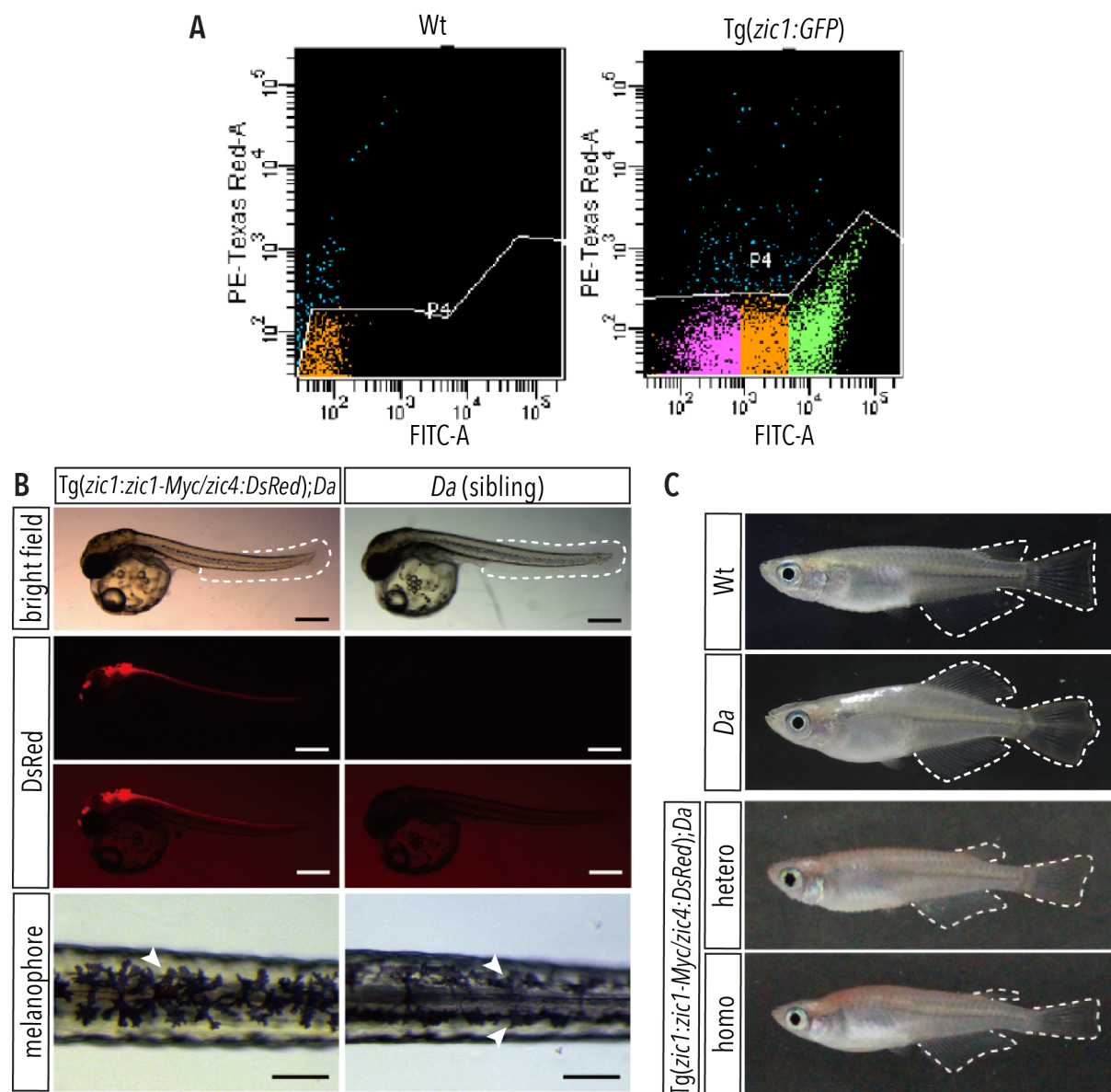
437   genes.

438

439

440

Figure 5 - figure supplement 1: In Tg(*zic1:zic1-Myc*);*Da* fish, the ventralized trunk

phenotype is rescued.

(A) A representative flow cytometry plot of GFP expression in somite cells. Cells in green

and magenta regions were collected as GFP positive and negative cells, respectively. PE-

Texas Red-A indicates dead cells by PI staining. FITC shows GFP signal intensity.

(B-C) *Da* phenotype is rescued in the transgenic line Tg(*zic1:zic1-Myc*);*Da*. DsRed is

expressed in the neural tube and dorsal somites, mimicking the endogenous expression of

zic1 and *zic4* (scale bar = 500 μm). *Da* mutant embryos display two rows of melanophores on

the dorsal midline of their trunk (arrowheads, scale bar = 100 μm). In Tg(*zic1:zic1-Myc*);*Da*

26

450   embryos, as in Wt embryos, a single row of melanophores (arrowhead) on the dorsal midline

451   is found. Ventralized phenotypes of adult *Da* mutants (including body shape, pigmentation,

452   fin shape (outlined)) are rescued in Tg(*zic1:zic1-Myc*);*Da* adult fish. (C) Images of Wt and

453   *Da* adult medaka were first shown in Figure 1B,D.

454

Figure 5 - figure supplement 2: Common ZIC family DNA motifs are enriched in Zic1 peaks.

456   (A) Top DNA motif enriched in Zic-Myc ChIPmentation peaks identified by HOMER.

458   (B) Known motifs matched to DNA motif in (A). For each match, the upper motif, identified

459   in this study (A), was compared to a known motif (lower motif). Matches were analyzed by

460   HOMER.

27

461

462   Supplementary table 1: Gene expression profiles and distances to nearest Zic1 ChIP peak

463

464   **_Wnt11r_ is a direct downstream target of Zic1 and down-regulated in the dorsal somites**

465   **of the _Da_ mutant**

466   To identify potential regulators of dorsal somite extension, we further investigated the

467   differentially expressed Zic1 target genes. Gene Ontology (GO) analysis indicated that both

468   dorsal-high and dorsal-low gene groups, regardless of whether they are Zic1 targets or non-

469   Zic1 targets, were significantly enriched in development related GO terms (Figure 6A, Figure

470   6 - figure supplement 1A, Supplementary table 2, 3). This indicates that Zic1 regulates a

471   number of developmental genes both directly and indirectly. These results are consistent with

472   the fact that Zic1 regulates various dorsal-specific morphologies of somite-derivatives

473   (Kawanishi _et al._, 2013).

474   In the dorsal-high Zic1 targets, GO terms related to cell migration showed higher

475   enrichment (e.g. "chemotaxis" (P=5.62E-10), "locomotion" (6.65E-15), "ameboidal-type cell

476   migration" (P=9.32E-10)) than dorsal-low genes or non-Zic1 target genes (Supplementary

477   table 3). Interestingly, Wnt signaling pathway genes (e.g.: _axin2, wnt11r, sp5, lrp5, fzd10,_

478   _prickle1a_) and semaphorin-plexin signaling pathway genes (e.g.: _sema3a, sema3c, sema3g,_

479   _plxna1, plxna2, plxnb2, plxnb3, nrp2_) were included in these gene groups. Indeed, the GO

480   terms "Wnt signaling pathway" and "Semaphorin-plexin signaling pathway" themselves were

481   also significantly enriched (P=4.45E-7 and 1.94E-8, respectively) in dorsal-high Zic1 target

482   genes (Supplementary table 2). This suggests that Zic1 directly regulates Wnt pathway,

483   semaphorin and plexin genes, possibly to regulate cell movement in the dorsal somites. We

484   also noticed that the terms "extracellular matrix organization" (P=7.74E-7; e.g.: _adamts20,_

485   *fbln1*), "cell communication" (P=1.95E-5; e.g.: *efna5, epha3*) are enriched, suggesting that

486   these genes also affect dorsal somite cell behavior.

487   Wnt signaling pathway components also ranked high among the dorsal-high Zic1 target

488   genes by pathway enrichment analysis (Figure 6B, Figure 6 - figure supplement 1B). Among

489   them, we focused on *wnt11r* for further analyses due to the following reasons: First, *wnt11r*

490   was one of the most differentially expressed genes in dorsal somites (Figure 5C,

491   Supplementary table 1). Second, previous studies implicated Wnt11 in protrusion formation

492   and cell migration (Ulrich *et al.*, 2003; De Calisto *et al.*, 2005; Garriock and Krieg, 2007;

493   Matthews *et al.*, 2008). This is particularly interesting since DM cells also exhibit protrusions

494   and migration activity during dorsal somite extension, which are defective in *Da* mutants.

495   At the *wnt11r* locus, peaks of the Zic1-ChIP overlapped with intergenic open chromatin

496   regions downstream of *wnt11r*. These sites were more accessible in dorsal somites than in

497   ventral somites, suggesting that Zic1 regulates *wnt11r* via enhancers (Figure 6C).

498   Additionally, *in situ* hybridizations against *wnt11r* performed on Wt and *Da* embryos

499   indicated that *wnt11r* expression is significantly reduced at the dorsal tip of *Da* dorsal

500   somites including the DM (Figure 6 - figure supplement 1C-D). Already when the *zic1*

501   expression becomes restricted in the dorsal somites (12 ss) and with proceeding development

502   when *zic1* expression gets further restricted to the most dorsal part of the somites and

503   mesenchymal DM cells (Ohtsuka *et al.*, 2004), the expression of *wnt11r* in the dorsal somites

504   of the *Da* mutant was reduced compared to Wt (Figure 6D-F, Figure 6 - figure supplement

505   1C-F').

506   Taken together, we identified *wnt11r* as promising downstream target gene of Zic1,

507   which could be a novel somite dorsalization factor and play a role in dorsal somite extension.

508

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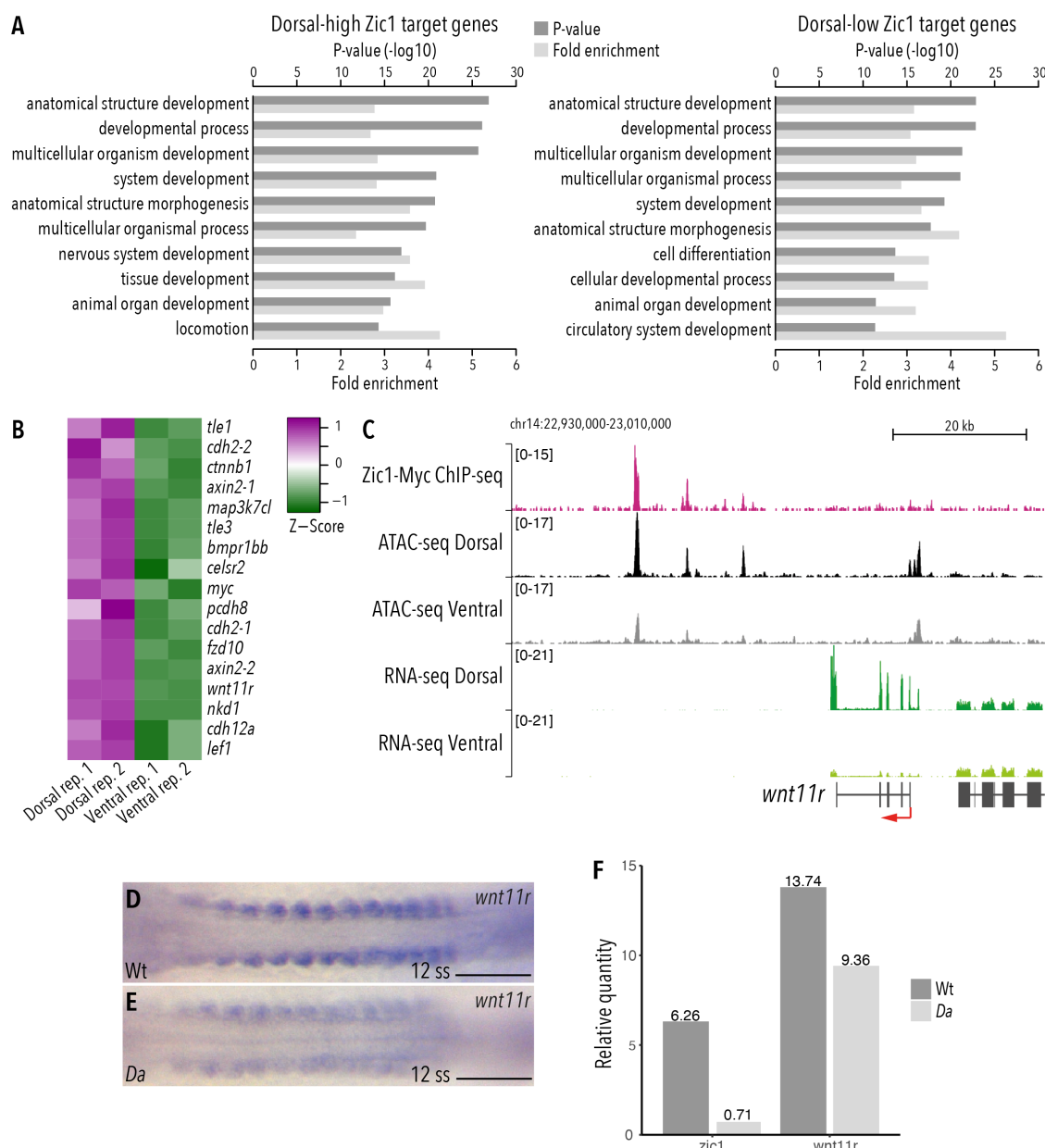
Figure 6: *Wnt11r* is a direct downstream target of Zic1 and down-regulated in the dorsal somites of the *Da* mutant.

(A-A') GO analysis of (A) dorsal-high and (A') dorsal-low Zic1 target genes.

(B) Pathway enrichment analysis indicated that genes associated with Wnt signaling pathway are specifically enriched in dorsal-high Zic1 target genes.

(C) Analysis of the *wnt11r* locus. Peaks form the ChIPmentation against Zic1 (magenta track) overlap with open chromatin regions in the dorsal somites (black track), while this genomic region is less open in ventral somites (grey track). RNA-seq data revealed that

30

518   *wnt11r* is highly expressed in dorsal somites (dark green track) and minimally in ventral

519   somites (light green track).

520   (D-E) Dorsal view of tails of whole-mount *in situ* hybridization against *wnt11r* performed in

521   (D) Wt and (E) *Da* 12 ss embryos. The expression of *wnt11r* is reduced in the *Da* mutant.

522   (F) RT-PCR performed on pooled tails of Wt and *Da* 12 ss embryos indicates that *zic1*

523   expression is reduced by 8.8-fold and *wnt11r* expression by 1.4-fold in the *Da* mutant.
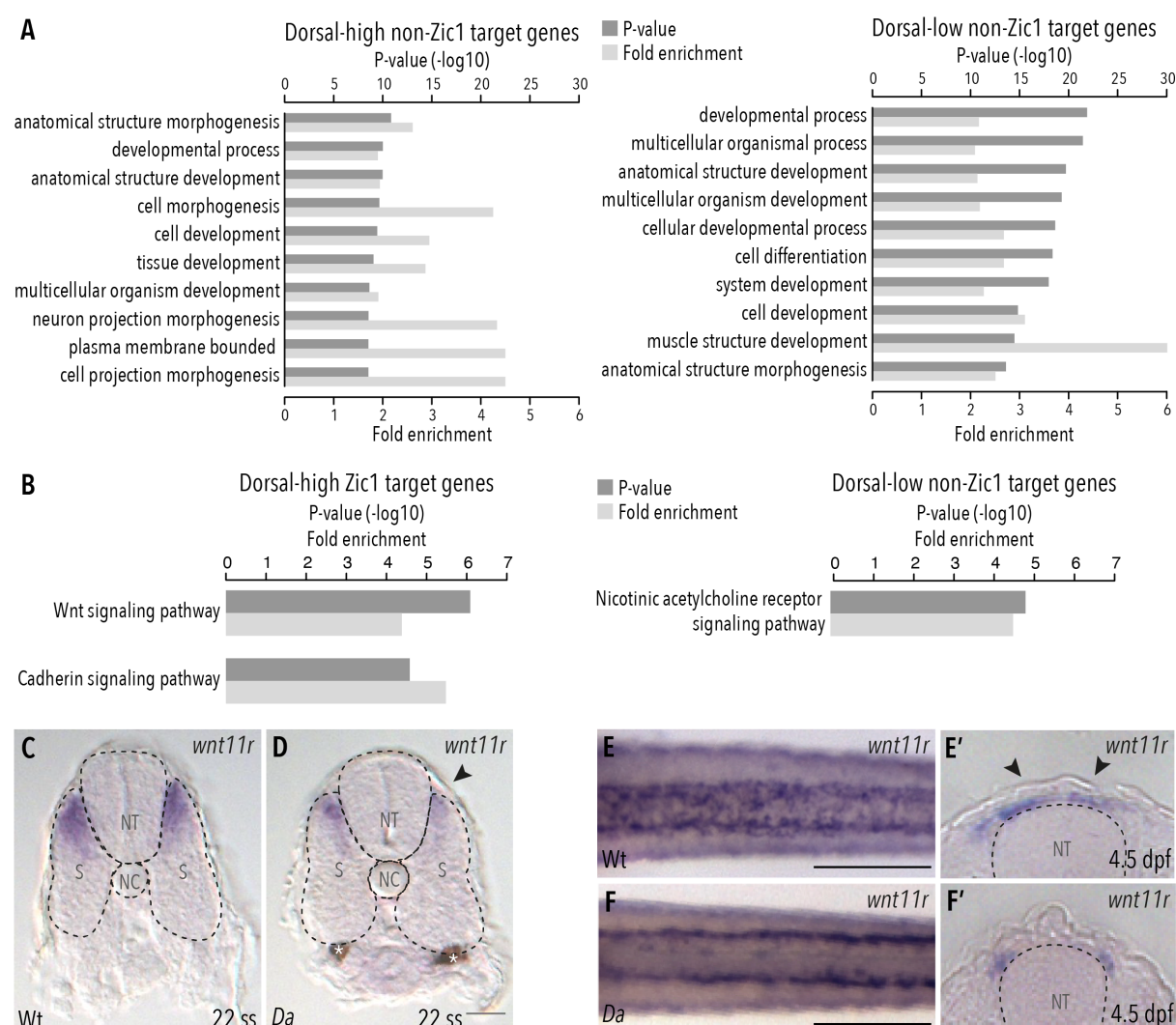
524   Anterior = left, scale bar = 100 μm.

525

526

527   Figure 6 - figure supplement 1: Wnt signaling pathway is enriched in dorsal-high Zic1 target

528   genes.

31

529   (A) GO analysis of dorsal-high and dorsal-low non-Zic1 target genes.

530   (B) Pathway enrichment analysis of dorsal-high Zic1 target genes and dorsal-low non-Zic1

531   target genes.

532   (C-D) Sections of the trunk of whole-mount *in situ* hybridization against *wnt11r* performed

533   on 22 ss (C) Wt and (D) *Da* mutant embryos. The expression of *wnt11r* is reduced in the

534   dorsal somites of the *Da* mutant (arrowhead). Asterisks mark melanophores, scale bar = 38

535   μm.

536   (E-F) Whole-mount *in situ* hybridization against *wnt11r* on (E-E') Wt and (F-F') *Da* embryos

537   at 4.5 dpf (dorsal view: E,F, cross-section: E', F'). In Wt *wnt11r* expression is further

538   restricted to the dorsal most part of the somites (E') and the mesenchymal DM cells (E-E',

539   arrowheads). The expression of *wnt11r* is reduced in the dorsal somites of the *Da* mutant (F-

540   F'). Scale bar = 100 μm

541   Dorsal = up, NC = notochord, NT = neural tube, S = somite, scale bar = 38 μm, sections = 40

542   μm.

543

544   Supplementary table 2: Full list of GO terms enriched in dorsal-high Zic1 target genes

545

546   Supplementary table 3: Full list of GO terms enriched in dorsal-low Zic1 target genes

547

548   **Wnt11r regulates cell behavior of dorsal DM**

549   Our previous RNA-seq dataset revealed that the expression of *wnt11r* starts before

550   gastrulation and increases as development proceeds (Figure 7 - figure supplement

551   1A)(Nakamura *et al.*, 2021). This makes it challenging to examine the role of *wnt11r* during

552   late embryonic development using loss-of-function experiments. We took two different

553   approaches to knock-down Wnt11r during dorsal somite extension. Firstly, we used a *wnt11r*

32

554 anti-sense morpholino (*wnt11r* MO) and determined a concentration which resulted in

555 maximal knock-down effects during dorsal somite extension with minimal gastrulation

556 phenotypes (we excluded any embryos showing gastrulation defects in our experiments).

557 Secondly, we performed temporally controlled knock-down of Wnt11r after gastrulation

558 using photo-cleavable Photo-Morpholinos (PhotoMOs) (Tallafuss *et al.*, 2012) (Figure 7A).

559 Strikingly, in *wnt11r* Photo-Morphants with severe phenotypes the dorsal myotome failed to

560 cover the neural tube (n = 7, Figure 7B-C), a phenotype similar to the *Da* mutant myotome.

561 Additionally, the myofibers of the Photo-Morphants at 9 dpf were shorter and less organized.

562 This phenotype is consistent with a previously reported role of Wnt11 during early

563 myogenesis, where it regulates the elongation and orientation of myoblasts (Gros, Serralbo

564 and Marcelle, 2008).

565   Next, we investigated the proliferative activity of dorsal DM cells in *wnt11r*

566 morphants and found that knock-down of *wnt11r* (either by conventional MO or by

567 PhotoMO) induced significantly more PH3-positive cells per somite, compared to control

568 embryos at 22 ss (Figure 7D). These findings are similar to the observations previously made

569 in the dorsal DM of *Da* mutants, and suggest that Wnt11r is negatively regulating

570 proliferation of dorsal DM cells.

571   We then explored whether the onset of dorsal somite extension in *wnt11r* morphants

572 is similarly impaired as in the *Da* by time-lapse *in vivo* imaging. Remarkably, we observed a

573 protrusion formation behavior of *wnt11r* morphant DM cells similar to *Da* DM cells, namely

574 delayed onset of protrusion formation and the formation of fewer, shorter protrusions (Figure

575 7E, Video 5). Quantification of protrusions indicated that *wnt11r* morphants have

576 significantly fewer large protrusions and protrusions in total, compared to control embryos

577 (Figure 7F-H'').

33

578   Overall, the knock-down of the Zic1 target gene Wnt11r recapitulated the phenotype

579   of *Da* DM cells (Figure 3G-G''), showing the essential role of Wnt11r in regulating

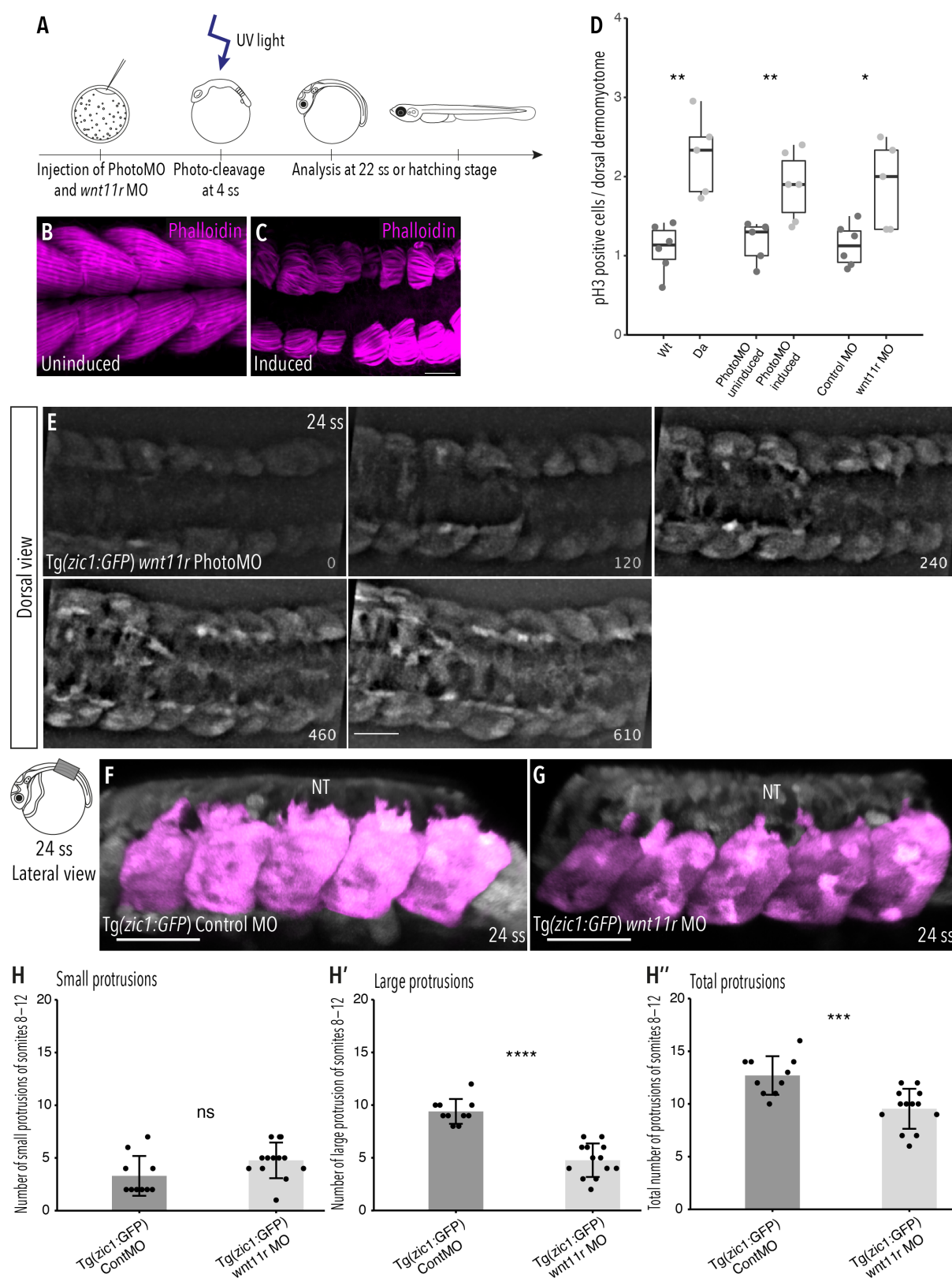580   protrusion formation of DM cells.

581

Figure 7: Knock-down of Wnt11r in Wt embryos recapitulates the *Da* dorsal somite phenotype.

(A) Schematic outline of PhotoMO mediated knock-down of Wnt11r.

586   (B,C) Dorsal view of maximum projection of whole-mount Phalloidin staining of 9 dpf

587   embryos injected with (B) PhotoMO but not photocleaved and (C) photo-cleaved. The

588   epaxial myotomes of the *wnt11r* morphant does not cover the neural tube.

589   (D) Quantification of PH3-positive cells in the dorsal DM of 22 ss embryos. Embryos with

590   reduced levels of Wnt11r in their dorsal somites (n = 40.5 somites from 5 *Da* embryos, n =

591   61.5 somites of 6 *wnt11r* PhotoMO-Morphant embryos and n = 52 somites from 5 *wnt11r*

592   morphant embryos) have significantly more PH3-positive cells in their dorsal DM compared

593   to the respective controls (n = 66 somites from 6 Wt embryos, n = 51 somites of 5 uninduced

594   PhotoMO embryos and n = 65 somites from 6 Control MO embryos) (median, first and third

595   quartiles, **$P_{Wt/Da}$ = 0.0035, **$P_{PhotoMO}$ = 0.0091, *P_{MO} = 0.031).

596   (E) Dorsal view of time-lapse imaging of 24 ss Tg(*zic1:GFP*) embryo injected with

597   PhotoMO-douplex and photo-cleaved at 4 ss. Z-stacks were recorded every 10min, time is

598   displayed in min, 15th somite is positioned in the center.

599   (F-G) Lateral view of 3D-reconstructions of dorsal somites (magenta) of Tg(*zic1:GFP*)

600   injected with (F) Control MO and (G) *wnt11r* MO.

601   (H-H'') Quantification of protrusions formed by the 8th-12th somite of Tg(*zic1:GFP*) injected

602   with Control MO (n = 10 embryos) or *wnt11r* MO (n = 13 embryos) (mean ± SD, $^{ns}P_{small}$

603    $_{protrusions}$ = 0.069, ****$P_{large\ protrusions}$ = 7.7e-08, ***$P_{total\ protrusions}$ = 0.00064).

604   Anterior = left, NT = neural tube, scale bar = 50 μm.

605

606   Video 5: Onset of dorsal somite extension in *wnt11r* morphant embryo.

607   Wnt11r was knocked-down using the PhotoMO approach (Figure 7A) in a Tg(*zic1:GFP*)

608   embryo. Dorsal view of time-lapse *in vivo* imaging of 24 ss Tg(*zic1:GFP*) embryo. 15th

609   somite is positioned in the center, z-stacks were imaged every 10 min, time is displayed in

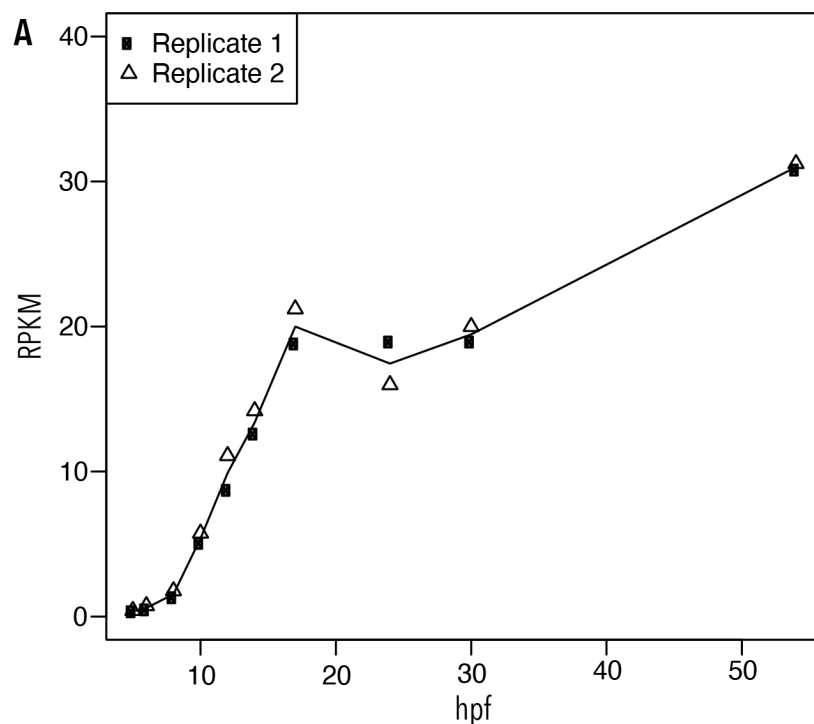610   min. Anterior = left, scale bar = 50 μm.

36

611

612

Figure 7 - figure supplement 1: *Wnt11r* expression starts before gastrulation and increases

with proceeding development.

(A) Reads per kilo base per million mapped reads (RPKM) of *wnt11r* at 5 hpf (hours post

fertilization, stage 9), 6 hpf (stage 10), 8 hpf (stage 11), 10 hpf (stage 12), 12 hpf (stage 13,

onset of gastrulation), 14 hpf (stage 14), 17 hpf (stage 15, gastrulation competed), 24 hpf

(stage 18), 30 hpf (6 ss, stage 21) and 54 hpf (24 ss, stage 27).

619

620   To further confirm the importance of Wnt11r during dorsal somite extension, we

performed rescue experiments in *Da* embryos. At 18 ss (2.1 dpf, stage 25), we injected a mix

of human recombinant Wnt11 (hrWnt11) protein (or BSA in the control group) and Dextran

Rhodamine onto the top of the 10th somite of Tg*(zic1:GFP);Da* embryos (Figure 8A, B).

Strikingly, the dorsal DM of *Da* embryos injected with Wnt11 formed significantly more

large protrusions and more protrusions in total compared to *Da* embryos injected with BSA

only (Figure 8C-C''). This indicates that the ventralized *Da* DM protrusion phenotype can be

37

627   partially rescued by Wnt11 protein injections and further emphasizes the importance of

628   Wnt11r in somite dorsalization and during dorsal somite extension.

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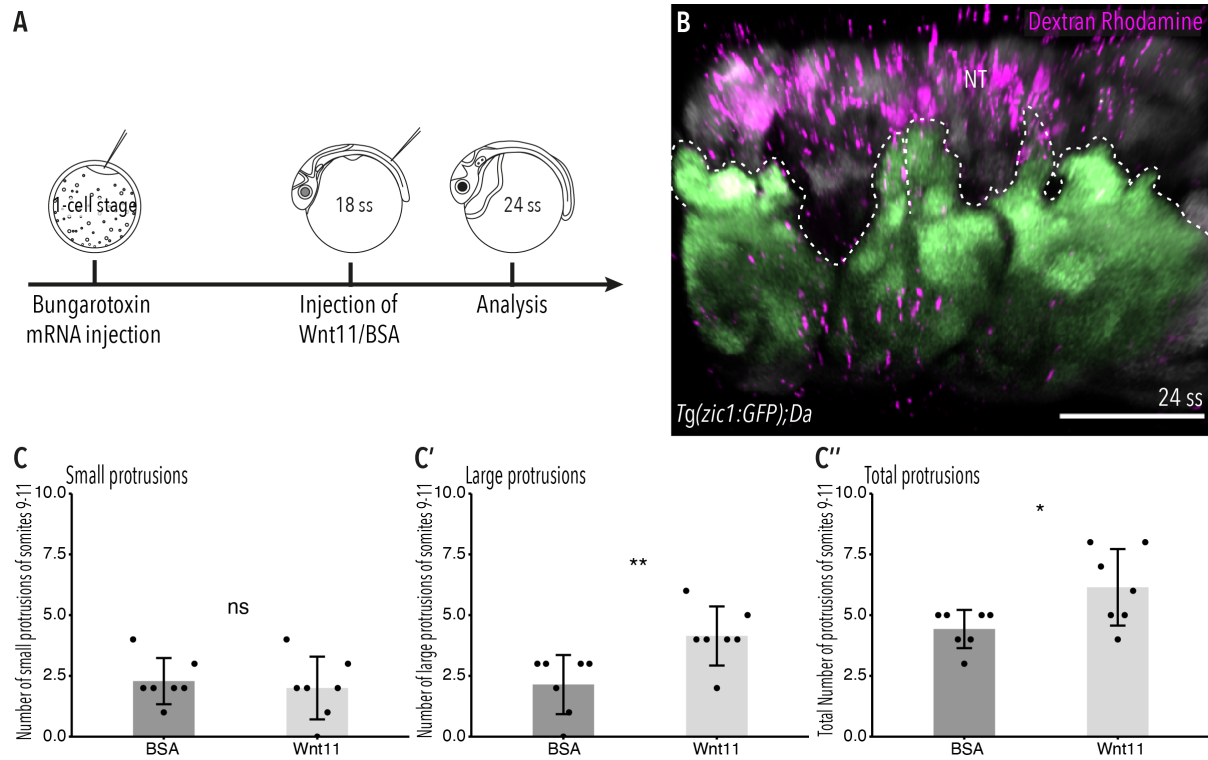
631   Figure 8: Wnt11 injections partially rescue the Tg(*zic1:GFP*);*Da* protrusion phenotype.

632   (A) Schematic representation of Wnt11 protein injection on top of the 10th somite of 18 ss

633   Tg(*zic1:GFP*);*Da)* embryos.

634   (B) Dorsal view of 3D-reconstruction of *in vivo* imaging of dorsal somites (green) of

635   Tg(*zic1:GFP*);*Da* embryos injected with Wnt11r-Dextran Rhodamine mix (magenta).

636   Protrusions are outlined.

637   (C-C'') Quantification of dorsal protrusions of the 9th – 11th somite (somite at injection site

638   plus adjacent somites) of embryos injected with BSA (n = 7 embryos) or hrWnt11(n = 7

639   embryos) (mean ± SD, $^{ns}P_{small\ protrusions} = 0.65$, $**P_{large\ protrusions} = 0.0095$, $*P_{total\ protrusions} = 0.03$).

640   Anterior = left, dorsal = up, NT = neural tube, scale bar = 50 μm.

641

642   **Wnt11r acts through the Wnt/Ca^{2+} signaling pathway at the onset of somite extension**

643   Finally, we investigated through which signaling pathway the non-canonical Wnt11r acts. In

644   *Xenopus*, Wnt11r acts through the Wnt/Ca^{2+} pathway regulating the migration of cells from

645   the dorsal somite and the neural crest into the dorsal fin fold (Garriock and Krieg, 2007). To

646   examine whether this signaling pathway also plays a role during dorsal somite extension, we

647   inhibited the Wnt/Ca^{2+} signaling pathway using KN-93 in Tg(*zic1:GFP*) embryos from 4 ss

648   (1.3 dpf, stage 20) to 22 ss (Figure 9A). KN-93 is known to inhibit CaMKII, a component of

649   the Wnt/Ca^{2+} pathway (Wu and Cline, 1998; Garriock and Krieg, 2007; Rothschild *et al.*,

650   2013). Embryos treated with KN-93 showed a higher number of PH3-positive dorsal DM

651   cells per somite, compared to embryos in the control group (Figure 9B). Furthermore, DM

652   cells at the tip of the dorsal somite of embryos treated with KN-93 formed significantly fewer

653   large and fewer protrusions in total, compared to embryos in the control group (Figure 9C-

654   C''), although the effect was less significant compared to the *wnt11r* morphants (Figure 7).

655   From these results, we suggested that Wnt11r potentially acts through the Wnt/Ca^{2+}

656   signaling pathway at the onset of somite extension. Wnt/Ca^{2+} signaling pathway is known to

657   regulate actin polymerization (Choi and Han, 2002; Kohn and Moon, 2005) which could

658   explain the dynamic protrusive activity of the DM cells (Figure 3), emphasizing the

659   importance of Wnt11r during epaxial myotome morphogenesis.
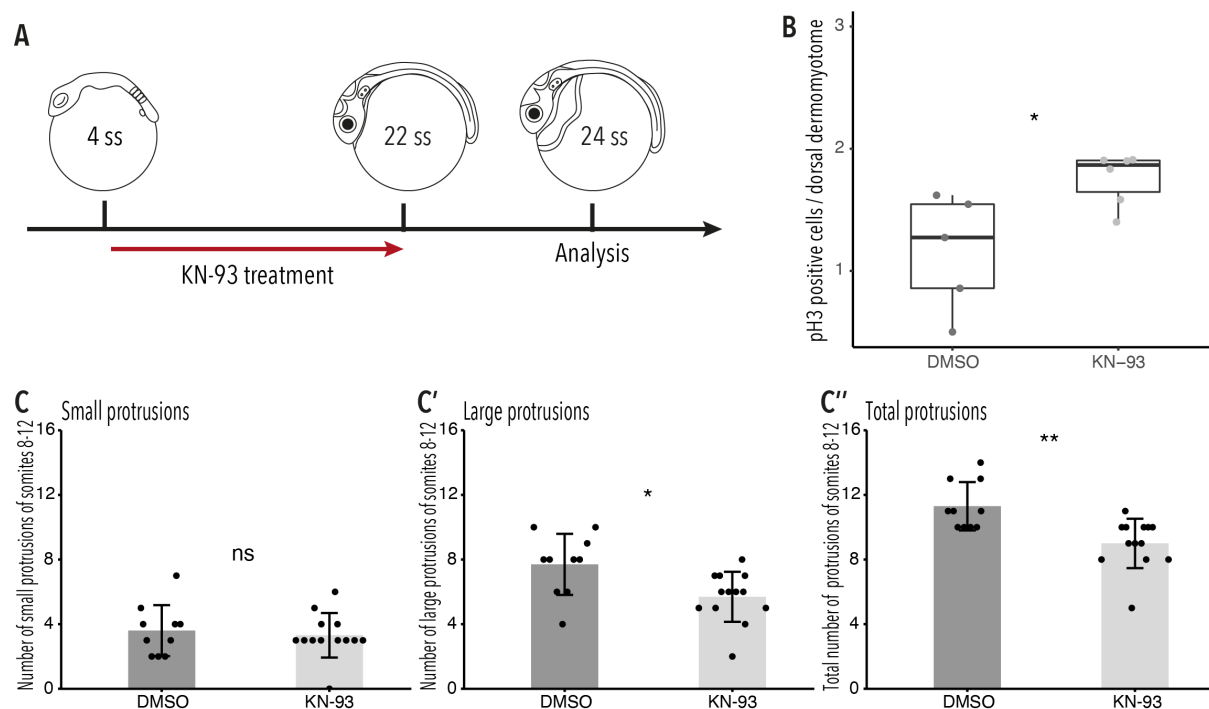
660

Figure 9: Wnt11r acts through the Wnt/Ca^{2+} signaling pathway during dorsal somite extension.

(A) Schematic representation of KN-93 treatment, embryos in the control group were treated with DMSO.

(B) Embryos treated with KN-93 (n = 65.5 somites from 6 embryos) have significantly more PH3-positive cell in the dorsal DM compared to embryos of the control group (n = 52.5 somites from 5 embryos) (median, first and third quartiles, *P = 0.045).

(C-C'') Quantification of protrusions formed by the 8th-12th somites of Tg(*zic1:GFP*) embryos treated with DMSO (n = 10 embryos) or KN-93 (n = 13 embryos)(mean ± SD, ^{ns}P$_{small\ protrusions}$ = 0.65, *P$_{large\ protrusions}$ = 0.014, **P$_{total\ protrusions}$ = 0.0017).

Discussion

Here, we elucidate the key developmental process underlying the epaxial myotome morphogenesis in teleost fish, medaka. At the initiation of dorsal somite extension, DM cells at the tip of the dorsal somite form unique large, motile protrusions, which extend dorsally,

677 and potentially play a pioneering role in guiding the myotome towards the top of the neural

678 tube. By analyzing dorsal somite extension in the ventralized *Da* mutant, we demonstrated

679 that *zic1* is essential for this process. DM cells of the *Da* dorsal somite have a higher

680 proliferative activity and form fewer and shorter protrusions during dorsal somite extension.

681 These altered cellular properties of *Da* dorsal DM cells, together with a delayed onset of

682 dorsal somite extension, potentially cause the incomplete coverage of the neural tube by the

683 epaxial myotome at the end of embryonic development.

684 Furthermore, we investigated the molecular background of dorsal somite extension

685 and identified a direct downstream target of Zic1, the non-canonical Wnt *wnt11r*, as a crucial

686 factor for dorsal somite extension. Wnt11r reduces the proliferative activity of DM cells in

687 the dorsal somites, but instead, promotes the formation of large, motile protrusions of dorsal

688 DM cells at the tip of the somite. Indeed, *wnt11r* morphants recapitulate the phenotype of the

689 *Da* mutant. Additionally, the protrusion phenotype of *Da* dorsal DM cells can be partially

690 rescue by injection of Wnt11 proteins. Based on these findings, we propose a model for

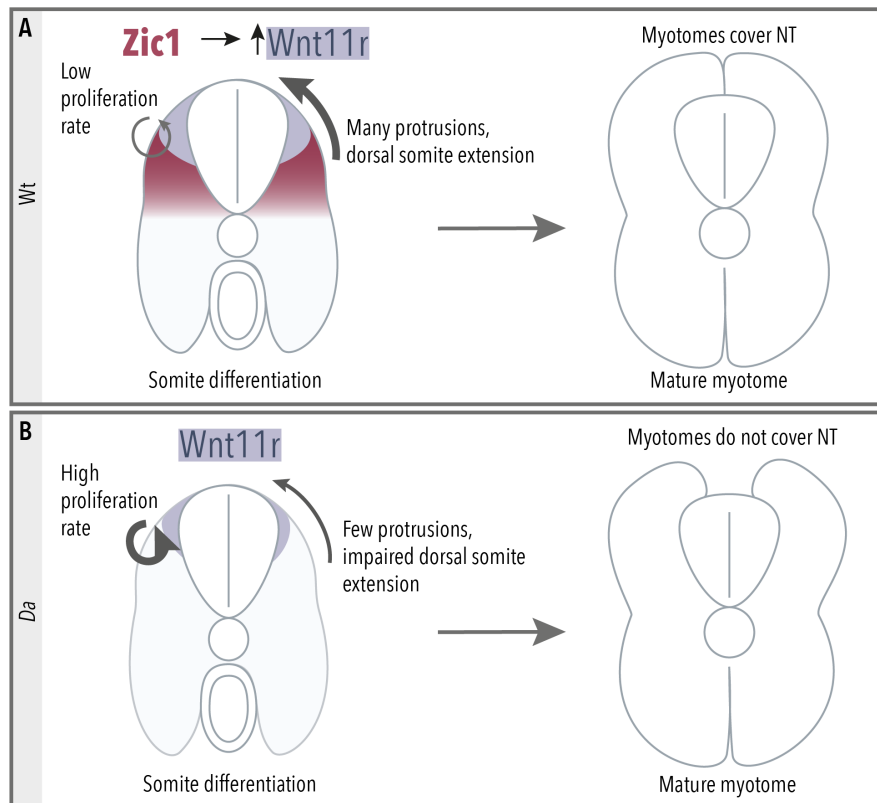691 dorsal somite extension shown in Figure 10.

692

693

694 Figure 10: Summary of dorsal somite extension in Wt and the *Da* mutant.

695 (A) Zic1 induces or maintains the expression of *wnt11r* during somite differentiation. This

696 leads to a reduced proliferative activity of the dorsal DM and increases protrusion formation

697 of the dorsal DM cells. Ultimately, the DM guides the epaxial myotomes dorsally where the

698 myotomes form a gapless muscle layer covering the neural tube at the end of embryonic

699 development.

700 (B) In the *Da* mutant *wnt11r* expression is reduced in the dorsal somites. The dorsal DM cells

701 show a high proliferative activity and reduced ability to form numerous large protrusions

702 causing insufficient dorsal somite extension. This results in the incomplete coverage of the

703 neural tube by the epaxial myotomes at the end of embryonic development.

704

705   In the present study, we described the characteristic behavior of dorsal DM cells and

706 suggest their guiding role for the epaxial myotome moving towards the top of the neural tube.

707 During dorsal somite extension, DM cells at the tip of the dorsal somites form large, motile

708 protrusions which contain multiple bundles of filipodia-like protrusions dynamically

709 branching out from the tip of the protrusions. These long protrusions could be beneficial for

710 invading the restricted open space between the neural tube and the ectoderm. Additionally,

711 previous studies showed that in migrating mesenchymal cells, the formation of lamellipodia

712 is associated with higher migratory speed, whereas filopodia play an exploratory role and are

713 associated with high directionality (Leithner *et al.*, 2016; Innocenti, 2018). Large protrusions

714 of wild-type DM cells consist of a lamellipodia-like core and multiple filopodia, which could

715 account for fast dorsal somite extension with high accuracy. The detailed structure and

716 function of the large protrusions needs to be further investigated in the future studies.

717 At later stages of dorsal somite extension, we observed that DM cells delaminate from

718 the tip of the dorsal somites and progressively occupy the space between the opposing dorsal

719 somites. These mesenchymal DM cells actively form protrusions towards neighboring

720 mesenchymal DM cells and DM cells of the somites, thus forming a dense cellular network

721 on top of the neural tube. This could provide a communication platform for the opposing

722 somites to meet at the right position, exactly on top of the neural tube.

723 The ability of the motile DM cells to guide the underlying myotome dorsally assures

724 complete coverage of the neural tube by the epaxial myotomes. Considering the fact that DM

725 cells are in close cell-cell contact with the underlying myotome, it is plausible that they are

726 biomechanically coupled with myotomal cells, facilitating dorsal myotome extension. A

727 similar mechanism has been investigated in the mouse neural tube formation. During mouse

728 neural tube closure, the surface ectoderm, overlying the neuroepithelium, forms cell

729 protrusions towards the midline. Disruption of membrane ruffles, a form of lamellipodia, in

730 the surface ectoderm results in incomplete fusion of the subjacent neuroepithelium (Rolo *et*

731 *al.*, 2016).

43

732   In medaka dorsal somites, Wnt11r exerts its effect through promotion of protrusion

733   formation and down-regulation of cell proliferation in the dorsal DM. Regarding the

734   protrusion formation, previous studies of the zebrafish mutant *silberblick* and migrating

735   neural crest cells consistently reported that Wnt11 is involved in the oriented elongation and

736   stabilization of protrusions (Ulrich, 2003; De Calisto *et al.*, 2005; Matthews *et al.*, 2008).

737   Similarly, myocardial cells in the heart of *Wnt11* mutant mice also form fewer protrusions

738   (Zhou *et al.*, 2007). Thus, regulation of cell protrusions is a conserved function of Wnt11,

739   observed in various developmental processes. However, the relationship between Wnt11 and

740   cell proliferation could be context-dependent, as it negatively regulates proliferation in mouse

741   neonatal hearts (Touma *et al.*, 2017), while it promotes cell proliferation in mouse intestinal

742   epithelial cell culture (Ouko *et al.*, 2004). Hence, the function of Wnt11r in medaka somites

743   is unique in that it promotes tissue elongation by regulating a balance between proliferative

744   and migrative activity.

745   By inhibiting the Wnt/Ca^{2+} signaling pathway we showed that Wnt11r probably acts

746   through this non-canonical Wnt signaling pathway during dorsal somite extension. Likewise,

747   previous studies in *Xenopus* have shown that Wnt11r acts through this pathway during cell

748   migration into the dorsal fin fold. There it regulates epithelial–mesenchymal transition in a

749   distinct dorsal somite cell population which, together with a population of neural crest cells,

750   contribute to the mesenchyme of the dorsal fin fold (Garriock and Krieg, 2007). Furthermore,

751   during convergent extension in vertebrate gastrulation, Wnt/$Ca2^{+}$ pathway can regulate cell

752   adhesion by promoting actin polymerization (Choi and Han, 2002; Kohn and Moon, 2005).

753   Importantly, our RNA-seq and ChIPmentation analyses revealed that Zic1 has diverse

754   downstream target genes including various developmental genes. This suggests that the

755   dorsal myotome is established via pleiotropic actions of Zic1; Wnt11r may not be a sole

756   factor for dorsal somite extension, although it was shown to be essential in the present study.

757   The semaphoring-plexin pathway may play a role, since previous studies suggested, besides

758   its implication in axon guidance and neural cell migration, a role in non-neural cell migration

759   (Alto and Terman, 2017). Furthermore, cell migration and tissue deformation are often linked

760   with the extracellular matrix (ECM), which provides guiding or restraining cues influencing

761   cell movements. Previous studies showed that during mouse epaxial myotome development,

762   ECM composition changes dynamically, which is tightly accompanied by epaxial muscle

763   morphogenesis; while the laminin content in the ECM decreases, increasing tenascin and

764   stable fibronectin contents potentially promotes the alignment of myofibers and their final

765   organization (Deries *et al.*, 2012). Furthermore, recent studies suggested that cells can

766   remodel the surrounding ECM and thereby increase their own motility. For example, during

767   zebrafish gastrulation, the metalloproteinase *mmp14* is expressed by migrating endoderm

768   cells and degrades laminin and fibronectin, components of the ECM (Hu et al., 2018). In this

769   context, of particular importance is our identification of *Adamts20*, encoding a

770   proteoglycanase, as a dorsal-high Zic1-target gene in the somites (Supplementary table 1).

771   Since Adamts20 is known to play a pivotal role in embryonic melanoblast migration by

772   remodeling the dermal ECM (Rao *et al.*, 2003; Silver *et al.*, 2008), Adamnts20 could thus

773   facilitate migration of Wnt11r-expressing DM cells through remodeling of the ECM. Further

774   functional analysis of dorsal-high Zic1-target genes identified in somites will provide useful

775   insight into the molecular network driving dorsal somite extension.

776   Finally, in vertebrates and especially in fish, body shape and muscle morphology are

777   closely linked, since a majority of the body mass consist of muscular tissue. Previous studies

778   in fish populations have shown that speciation and adaptation to a specific aquatic habitat are

779   associated with a changes in body depth, a measurement of the trunk dorsoventral axis

780   (Tobler *et al.*, 2008; Elmer *et al.*, 2010; Weese, Ferguson and Robinson, 2012; Fruciano *et*

781   *al.*, 2016). In this context, the external appearance of the adult *Da* mutant is intriguing, in that

782   it exhibits a teardrop body shape (typical for fish swimming in the middle layer like tuna),

783   instead of a dorsally flattened one (typical for surface swimming fish like medaka). Since our

784   study shows that the activity of *wnt11r* could influence the body shape by regulating cell

785   proliferation and the behavior of muscle progenitor cells, Wnt11r could be one of the crucial

786   factors in evolution and diversity of body shape in fish. Furthermore, the expression of *zic1*

787   (Ohtsuka *et al.*, 2004; Sun Rhodes and Merzdorf, 2006; Houtmeyers *et al.*, 2013) and *wnt11r*

788   (Marcelle, Stark and Bronner-fraser, 1997; Olivera-Martinez, Thelu and Dhouailly, 2004;

789   Garriock *et al.*, 2005; Matsui *et al.*, 2005) are strongly conserved among vertebrates, and we

790   hypothesize that Wnt11r-mediated morphogenesis of the somites represents an evolutionarily

791   conserved mechanism that acts across vertebrates.

792   **Materials and Methods**

793   **Key resource table**

794

795   **Animals and transgenic lines**

796   Fish were raised and maintained under standard conditions. All experimental procedures and

797   animal care were performed according to the animal ethics committee of the University of

798   Tokyo. Sex was randomly assigned to experimental groups. Medaka d-rR stain was used as

799   wild-type, the *Da* mutant used in this study was previously described (Ohtsuka *et al.*, 2004).

800   The pre-existing transgenic line Tg(*zic1:GFP/zic4:DsRed*) (Kawanishi *et al.*, 2013) was used,

801   and the transgenic line Tg(*zic1:GFP/zic4:DsRed*);*Da* was created by crossing *Da* mutants

802   with Tg(*zic1:GFP/zic4:DsRed*). The transgenic line Tg(*zic1:zic1-Myc/zic4:DsRed*);*Da* was

803   generated by modifying the BAC used to generate Tg(*zic1:GFP/zic4:DsRed*) (Kawanishi *et*

804   *al.*, 2013), by replacing the ORF of *GFP* with the ORF of *zic1* containing a sequence for a

46

805   Myc-tag fused to its C-terminus. To establish the transgenic line, the BAC(*zic1:zic1-*

806   *Myc/zic4:DsRed)* was co-injected with I-SceI Meganuclease (NEB) into 1-cell stage *Da*

807   embryos, as previously described (Thermes *et al.*, 2002).

808

Visualization of actin skeleton of protrusions

810   The AC-TagGFP2 sequence from the Actin-Chromobody plasmid (TagGFP2)

811   (Chromotek) was cloned into the pMTB vector for mRNA generation. To investigate the

812   actin skeleton of protrusions, cells of Wt and *Da* embryos were mosaically labelled using

813   *Actin-Chromobody-GFP* (*AC-GFP*) mRNA. Embryos were injected at 1-cell stage with 152

814   ng/µl *membrane-mCherry* mRNA. At 4-cell stage, one cell was injected with 184 ng/µl *AC-*

815   *GFP* mRNA.

816

Lineage tracing of mesenchymal DM cells

818   Mosaic labelling of cells was achieved by co-injecting 20 ng/µl pMTB-memb-mTagBFP2

819   plasmid with Tol2 into a 1-cell stage embryo of the transgenic line

820   Tg(*zic1:GFP/zic4:DsRed*). Embryos were raised until 5 dpf and labelled cells were

821   continuously observed until 8 dpf.

822

Morpholino injection

824   Microinjections into medaka embryos were performed using 12.5 µM *wnt11r* Morpholino

825   antisense oligonucleotides (MO). Injections were performed into 1-cell stage embryos.

826

Photo-Morpholino mutagenesis

828   *Wnt11r* Sense-Photo-Morpholino (PhotoMO) and *wnt11r* antisense Morpholino were

829   annealed in a ratio 2:1. Microinjection of 25 µM of the annealed oligonucleotides was

47

830   performed into 1-cell stage embryos and embryos were raised until 4 ss in the dark. Photo-

831   cleavage was performed using the 10x objective and the DAPI filter of a Keyence BZ-9000

832   Biorevo microscope (Keyence). Embryos were mounted, dorsal side facing up, in 1 %

833   Methylcellulose in a glass bottom dish (Wako) and illuminated for 30 min. After Photo-

834   cleavage, embryos were dechorionated and raised until the desired stage for subsequent

835   analysis.

836

837   **Injection of human recombinant Wnt11 protein into *Da* mutant somite**

838   To immobilize embryos, embryos from the Tg(*zic1:GFP/zic4:DsRed*);*Da* transgenic line

839   were injected with 25 ng/µl *α-bungarotoxin* mRNA at 1-cell stage. At 18 ss embryos were

840   mounted in 1 % low melting agarose in 1x Yamamoto's Ringer Solution and oriented with

841   the dorsal side facing upwards. Embryos were injected on top of the 10th somite with a mix

842   containing Dextran Rhodamine (Thermo Fisher) and 1.7 ng hrWnt11 protein (R&D Systems)

843   or BSA (Sigma-Aldrich) and raised to 24 ss, followed by *in vivo* imaging and analysis.

844

845   **KN-93 treatment**

846   Dechorionated 4 ss embryos were treated with 30 µM KN-93 (Wako, Japan) or DMSO

847   (Sigma Aldrich, Germany) in 1x Yamamoto's Ringer Solution at 28 °C, in the dark until 22

848   ss was reached.

849

850   ***In vivo* imaging and *in vivo* time-lapse imaging**

851   To immobilize embryos for *in vivo* imaging, embryos were injected at 1-cell stage with 25

852   ng/µl *α-bungarotoxin* mRNA (Swinburne *et al.*, 2015; Lischik, Adelmann and Wittbrodt,

853   2019). Embryos were mounted in 1 % Low melting agarose (Sigma Adrich) in 1x

854   Yamamoto's Ringer Solution in a glass-bottomed petri dish (IWAKI) and oriented with the

855   dorsal side facing down. Imaging was performed using a Zeiss LSM 710 confocal

856   microscope system (Zeiss) equipped with an inverted stand and a Zeiss AXIO Observer Z1

857   and a T-PMT detector. The embryos were positioned with the 5th or 10th somite in the center

858   and images were acquired using a 40x water objective. For the *in vivo* time-lapse imaging,

859   the 10th or 15th somite was positioned in the center, z-stacks were imaged in a 600-sec

860   interval for 10-15 h. Image analysis was performed in Fiji using the "Image Stabilizer"

861   Plugin, the FFT Bandpass filter and the "Draw_arrows" Plugin to draw customized arrows

862   (Li, 2008; Daetwyler, Modes and Fiolka, 2020).

863

Whole mount *in situ* hybridization

865   Whole mount *in situ* hybridization was performed as previously described with the following

866   modifications (Takashima *et al.*, 2007). Embryos were fixed in 4 % PFA/1.5x PTW at 4 °C,

867   overnight. Hybridization was performed at 65 °C, overnight. Samples were treated with

868   alkaline-phosphatide anti-DIG-AP Fab fragments (1:2000, Roche). Signals were developed

869   using 4-nitro blue tetrazolium chloride (NBT, Roche) and 5-bromo-4-chloro-3-indolyl

870   phosphate (BCIP, Roche).

871

Whole-mount immunohistochemistry

873   Embryos were fixed in 4 % PFA/PBS for 2 h at room temperature or at 4 °C overnight.

874   Samples were permeabilized with 0.5 % TritonX-100 (Wako) in 1x PBS for 1-2 h and

875   blocked in blocking solution (2 % BSA (Sigma-Aldrich), 1 % DMSO, 0.2 % TritonX-100 in

876   1x PBS) for 2-4 h at room temperature. Samples were incubated with respective primary

877   antibody diluted in blocking solution at 4 °C, overnight. After an additional 4 h blocking step,

878   samples were incubated with respective secondary antibodies diluted in blocking solution, at

879   4 °C, overnight. Samples were stored in 1x PBS at 4 °C until imaging.

880

881 **Antibodies**

Primary antibody	Species	Concentration	Company
anti-Pax3/7 (DP312)	mouse	1:100	Davis et al., 2001
anti-Ph3	rabbit	1:200	Millipore, 06-570
anti-GFP	rabbit	1:200	Clontech, 632593

882

Secondary antibody	Species	Concentration	Company
anti-mouse Alexa 555	goat	1:500	Thermo Fisher, A-21422
anti-rabbit Alexa 488	donkey	1:500	Life Technnologies, A-11008

883

884 **Vibratome sectioning**

885 Samples were mounted in 4 % agarose in 1x PBS. 40 μm or 200 μm sections were obtained

886 by a Vibratome (Leica, Vibratome). The sections were mounted on a glass slide (Matsunami)

887 in 60 % glycerol (Merck, Wako) and stored at 4 °C until imaging. Images were acquired

888 using the 40x water objective of a Zeiss LSM 710 confocal microscope.

889

890 **Histological sections**

891 Dechorionated embryos were fixed in Bouin's solution overnight, followed by a gradual

892 dehydration using ethanol. Samples were embedded in Technovit 7100 (Heraeus Kulzer) and

893 sectioned into 5-6 μm thick sections. Sections were stained with hematoxylin (Wako) and

894 imaged using the 1.6x objective of a Leica M165 FC fluorescent stereo microscope.

895

50

Image processing and statistical analysis

Image processing was performed with the image processing software Fiji. The 3D-recreation of *in vivo* imaging date was created using FluoRender (Wan *et al.*, 2009). Measurements of morphological features (distance between myotome tips, area of cross-section of dorsal somites, diameter of myofibers, distance between dorsal somite tip and the tip of neural tube, somite height) was performed by averaging the analysis of the feature from 3 consecutive Y planes. RStudio was used for the statistical analysis and representation of the data. In bar plots mean and error limits, defined by the standard deviation, are indicated. In box plots median first and third quantiles are indicated. Statistical significance was determined by un-paired t-tests, a p-value $p < 0.05$ was considered as significant. In the figure legends sample size (n) and number of individuals used in the experiment are stated. Sample sizes were not predetermined using statistical methods, but the sample sizes used are similar to those generally used in the field. To compare experimental groups, the allocation was performed randomly, without blinding.

Isolation of dorsal and ventral somite cells for ATAC-seq and RNA-seq

Yolk and head region were removed from 22 ss Tg(*zic1:GFP/zic4:DsRed*) embryos and embryos were incubated with 10 mg/ml pancreatin (Wako) at room temperature for 5-10 min. Epidermis and intestinal tissues were removed and somites were isolated from the neural tube. Cells were dissociated in 0.5% (w/v) Trypsin (Nacalai Tesque) at 37 °C for 10 min, the dissociation was stopped by adding the same volume of 15% (v/v) FBS / Leiboviz's L-15 (Life Technologies). Dissociated cells were washed with PBS, and sorted into GFP positive (dorsal) and negative (ventral) cells using FACSAria III (BD Biosciences). Dead cells were detected by Propidium iodide (Life Technologies) and removed.

921   **RNA-seq**

922   Total RNA was extracted from sorted somite cells using RNeasy Mini kit (Qiagen). mRNA

923   was enriched by poly-A capture and mRNA-seq libraries were generated using KAPA

924   Stranded mRNA-seq Kit (KAPA Biosystems). Libraries were generated from two biological

925   replicates, and sequenced using the Illumina HiSeq 1500 platform.

926

927   **RNA-seq data processing**

928   The sequenced reads were pre-processed to remove low-quality bases and adapter derived

929   sequences using Trimmomatic v0.32 (Bolger, Lohse and Usadel, 2014), and aligned to the

930   medaka reference genome (HdrR, ASM223467v1) by STAR (Dobin *et al.*, 2013). Reads with

931   mapping quality (MAPQ) larger than or equal to 20 were used for the further analyses.

932

933   **ATAC-seq**

934   ATAC-seq was performed as previously described (Buenrostro *et al.*, 2013) with some

935   modifications. Approximately 4,000 sorted somite cells were used for each experiment. After

936   washing with PBS, cells were resuspended in 500 μl cold lysis buffer (10 mM Tris-HCl pH

937   7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% Igepal CA-630), centrifuged for 10 min at 500 g,

938   supernatant was removed. Tagmentation reaction was performed as described previously

939   (Buenrostro *et al.*, 2013) with Nextera Sample Preparation Kit (Illumina). After tagmented

940   DNA was purified using MinElute kit (Qiagen), two sequential PCRs were performed to

941   enrich small DNA fragments. First, a 9-cycle PCR was performed using indexed primers

942   from Nextera Index Kit (Illumina) and KAPA HiFi HotStart ReadyMix (KAPA Biosystems),

943   amplified DNA was size selected for a size less than 500 bp using AMPure XP beads

944   (Beckman Coulter). A second 7-cycle PCR was performed using the same primers as for the

945   first PCR. PCR product was purified by AMPure XP beads. Libraries were generated from

946   two biological replicates, and sequenced using the Illumina HiSeq 1500 platform.

947

948   **ChIPmentation**

949   Yolk and head region were removed from 22 ss Tg(*zic1:zic1-Myc/zic4:DsRed*);*Da* embryos,

950   followed by an incubation with 10 mg/ml pancreatin (Wako) at room temperature for 5-10

951   min. Epidermis and intestinal tissues were removed and somites were isolated from the

952   neural tube. ChIP was performed as previously described with the following modifications

953   (Nakamura *et al.*, 2014). Isolated somites were fixed with 1% formaldehyde for 8 min at

954   room temperature then quenched by adding glycine (200 mM final) and incubating on ice for

955   5 min. After washing with PBS, cell pellets were stored at -80 °C. Approximately 1.8×10^6

956   cells were thawed on ice, suspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM

957   EDTA, 1% SDS, 20 mM Na-butyrate, complete protease inhibitors, 1 mM PMSF) and

958   sonicated 10 times using a Sonifier (Branson) at power 5. Chromatin lysates were collected

959   by centrifugation and diluted 10-fold with RIPA ChIP buffer (10 mM Tris-HCl (pH 8.0), 140

960   mM NaCl,1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.2% sodium

961   deoxycholate, 20 mM Na-butyrate, complete protease inhibitors, 1 mM PMSF) followed by

962   an incubation with antibody/protein A Dynabeads (Invitrogen) complex at 4 °C, overnight,

963   while rotating. Immunoprecipitated samples were washed three times with RIPA buffer (10

964   mM Tris-HCl (pH 8.0), 140 mM NaCl,1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1%

965   SDS, 0.2% sodium deoxycholate) and once with TE buffer. After the washing steps 150 μl of

966   Tris-HCl was added.

967   Library preparation for ChIPmentation was performed as previously described

968   (Schmidl *et al.*, 2015) with the following modifications. 24 μl of Tagmentation reaction mix

969   (10 mM Tris-HCl pH8.0, 5 mM MgCl$_2$, 10%(v/v) N,N-dimethyl formamide) and 1 μl of

53

970   Tagment DNA Enzyme from Nextera Sample Preparation Kit (Illumina) were added to the

971   DNA-beads complex and incubated for 70 sec at 37 °C. 150 μl ice-cold RIPA buffer was

972   added and incubated for 5 min on ice. The DNA-beads complex was washed with RIPA

973   buffer and TE buffer, suspended in 50 μl lysis buffer and 3 μl of 5 M NaCl, and incubated at

974   65 °C, overnight. The sample was incubated for 2 h with 2 μl of 20 mg/ml ProteinasK

975   (Roche), and subjected to AMPure XP beads (Beckman Coulter) purification. The library

976   was amplified by 18-cycle PCR using indexed primers from Nextera Index Kit (Illumina) and

977   KAPA HiFi HotStart ReadyMix (KAPA Biosystems). For the input chromatin, tagmentation

978   reaction was performed after DNA purification. Libraries were generated from two biological

979   replicates, and sequenced using the Illumina HiSeq 1500 platform.

980

981

982   **ChIPmentation and ATAC-seq data processing**

983   The sequenced reads were pre-processed to remove low-quality bases and adapter derived

984   sequences using Trimmomatic v0.32 (Bolger, Lohse and Usadel, 2014) and aligned to the

985   medaka reference genome (HdrR, ASM223467v1) by BWA (Li and Durbin, 2009). Reads

986   with mapping quality (MAPQ) larger than or equal to 20 were used for the further analyses.

987   MACS2 (version 2.1.1.20160309) (Zhang *et al.*, 2008) was used to call peaks and generate

988   signals per million reads tracks using following options; ChIPmentation: -g 600000000 -B --

989   SPMR --keep-dup 2, ATAC-seq: --nomodel --extsize 200 --shift -100 -g 600000000 -q 0.01 -

990   B --SPMR.

991   For ChIPmentation, peak regions called by two biological replicates were used as

992   reliable peaks.

993

994   **Motif analyses of Zic1 ChIPmentation peaks**

995   Motifs enriched at reliable ChIPmentation peaks were analyzed by findMotifsGenome

996   command of HOMER (Heinz *et al.*, 2010) using default parameters.

997

998   **Identification of Zic1 target genes**

999   Differentially expressed genes were identified using DESeq2 (padj < 0.01). Each reliable

1000   ChIP peak was associated to the nearest TSS, and the gene was defined as Zic1-target gene if

1001   the distance between the peak and the TSS was closer than 50 kb.

1002

1003   **Gene ontology and pathway analyses**

1004   The gene ontology enrichment analyses and pathway enrichment analyses were performed

1005   using the Gene Ontology Resource (Ashburner *et al.*, 2000; Gene Ontology Consortium,

1006   2021).

1007

1008   **RT-PCR of cDNA generated from embryonic tails**

1009   To investigate the gene expression in tails of embryos, tails were dissected anterior from the

1010   first somite. Ten tails were pooled together and RNA was isolated using Isogen (Nippon

1011   Gene). RNA was purified using the RNeasy Mini kit (Qiagen) and reverse transcribed to

1012   cDNA using the Super Script III Kit (Invitrogen). RT-PCR was performed using the

1013   Thunderbird Sybr qPCR Mix (Toyobo) following manufacturer's instructions and run in the

1014   Agilent Mx3000P qPCR System (Agilent). Normalization of relative quantities was

1015   performed against *gapdh* expression, followed by analysis with excel and RStudio.

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