A fibronectin mechanotransduction pathway translates segmentation clock oscillations into periodic somite formation

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Abstract:

Somitogenesis starts with cyclic waves of expression of segmentation clock genes in the presomitic mesoderm (PSM) and culminates with periodic budding of somites in its anterior-most region. How cyclic clock gene expression is translated into timely morphological somite formation remains unclear. A posterior to anterior gradient of increasing fibronectin matrix complexity surrounds the PSM, suggesting that the mechanical properties of the matrix may play a role in regulating this transition. Here we interfered with the chick PSM fibronectin mechanotransduction pathway by perturbing (1) actomyosin-mediated contractility, (2) Rho-associated protein kinase function, (3) integrin-fibronectin binding and (4) fibronectin matrix assembly. Each treatment perturbed segmentation clock expression dynamics, *meso1* expression and resulted in defective somitic clefts. A model is presented where the fibronectin matrix gradient participates in the PSM wavefront of maturation by activating a mechanotransduction signaling cascade ensuring the correct spatio-temporal conversion of cyclic segmentation clock gene expression into periodic somite formation.

Keywords:

Fibronectin, Mechanotransduction, Segmentation Clock, Cleft Formation, Somitogenesis

38 **Introduction:**

39 Cells in the developing embryo are constantly receiving and integrating information,
40 including tension-derived signals generated by the adhesion to neighboring cells and/or the
41 surrounding extracellular matrix (ECM). Cell-surface integrins bind to ECM ligands in the
42 extracellular space and connect the ECM to the intracellular actomyosin cytoskeleton through
43 adaptor proteins (Campbell and Humphries, 2011; Wolfenson et al., 2013). These multimolecular
44 complexes (i.e. focal adhesions) act as mechanotransduction centers allowing cells to perceive the
45 chemical and physical properties of the ECM, such as its molecular composition, density and
46 stiffness. While morphogens have been extensively studied as major chemical regulators of
47 developmental processes (Marek and Kubícek, 1981; Slack, 1987; Tiedemann, 1976), the
48 importance of mechanical forces in embryo development has, until recently, been largely
49 overlooked (Chan et al., 2017; Merle and Farge, 2018). In fact, the ability of cells to sense and
50 respond to mechanical signals has been shown to regulate critical developmental processes,
51 including cell proliferation, migration and differentiation (Barriga et al., 2018; Cosgrove et al.,
52 2016; Smutny et al., 2017; Wolfenson et al., 2016).

53 One of the most conspicuous morphogenetic events during early vertebrate
54 embryogenesis is the formation of somites, which are the source of axial skeleton and skeletal
55 muscle precursor cells (Christ et al., 2007). Somites are spheres of epithelioid cells and are formed
56 periodically from the anterior portion of the mesenchymal presomitic mesoderm (PSM), bilateral
57 to the axial structures (Bailey and Dale, 2015). Temporal control of somite formation is dependent
58 on cyclic waves of expression of segmentation clock genes, many of which are targets of the Notch
59 signaling pathway (Dequéant et al., 2006; Masamizu et al., 2006; Palmeirim et al., 1997). These
60 waves periodically sweep the PSM in a posterior to anterior direction (Aulehla and Pourquié, 2010)
61 and, as they reach the anterior PSM, oscillations first slow down and then arrest (Morimoto et al.,
62 2005; Shih et al., 2015). The transcription factor Mesp2/Meso1 is upregulated downstream of the
63 segmentation clock in the anterior PSM, leading to Eph/Ephrin signaling and somitic cleft

64 formation (Barrios et al., 2003; Nakajima et al., 2006; Saga, 2012; Watanabe et al., 2009), followed
65 by progressive cell rearrangements into a somite (Martins et al., 2009; Morimoto et al., 2005; Shih
66 et al., 2015).

67 The PSM of vertebrate embryos is surrounded by a fibronectin-rich ECM, and both
68 fibronectin and its integrin receptors are essential for somite formation (George et al., 1993;
69 Georges-Labouesse et al., 1996; Goh et al., 1997; Jülich et al., 2005; Koshida et al., 2005; Kragtorp
70 and Miller, 2007; Rifes et al., 2007; Takahashi et al., 2007; Yang et al., 1999, 1993). Moreover,
71 perturbing fibronectin binding to its integrin receptors, by substituting the fibronectin RGD binding
72 site with an RGE sequence (mouse *Fn1*$^{RGE/RGE}$), causes dramatic somitogenesis defects (Girós et al.,
73 2011; Takahashi et al., 2007). Indeed, fibronectin provides the structural support for cells to attach,
74 polarize, change shape and position, required for somite epithelialization (Martins et al., 2009).

75 We have previously shown that the fibronectin matrix assembled around the chick PSM
76 becomes progressively denser and more complex as the tissue matures (Rifes and
77 Thorsteinsdóttir, 2012), and have proposed that this posterior to anterior gradient culminates in
78 a fibronectin-dependent matrix assembly state that supports somite epithelialization in the
79 anterior PSM (Rifes et al., 2007; Rifes and Thorsteinsdóttir, 2012). More recently, adhesion to a
80 fibronectin substrate was noted as a regulator of *Lfng* oscillations in cultured mouse tailbud cells
81 (Hubaud et al., 2017; Lauschke et al., 2013) and cell adhesion to fibronectin was linked to
82 dampening and eventual arrest of *Lnfg* oscillations (Hubaud et al., 2017), reminiscent of what is
83 observed in the anterior PSM prior to somite formation. Nevertheless, how PSM cells sense and
84 respond to an increasing fibronectin matrix complexity gradient, and how it affects segmentation
85 clock oscillations *in vivo* remains unknown.

86 In this study, we addressed the involvement of the fibronectin ECM and its downstream
87 mechanotransduction pathway in the regulation of both segmentation clock dynamics and
88 subsequent somite formation *in vivo*, using the chick embryo as a model. We experimentally
89 perturbed (1) non-muscle myosin II (NM II) ATPase activity (2), Rho-associated protein kinase

90 (ROCK), (3) integrin-fibronectin binding through RGD and (4) extracellular fibronectin matrix
91 assembly, and found that all these treatments resulted in abnormal segmentation clock
92 oscillations, mis-positioning of *meso1* expression in the rostral PSM and perturbations in somite
93 morphogenesis. These results strongly suggest that tissue tension generated by the fibronectin
94 matrix surrounding the PSM plays an important role in converting segmentation clock oscillations
95 into periodic somite formation.
96

97 **Results:**

98 <u>The intracellular mechanotransduction machinery of PSM cells regulates segmentation clock</u>
99 <u>oscillations and *meso1* activation</u>

100 In the chick embryo, sequential pairs of somites bud off from the anterior PSM every 90
101 min, which corresponds to the period of segmentation clock oscillations (Figure 1 A). To investigate
102 the involvement of intracellular actomyosin contractility in this process, the expression of the
103 segmentation clock gene *hairy1* (Palmeirim et al., 1997), a target of Notch signaling, was analyzed
104 in the PSM of embryo half explants cultured in the presence of either Blebbistatin, which directly
105 inhibits the ATPase activity of NM II (direct inhibition) and consequently all actomyosin
106 contractility, or RockOut (indirect inhibition), a chemical inhibitor of ROCK I and II (ROCK I/II)
107 enzymes involved in activating NM II (Figure 1 B, C; Ringer et al., 2017; Straight et al., 2003; Yarrow
108 et al., 2005). The contralateral control sides were cultured with an equal volume of DMSO.

109 Explants cultured for 6 hours in each experimental condition presented significantly
110 altered *hairy1* expression. *hairy1* expression was either absent or in a different phase of the cycle
111 relative to the contralateral control in 80% of the Blebbistatin- (n=7/9) and RockOut-treated
112 explants (n= 8/10; Figure 1 D, a-c), suggesting that temporal control of *hairy1* oscillations requires
113 the generation and transduction of tensional cues mediated by NM II and ROCK I/II activity.

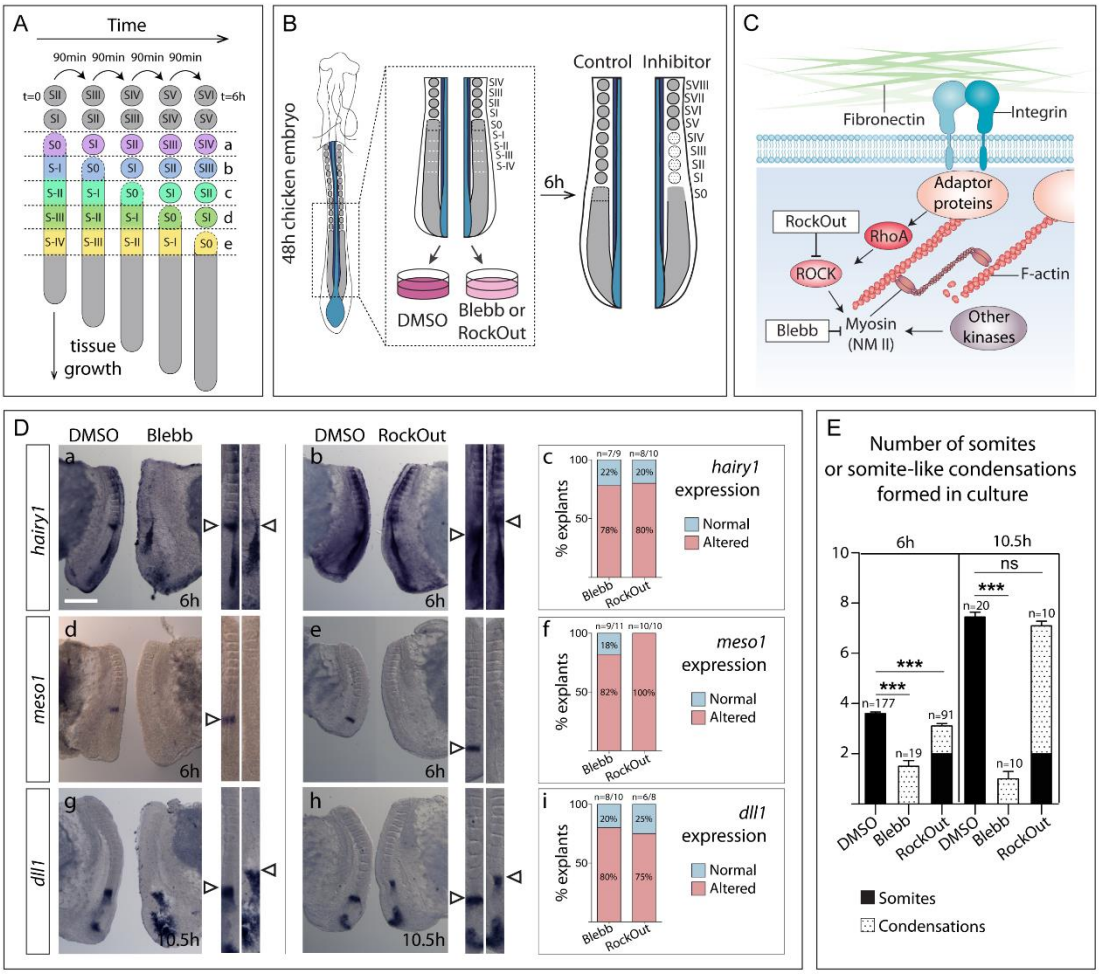

Figure 1. Blocking NM II or ROCK I/II activity leads to misregulation of *hairy1*, *meso1* and *dll1* expression and to defects in somite formation.

(A) Schematic representation of chick PSM maturation and somite formation over time. A new pair of somites buds off from the anterior PSM every 90 min. This is also the period of segmentation clock oscillations in the chick embryo. With each new pair of somites, the previously formed somites mature (SI becomes SII, SII becomes SIII, etc). (**B**) Schematic representation of the explant culture system. Posterior explants of HH11-14 chick embryos were bisected along the midline and cultured for 6 (or 10.5) hours. One side of the explant was cultured with either Blebbistatin (Blebb) or RockOut, while the contra-lateral half was cultured with an equal volume of DMSO. **(C)** Schematic representation of the action of Blebbistatin and RockOut. Blebbistatin inhibits NM II ATPase activity directly while RockOut inhibits ROCK I/II-mediated phosphorylation of myosin light chain, thus indirectly decreasing NM II ATPase activity. (**D**) *In situ* hybridization for *hairy1* (a, b) *meso1* (d, e) and *dll1* (**g, h**) after 6 (a, b, d, e) or 10.5 hours of culture (g, h) in Blebbistatin (Blebb; a, d, g) and in RockOut-containing (b, e, h) media. Straightened images of the respective explant pairs (right) were aligned by SIV. Rostral is on top. Percentage of Blebbistatin- or RockOut-treated explants with altered *hairy1*, *meso1* and *dll1* expression compared to the contralateral controls is shown in c, f and i, respectively. **(E)** Number of somites (black bars) or somite-like condensations (dotted bars) formed in cultured explants. Explants cultured with DMSO formed sharp somite boundaries and clearly individualized somites. Blebbistatin-treated explants only formed 1-2 somite-like condensations. In RockOut treated explants the first two somites were normal while the remaining ones were cell condensations with poorly defined boundaries. ns – not significant, *** – p<0.01. Scale bar in D**:** 500 µm. Bars - standard error of the mean.

136 Segmentation clock oscillations are required for the correct spatial and temporal
137 upregulation of *Mesp2* in the anterior PSM (Niwa et al., 2011; Saga and Takeda, 2001; Sato et al.,
138 2002), which regulates downstream targets required for the formation of the somitic cleft (Saga,
139 2012). The expression of the chick *Mesp2* homolog, *meso1*, was altered in Blebbistatin- (n=9/11)
140 and RockOut-treated explants (n=10/10; Figure 1 D, d-f). *meso1* expression was either absent,
141 located more rostrally or presented a different number of bands of expression, clearly indicating
142 that the normal cycles of activation and suppression of *meso1* in the rostral PSM were altered.
143 Importantly, *meso1* expression was also perturbed after 3 hours in culture with Blebbistatin or
144 RockOut (n=8/9 and 5/5, respectively; Supplementary Figure 1 A-B), corresponding to an effect
145 within two segmentation clock cycles. Furthermore, timely downregulation of *dll1* in the anterior-
146 most PSM (Palmeirim et al., 1998), which normally occurs downstream of Meso1/Mesp2 activity
147 (Takahashi et al., 2000; Takahashi et al., 2003), was not observed in either Blebbistatin- (80%,
148 n=8/10) or RockOut-treated (75%, n=6/8) explants after 10.5h of culture (Figure 1 D, g-i). Together
149 these data indicate that interfering with actomyosin contractility perturbs three sequential events:
150 the spatio-temporal expression dynamics of *hairy1*, timely *meso1* expression and the
151 downregulation of *dll1* expression in the anterior-most PSM.

152 Alterations in somite formation were observed concomitantly with the perturbations in
153 *hairy1* and *meso1* expression. Control explants formed an average of 3.6 somites after 6 hours,
154 consistent with a 90 min periodicity (Figure 1 E), while contralateral RockOut-treated explants
155 formed 3.1 somites, of which only the first two somites were clearly individualized, while
156 subsequent somite-like condensations were poorly defined (Figure 1 E). After 10.5 hours, control
157 explants had formed an average of 7.5 somites, while RockOut-treated explants formed 7.1
158 somites of which the first two appeared normal, but the remaining ones were ill-defined (Figure 1
159 E). Importantly, explants cultured with Blebbistatin were unable to form more than 1-2 somite-
160 like aggregates after 6 hours, or even 10.5 hours, of culture (Figure 1 E), evidencing an absolute

161 requirement for NM II ATPase activity in somite formation. These effects were not due to an
162 increase in apoptosis (Supplementary Figure 2 A-D).

163 Our data reveal a previously unknown role for NM II- and ROCK I/II- mediated
164 mechanotransduction in the temporal regulation of the segmentation clock, *meso1* expression,
165 *dll1* downregulation and somite formation.

166

167 <u>NM II activity is required for somite cleft formation and cell polarization</u>

168 Somite formation involves a mesenchymal-to-epithelial transition (MET) of anterior PSM
169 cells (Martins et al., 2009; Saga, 2012). To determine to what extent this process is impaired upon
170 NM II or ROCK I/II inhibition, we performed a detailed analysis of the morphology of S0 to SIII in
171 explants after a 6 hour culture period (regions e-b in Figure 1 A).

172 In control explants, S0 showed apically enriched N-cadherin (Figure 2 A, D) and some
173 *zonula occludens* protein 1 (ZO-1) accumulation was observed apically (Figure 2 B, D, arrowheads).
174 Peripheral cell alignment occurred (Figure 2 C, D) and fibronectin matrix was detected in the
175 nascent somitic clefts (Figure 2 E, F, arrows). In contrast, in explants cultured with Blebbistatin, N-
176 cadherin was homogeneous (Figure 2 G, J), ZO-1 immunostaining was absent (Figure 2 H, J) and
177 neither peripheral cell alignment (Figure 2 I, J) nor fibronectin matrix accumulation within the
178 tissue was observed (Figure 2 K, L). Furthermore, the continuous and dense fibronectin matrix
179 observed surrounding the rostral PSM and S0 in control explants was disrupted in Blebbistatin-
180 treated explants (compare Figure 2 E and K, arrowheads). Moreover, the characteristic nuclear
181 alignment and F-actin apical enrichment observed in control SI (Supplementary Figure 3 A-C) was
182 absent in Blebbistatin-treated explants and no signs of somitic boundaries could be detected
183 (Supplementary Figure 3 D-F). We conclude that exposure of the S-IV and S-III regions of the PSM
184 (regions e and d in Figure 1 A) to Blebbistatin for 6 hours completely blocks their capacity to form
185 somites.

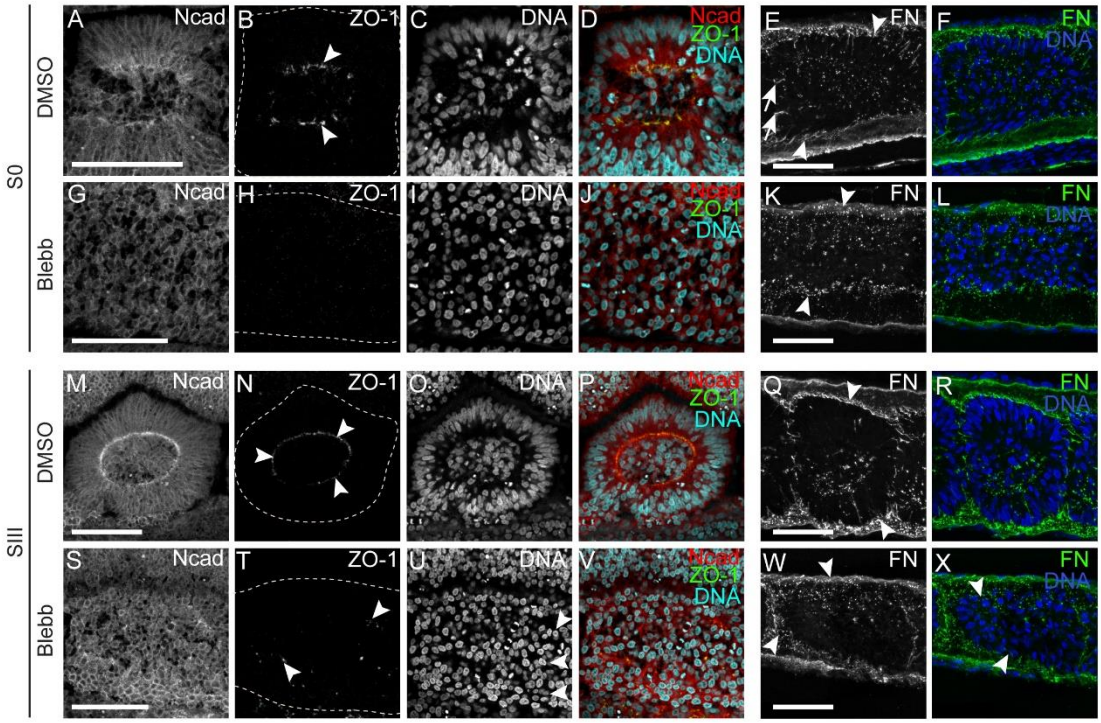

Figure 2. NM II inhibition abolishes N-cadherin and ZO-1 polarization and impairs fibronectin fibrillogenesis.

(A-X) Sagittal views of control explants (A-F, M-R) and their Blebbistatin-treated contralateral halves after 6 hours of culture, immunostained for N-cadherin (Ncad), ZO-1, fibronectin (FN), stained for DNA and imaged at S0 (A-L; region e in Figure 1 A) and SIII (M-X; region b in Figure 1 A) levels. S0 of DMSO-treated explants shows apically enriched N-cadherin (A) and ZO-1 (B, arrowheads) and peripheral nuclei are aligned (C, D), while no signs of polarized cell-cell adhesions (G, H) or nuclear alignment (I, J) are found in the contralateral Blebbistatin-treated explant. Apical polarization of N-cadherin and ZO-1 is maintained in SIII cells of DMSO-treated explants (M, N, arrowheads), but no polarized N-cadherin (S) or ZO-1 (T, arrowheads) are observed in contralateral Blebbistatin-treated halves. Nuclear alignment (U, arrowheads) and fibronectin assembly around somites (K, W, arrowheads) are also deficient in Blebbistatin-treated explants compared to contralateral controls (E, Q, arrowheads). Arrows in E show fibronectin assembly in the nascent somitic cleft. Rostral to the left and dorsal on top. Dashed lines mark borders of S0 (B, H) and SIII (N, T). FN – fibronectin. Ncad – N-cadherin. ZO-1 – *Zonula occludens* 1. Blebb – Blebbistatin. Scale bars: 50 µm.

We next turned our attention to SII and SIII somites after 6 hours of culture. These were at stage S-II and S-I in the PSM, respectively (regions c and b in Figure 1 A), when the explants were placed in culture and had thus already upregulated *meso1* (Buchberger et al., 1998). As before, in the presence of Blebbistatin, apical enrichment of N-cadherin failed to occur (Figure 2 S, V), ZO-1 was only detected in a few small foci (Figure 2 T, V) and, although a fibronectin matrix was present, it appeared less dense (Figure 2 W, X). An incipient nuclear alignment was sometimes observed (Supplementary Figure 3 K, arrowheads), but cells did not polarize their F-actin into apically

209 enriched adhesion belts (compare Supplementary Figure 3 G-I with J-L). Epithelial tissues other
210 than somites (e.g. ectoderm and neural tube) did not present significant alterations after
211 incubation with Blebbistatin (Supplementary Figure 4). Altogether, these results point to an
212 indispensable role for NM II activity for the MET underlying somite formation.

213 RockOut-treated explants also showed perturbations in somite formation, although to a
214 lesser extent (Figure 1 E). When compared to control explants (Figure 3 A-D; Supplementary Figure
215 5 A-C), RockOut treatment resulted in incomplete somitic clefts, such that S0 shared the
216 somitocoel with SI and sometimes also with SII (Fig 3 E-H, arrows, Supplementary Figure 5 D-F,
217 arrows). In contrast to the accumulation of fibronectin in the nascent clefts in controls (Figure 3 B,
218 arrow), no fibronectin was observed in the incipient somitic clefts of RockOut-treated explants
219 (Figure 3 F, arrows). These results suggest that ROCK I/II activity in the S-IV and S-III regions of the
220 PSM is required for the formation of individualized somites. In contrast, when the rostral-most
221 PSM (stage S-I before culture, region b in Figure 1 A) was exposed to RockOut for 6 hours, it was
222 undistinguishable from control explants, showing apical accumulation of ZO-1 (Figure 3 I, M,
223 arrowhead) and N-cadherin (Supplementary Figure 5, G, J), nuclear alignment (Figure 3, K, O,
224 Supplementary Figure 5, H, K) and a complete, fibronectin matrix-containing cleft (Figure 3 J, N).
225 This indicates that ROCK I/II activity is not required for S-I to develop into a somite.

226 Altogether, our data indicate that the intracellular mechanotransduction machinery of
227 PSM cells is required for periodic somite cleft formation, and that ROCK I/II- independent NM II
228 activity is essential for MET.

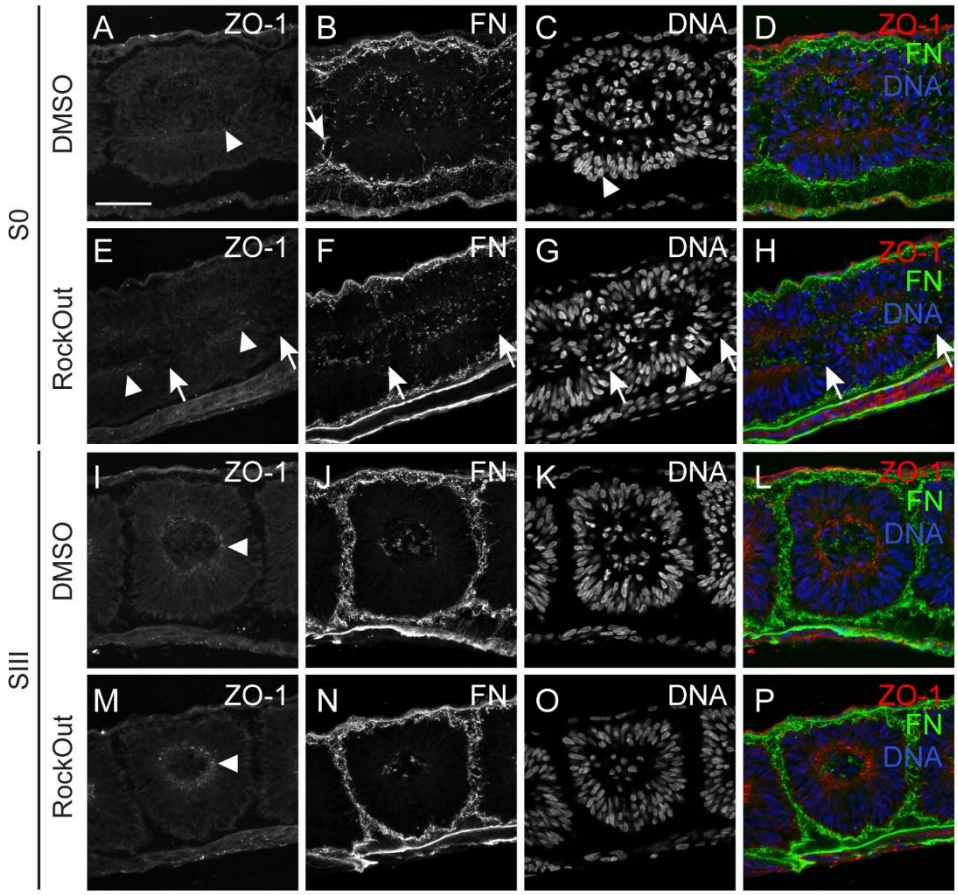

Figure 3. ROCK I/II inhibition impairs morphological somite formation, leading to deficient ZO-1 polarization and fibronectin assembly.

(A-P) Sagittal views of explants cultured in control (DMSO) medium (A-D, I-L) and their contralateral RockOut-treated halves (E-H, M-P) at S0 (A-H; region e in Figure 1 A) and SIII (I-P; region b in Figure 1 A) levels, immunostained for ZO-1 (first column), fibronectin (second column), stained for DNA (third column) and the respective merged image (fourth column). Explants were cultured for 6 hours. S0 in control explants show normal accumulation of ZO-1 (A, arrowhead), fibronectin assembly in the nascent cleft (B, arrow) and nuclear alignment (C, arrowhead). In contrast, S0 in contralateral RockOut-treated explants fails to form a clear cleft (E-H, arrows), although ZO-1 is generally polarized (E, arrowhead) and nuclei are aligned (G, arrowhead). At SIII level, both explants show normal ZO-1 polarization (I, M, arrowheads), fibronectin assembly (J, N) and nuclear alignment (K, O). Rostral on the left and dorsal on top. FN – fibronectin. ZO-1 – *Zonula occludens* protein 1. Scale bar: 50 µm.

<u>Integrin-fibronectin binding regulates segmentation clock oscillations and is required for somitic cleft positioning and morphogenesis</u>

Fibronectin signaling through α5β1 integrin plays a crucial role during somitogenesis (Takahashi et al., 2007). To evaluate if this interaction could be regulating segmentation clock oscillations and somite formation upstream of the intracellular mechanotransduction pathway,

249 embryo half explants were cultured in the presence of the RGD peptide which competes with
250 fibronectin for integrin binding (Figure 4 A, B). RGD-treated explants formed ill-defined somite-like
251 condensations, although in approximately the same number as the contralateral control (Figure 4
252 C). This was not due to cell death (Supplementary Figure 2 E-F). Concomitantly, RGD-treated
253 explants displayed alterations in *hairy1* (Figure 4 D, a-c), *meso1* (Figure 4 D, d-f) and *dll1* expression
254 patterns (Figure 4 D, g-i), evidencing that integrin-RGD interactions are required for proper
255 segmentation clock oscillations, *meso1* positioning and timely downregulation of *dll1* in the
256 anterior PSM.

257 When compared to the contralateral control, the area corresponding to S0 after 6 hours
258 of culture with RGD (region e in Figure 1 A) showed deficient nuclear alignment (Figure 5 B, E, H,
259 K, arrowheads) and N-cadherin polarization (Figure 5 A, G, arrowheads), accompanied by deficient
260 fibronectin assembly in the nascent cleft (Figure 5 D, J arrowheads). At the level of SII (region c in
261 Figure 1 A), complete somite individualization was impaired in RGD-treated explants (Figure 5 N,
262 Q, T, W, arrowheads) and, although N-cadherin polarization appeared normal (Figure 5 M, S,
263 arrowheads), cleft formation (Figure 5 Q, W, arrowheads; R, X) and fibronectin assembly between
264 adjacent somites was deficient (Figure 5 P, V, arrowheads; R, X).

265 These findings implicate cell-ECM interactions, mediated by integrin-fibronectin binding,
266 in temporal control of *hairy1* oscillations, correct positioning of *meso1* expression, downregulation
267 of *dll1* in the anterior PSM and somite morphogenesis.

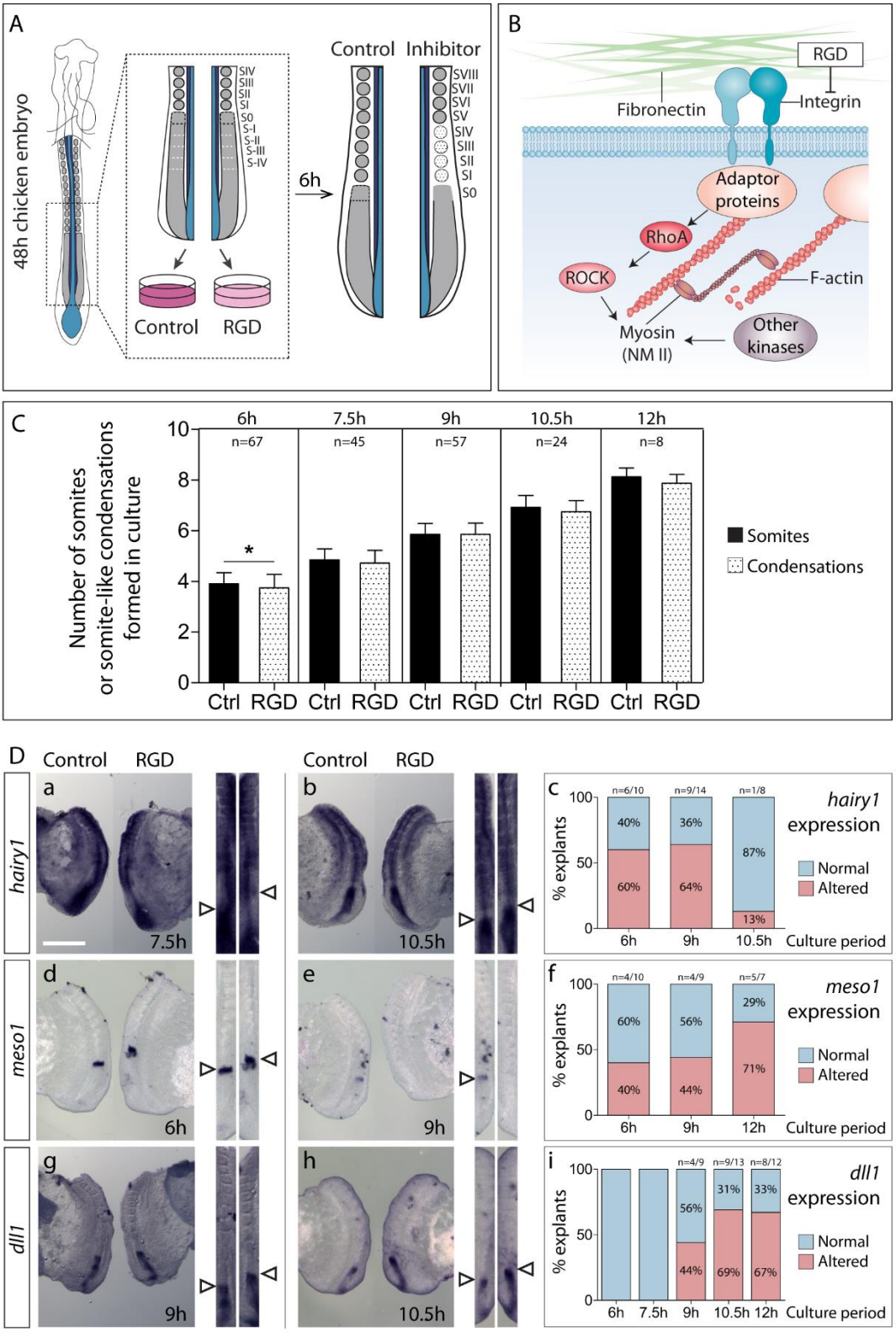

Figure 4. Integrin-fibronectin binding through RGD is required for timely *hairy1* and *meso1* expression and *dll1* downregulation.

(A) Schematic representation of the explant culture system. Posterior explants of HH11-14 chick embryos were bisected along the midline and cultured for 6 to 12 hours. One side of the explant was cultured with RGD, while the contralateral half was cultured in control medium. **(B)** Schematic representation of the action of RGD. RGD competes with the RGD-binding pockets of integrins, interfering with their binding to the ECM. **(C)** Number of somites (black bars) or somite-like condensations (dotted bars) formed in culture in control

and RGD-treated explants. *p<0.05. **(D)** Expression of *hairy1* (a, b), *meso1* (d, e) and *dll1* (g, h) in RGD-treated explants and contralateral controls at representative timepoints of culture. Straightened images of respective explant pairs (right) aligned by SIV. Rostral is on top. Percentage of RGD-treated explants with altered *hairy1*, *meso1* and *dll1* expression compared to the contralateral controls is shown in c, f and i, respectively. Impairing integrin-fibronectin binding with RGD alters *hairy1* and *meso1* expression relative to contralateral controls at 6 hours of culture onwards (a-f, arrowheads). *dll1* expression was altered at 9 hours of culture onwards (g-i, arrowheads). Scale bar: 500 µm.

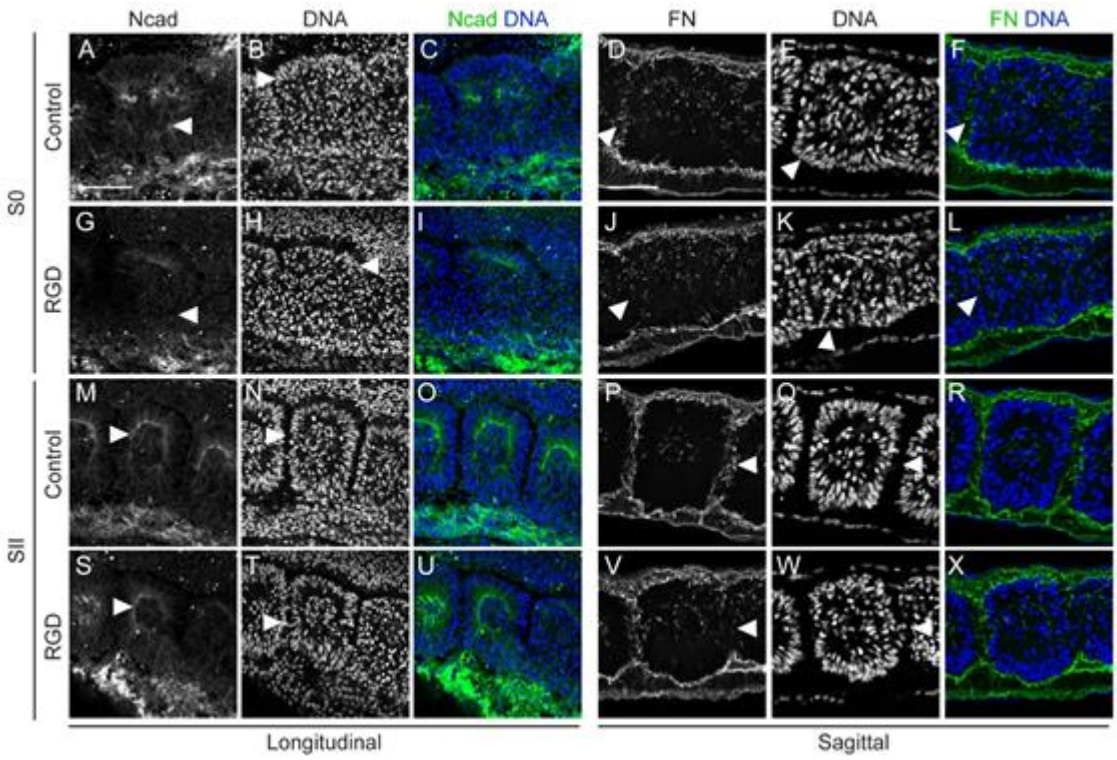

Figure 5. Inhibition of integrin-fibronectin binding impairs somitic cleft formation.
(A-X) Longitudinal (left) and sagittal (right) views of explants cultured in control medium (A-F, M-R) and their contralateral RGD-treated halves (G-L, S-X) at S0 (A-L; region e in Figure 1 A) and SII (M-X; region c in Figure 1 A) levels, immunostained for N-cadherin (first column), fibronectin (fourth column) and stained for DNA (second and fifth columns). Third and sixth columns show the respective merged images. Explants were cultured for 6 hours. At the S0 level, control explants show normal apical accumulation of N-cadherin (A, arrowheads) and nuclear alignment (B, E, arrowheads) in peripheral somitic cells as well as fibronectin assembly in the nascent cleft (D, F, arrowheads). All of these are deficient in their RGD-treated contralateral halves (G-L, arrowheads). At the level of SII, N-cadherin accumulation and nuclear alignment occurs normally in both control and RGD-treated explants (M, N, S, T, arrowheads), but fibronectin assembly between adjacent somites (P, V, arrowheads) and cleft formation (Q, W, arrowheads) are deficient in RGD-treated explants compared to contralateral controls. Ncad – N-cadherin; FN – fibronectin. Scale bars: 50 µm.

298 Impaired fibronectin assembly results in altered segmentation clock dynamics, *meso1* expression
299 and defects in somite morphogenesis

300 To interrogate the importance of the mechanical properties of the extracellular fibronectin
301 matrix on somite formation, we perturbed fibronectin assembly in the PSM and somites by
302 electroporating primitive streak-stage embryos with a construct expressing the 70kDa fibronectin
303 fragment, a dominant-negative inhibitor of fibronectin assembly (Figure 6 A, B; McKeown-Longo
304 and Mosher, 1985; Sato et al., 2017). 70kDa-electroporated embryos exhibited a disrupted
305 fibronectin matrix, composed of thinner fibrils when compared with control pCAGGs-
306 electroporated embryos (Supplementary Figure 6 A; also see Figure 7 A, B), and displayed multiple
307 morphological defects, including kinked neural tube and detached tissues as well as perturbations
308 in somite morphogenesis, which are all reminiscent of phenotypes obtained in previous studies
309 interfering with fibronectin-integrin binding (Supplementary Figure 6 B, C; Drake et al., 1992;
310 Drake and Little, 1991; George et al., 1993; Girós et al., 2011; Takahashi et al., 2007). Although the
311 average number of somite-like structures formed in 70kDa-electroporated embryos was similar to
312 the number of somites in controls (Figure 6 C) they were ill-defined, often appearing fused or
313 crammed (Supplementary Figure 6 B e-f, C), and thus resemble the somite-like condensations
314 formed in RockOut- and RGD-treated explants (Figures 3 and 5).

315 Next, we sought to evaluate the impact of inhibiting fibronectin matrix assembly on the
316 molecular machinery underlying somite formation by using unilateral electroporation of the 70kDa
317 fragment (Figure 6 A) to allow the direct comparison of gene expression patterns within the same
318 embryo. We observed a significant increase in the frequency of perturbations in the expression of
319 embryonic clock genes *hairy1* ($p<0.01$) and *hairy2* ($p<0.05$), as well as in *meso1* ($p<0.01$) and *dll1*
320 ($p<0.01$), when compared with embryos electroporated with pCAGGs alone (Figure 6 D).

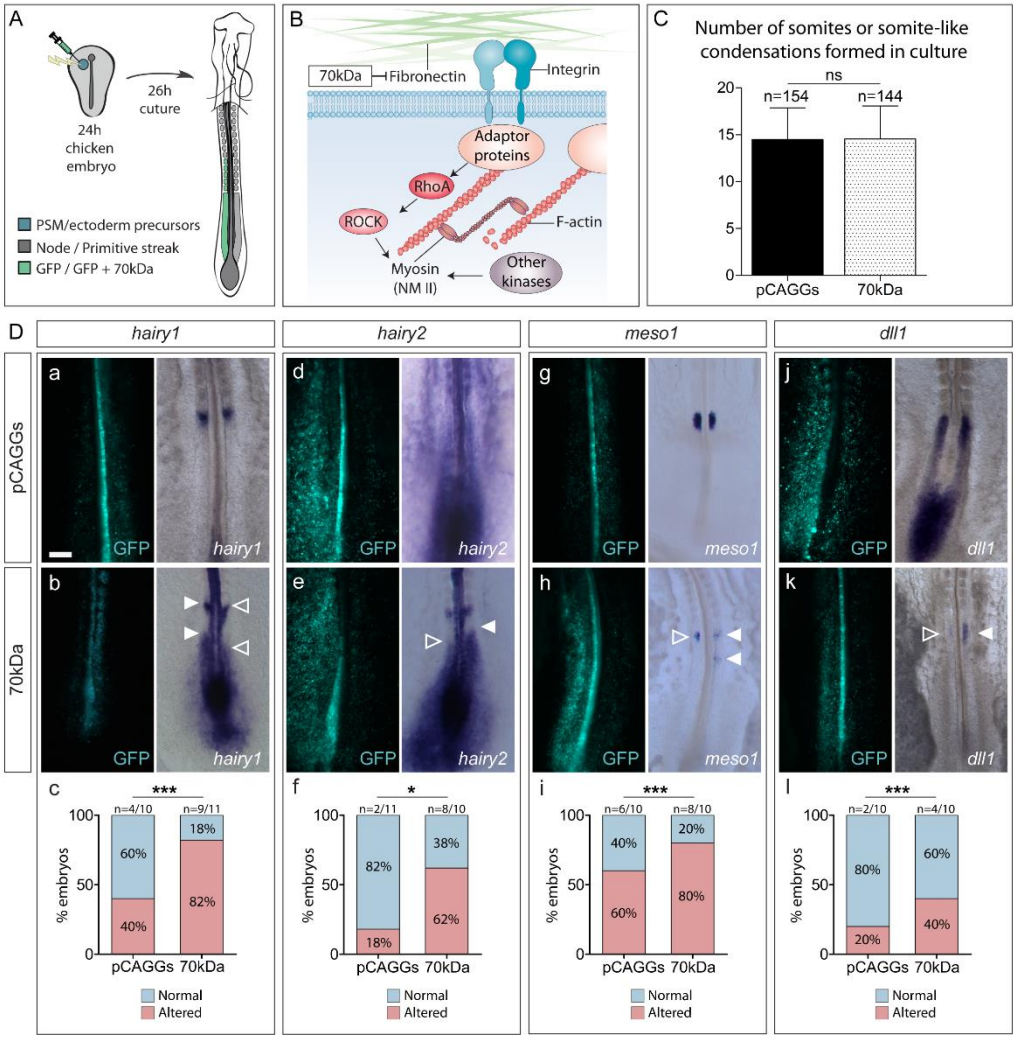

Figure 6. Impairing fibronectin assembly by electroporation with a 70kDa expressing vector perturbs segmentation clock oscillations, timely *meso1* expression and *dll1* downregulation.

(**A**) Schematic representation of the electroporation strategy. PSM/ectoderm progenitors of primitive-streak stage embryos were electroporated with either a pCAGGs GFP-expressing vector (pCAGGs) alone, or co-electroporated with a 70kDa-expressing vector (70kDa) and were incubated for about 26 hours. **(B)** Schematic representation of the action of 70kDa. 70kDa disrupts the assembly of fibronectin matrix by competitively binding to the N-terminal self-assembly domains of the protein, impairing fibronectin fibril formation. **(C)** Number of somites (black bars) or somite-like condensations (dotted bars) formed in pCAGGs- and 70kDa-electroporated embryos after 26 hours. **(D)** Examples of the expression of *hairy1* (a-b), *hairy2* (d-e), *meso1* (g-h) and *dll1* (j-l) in pCAGGs- (top row) and 70kDa-electroporated embryos (middle row). Electroporated side is on right (a, b, g) or left (d, e, h, j, k) Perturbing the assembly of fibronectin on one side of the PSM leads to an asymmetric pattern of *hairy1* (b, arrowheads), *hairy2* (e, arrowheads), *meso1* (h, arrowheads) and *dll1* expression (k, arrowheads) in a higher percentage of embryos than in controls. Percentage of pCAGGs- and 70kDa-electroporated embryos with asymmetric expression of *hairy1*, *hairy2*, *meso1* and *dll1* between the electroporated PSM and the contralateral non-electroporated control PSM is shown in c, f, i and l, respectively. The number of embryos with an asymmetric pattern was significantly higher in 70kDa-electroporated embryos for all four genes studied. * $p<0.05$, *** $p<0.01$. Rostral is on top. Scale bar: 200 µm.

340 Consistent with these data, 70kDa-electroporated embryos have deficiencies in somite
341 morphogenesis which, in severe cases, leads to incomplete somitic clefts (Figure 7 A, B k, w,
342 arrows). Peripheral cells of nascent somites of 70kDa-electroporated embryos did, however,
343 accumulate ZO-1 apically (Figure 7 B e, h, i, l) which was maintained as the somites matured (Figure
344 7 B q, u, t, x). Nevertheless, these somites were abnormal in shape and appeared smaller in
345 severely affected embryos (Figure 7 B, i-l, u, x). In fact, the SI of the electroporated sides of 70kDa-
346 treated embryos were significantly smaller in width than those of the contralateral control, while
347 SV was significantly shorter in length (n=151; Supplementary Figure 7). In addition to defects in
348 somite morphology, the ectoderm and endoderm were separated from the paraxial mesoderm in
349 70kDa-electroporated embryos, indicating that their fibronectin matrix was insufficient to hold
350 these tissues together (brackets in Figure 7 B g, k, s, w).
351 We conclude that unperturbed fibronectin assembly in the PSM is required for tuning clock
352 gene expression dynamics, positioning of *meso1* expression and timely *dll1* downregulation in the
353 rostral-most PSM, as well as for the complete separation and morphogenesis of somites.

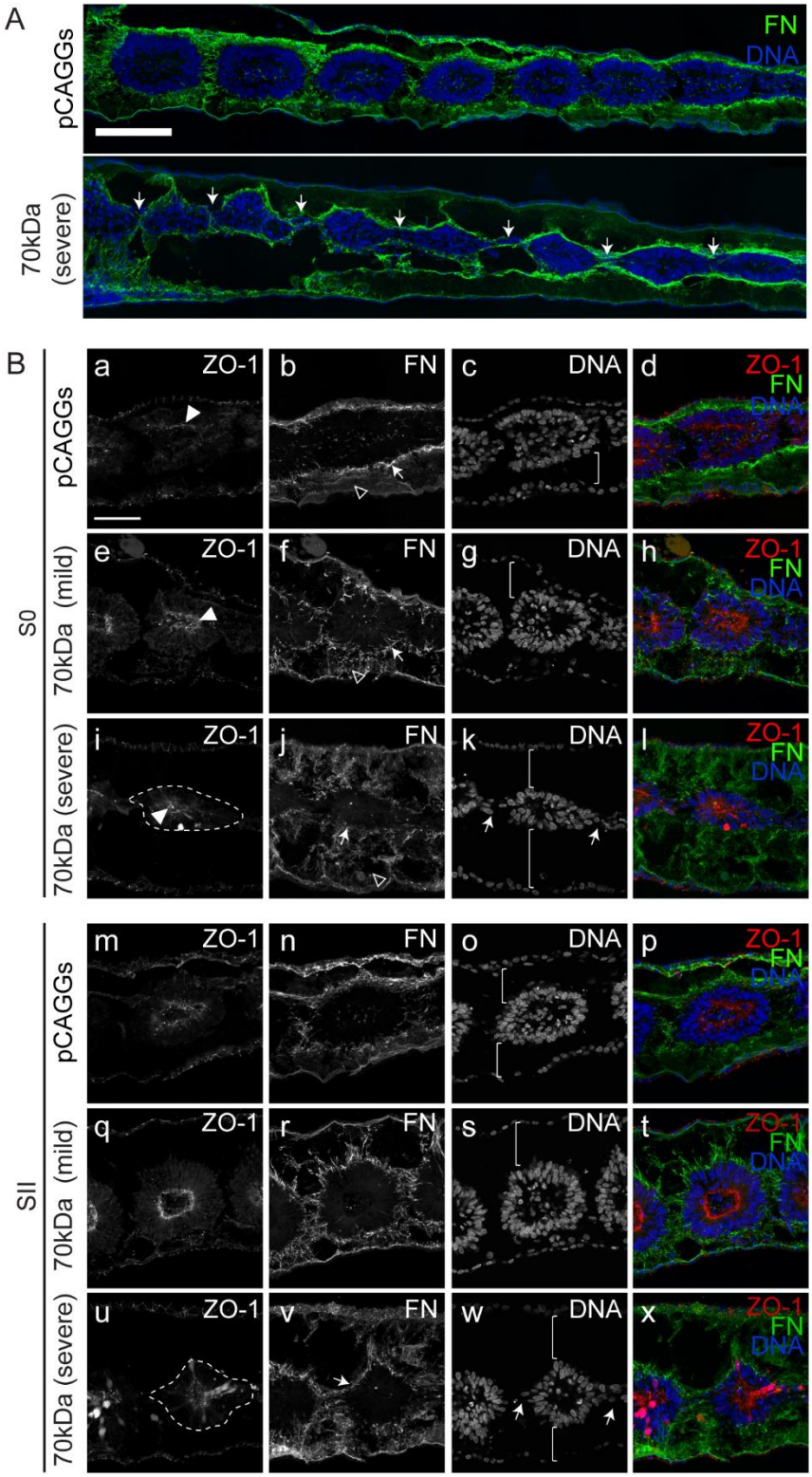

Figure 7. Somite morphology of 70kDa-electroporated embryos is severely compromised.
(A) Sagittal sections of embryos electroporated with pCAGGs and 70kDa, the latter with a severe phenotype. Arrows point to deficient somitic clefts. Rostral to the left and dorsal on top. **(B)** Sagittal views of embryos electroporated with pCAGGs (a-d, m-p) and 70kDa (e-l, q-x) with either mild (e-h, q-t) or severe (i-l, u-x) phenotypes, at S0 (a-l) and SII (m-x) levels, immunostained for ZO-1 (first column) and fibronectin (second column) and stained for DNA (third column). Fourth column shows the merge of the respective channels. S0 of pCAGGs- and 70kDa-electroporated embryos all polarize ZO-1 normally (a, e, i, arrowheads) and this is

362 maintained at SII levels (m, q, u), but the fibronectin matrix surrounding the somites of 70kDa-treated
363 embryos is disrupted compared to pCAGGs-electroporated embryos (b, f, j, v, arrows). Somites of 70kDa-
364 electroporated embryos are also severely detached from both the ectoderm and endoderm compared to
365 embryos electroporated only with pCAGGS (third column, brackets), and the fibronectin matrix connecting
366 the endoderm to the somites is severely compromised (second column, empty arrowheads). Somites of
367 embryos electroporated with 70kDa with more severe defects also fail to fully detach from adjacent somites
368 (k, w, arrows). Rostral to the left and dorsal to the top. Dashed lines indicate altered somite morphology. FN
369 – fibronectin. ZO-1 – *Zonula occludens* protein 1. Scale bars: 50 µm.
370

371

372 **Discussion:**

373 Fibronectin matrix and its downstream mechanotransduction pathway coordinate segmentation
374 clock dynamics and segment boundary formation

375 We have identified a mechanotransduction pathway involving a fibronectin – integrin –
376 ROCK – NM II axis, which regulates the dynamics of segmentation clock gene expression in the
377 chick embryo. Four independent treatments interfering with different levels of this pathway (from
378 intracellular actomyosin contractility to fibronectin matrix assembly; Figure 8 A), all lead to altered
379 segmentation clock dynamics, misregulated *meso1* expression, impaired or deficient cleft
380 formation and alterations in somite morphology (Figure 8 B).

381 The similarity of the phenotypes obtained in experiments blocking ROCK I/II and impairing
382 normal cell-fibronectin interactions with RGD or 70kDa (Figure 8 B) suggest that the fibronectin
383 matrix is transducing mechanical signals via ROCK and α5β1 integrin, the major fibronectin
384 receptor in the PSM (Jülich et al., 2005; Rifes et al., 2007; Yang et al., 1999), and that these signals
385 affect segmentation clock oscillations. Indeed, the fibronectin – α5β1 integrin – ROCK – NM II axis
386 is believed to play key roles in cellular force generation in a variety of cell types (Bharadwaj et al.,
387 2017; Daley et al., 2011; Schiller et al., 2013; Torr et al., 2015). Our data show that blocking
388 different elements of this axis leads to asymmetric patterns of *hairy1* expression as well as
389 incorrect positioning of *meso1* expression on the experimental versus the control sides of the same
390 embryo. Moreover, they suggest that the mechanical environment is regulating Notch signaling in

391 the PSM. This is in agreement with a previous report, evidencing that β1 integrins are needed for
392 correct Notch signaling in neural progenitors (Campos et al., 2006). Combined roles of integrin
393 α5β1 and Notch are also required for zebrafish somitogenesis (Jülich et al., 2005) and chicken
394 embryos electroporated with RNAi constructs against integrin β1 show alterations in *hairy2, lfng*
395 and *meso1* expression in the PSM (Rallis et al., 2010). Finally, mouse embryos expressing
396 fibronectin where the RGD sequence was substituted with RGE (*Fn1*$^{RGE/RGE}$ embryos) showed
397 asymmetric and/or dampened expression of *Lnfg* and *Hes7* in the PSM (Girós et al., 2011) and
398 *EphA4*, a direct target of Mesp2 in the anterior PSM (Nakajima et al., 2006), was diffusely
399 expressed or absent (Girós et al., 2011).

400 Exactly how the mechanical environment regulates segmentation clock oscillations is
401 unknown. However, it is becoming increasingly clear that mechanics play a crucial role in Notch
402 activation (Gordon et al., 2015; Luca et al., 2017; Meloty-Kapella et al., 2012) and it was recently
403 demonstrated that sustained Notch signaling in the *Drosophila* notum requires actomyosin
404 contractility in both signal sending and receiving cells (Hunter et al., 2019). It is thus possible that
405 cells in the PSM become detached from their neighbors when their mechanical environment or
406 intracellular contractility is impaired. This may place Notch receptors and ligands of adjacent PSM
407 cells further apart, leading to a reduction in the incidence or duration of Notch signaling events.

408 Altogether, our results strongly suggest the existence of a link between the fibronectin –
409 α5β1 integrin – ROCK – NM II pathway and Notch signaling in the PSM. When perturbed, this leads
410 to a dysregulation of segmentation clock oscillations and the mispositioning of the segmental
411 border.

412

413 <u>Somite cleft formation and cell epithelialization have different mechanical requirements</u>

414 In the rostral PSM, Mesp2/Meso1 activates the expression of EphA4, which interacts with
415 EphrinB2 in cells rostral to the presumptive cleft, causing cell-cell repulsion and the formation of
416 an incipient cleft (Nakajima et al., 2006; Watanabe et al., 2009). Then, fibronectin matrix assembly

417 stabilizes it (Jülich et al., 2015; Rifes and Thorsteinsdóttir, 2012) and promotes the epithelialization
418 of cells rostral to the cleft (Martins et al., 2009). This can be defined as the first step of
419 morphological somite individualization. The second step is defined as the complete
420 epithelialization of the remaining cells of the nascent somite and lasts until SII, when all somitic
421 cells have acquired a spindle-like shape and are organized into a rosette (Martins et al., 2009).

422 RockOut-, RGD- and 70kDa-treated embryos (Figure 8 A, B) all show perturbations in
423 segmentation clock gene expression, abnormal positioning of *meso1* and defects in somite
424 boundary development. Although somite morphology is also perturbed, the acquisition of the
425 spindle-shape cell morphology which occurs as S0 develops into SII does not appear to be
426 significantly perturbed. Thus, the first step of morphological somite formation is affected but the
427 second step is not. In contrast, Blebbistatin-treated explants not only have the defects listed
428 above, but cells that had already upregulated *meso1* before the addition of the drug and formed
429 an incipient cleft during culture, were completely unable to epithelialize. In fact, Blebbistatin-
430 treated explants formed only 1 or 2 somites, and their cells did not acquire the elongated, spindle-
431 shape typical of SI and SII somites. Hence, Blebbistatin affects both steps of morphological somite
432 formation.

433 RockOut targets NM II activity indirectly by inhibiting ROCK I and II, two of the kinases that
434 activate NM II (Newell-Litwa et al., 2015). In contrast, Blebbistatin directly targets the NM II
435 ATPase. Our results thus raise the possibility that the acquisition of the spindle-shaped
436 morphology of cells is dependent on another NM II activator. Interestingly, Ca^{++}/calmodulin
437 signaling can activate NM II and inhibiting calmodulin was shown to block the acquisition of this
438 morphology during chick somitogenesis (Chernoff and Hilfer, 1982).

439

440 <u>Fibronectin matrix complexity gradient as a player in the PSM wavefront of maturation</u>

441 While the waves of Notch oscillatory activity travel through the entire length of the PSM,
442 they are only translated into segments in the anterior-most region of the tissue. Opposing

443 gradients of Fgf/Wnt and Retinoic acid (RA) in the PSM are thought to define the wavefront, which
444 marks the region where PSM cells become competent for somite formation (Hubaud and
445 Pourquié, 2014). Rostral to the wavefront, segmentation clock oscillations progressively slow
446 down until they reach a halt and cells become part of a somite (Palmeirim et al., 1997).

447 Herein, we propose a model (Figure 8 C) where the posterior to anterior gradient of
448 fibronectin matrix complexity is interpreted by the PSM cells as an increasing gradient of
449 mechanical tension (Figure 8 C). We propose that the tensional state created by the fibronectin
450 matrix surrounding the PSM at the level of the wavefront acts as a threshold which activates a
451 mechanotransduction signaling cascade ensuring the correct spatio-temporal conversion of the
452 cyclic expression of segmentation clock genes into periodic *meso1* expression which defines the
453 next somitic cleft (Figure 8 C). In support of this hypothesis, a gradient of increasing paraxial
454 mesoderm stiffness from the tail to rostral somites has recently been identified in the chick
455 embryo (Marrese et al., 2019). The PSM thus integrates a combination of chemical and mechanical
456 signals, namely gradients of Fgf/Wnt and RA (Aulehla and Pourquié, 2010) and, simultaneously, an
457 increase in fibronectin matrix complexity and stiffness (Figure 8 C). The fibronectin matrix would
458 thus be a key contributor to the PSM wavefront of maturation in that it regulates where the next
459 somitic cleft is positioned while simultaneously supporting a cohort of competent cells as they bud
460 off from the anterior PSM as somites (Figure 8 C). Hence, we propose that the
461 mechanotransduction pathway downstream of fibronectin plays a major role in the translation of
462 cyclic waves of expression of segmentation clock genes into the periodic morphogenesis of
463 somites.

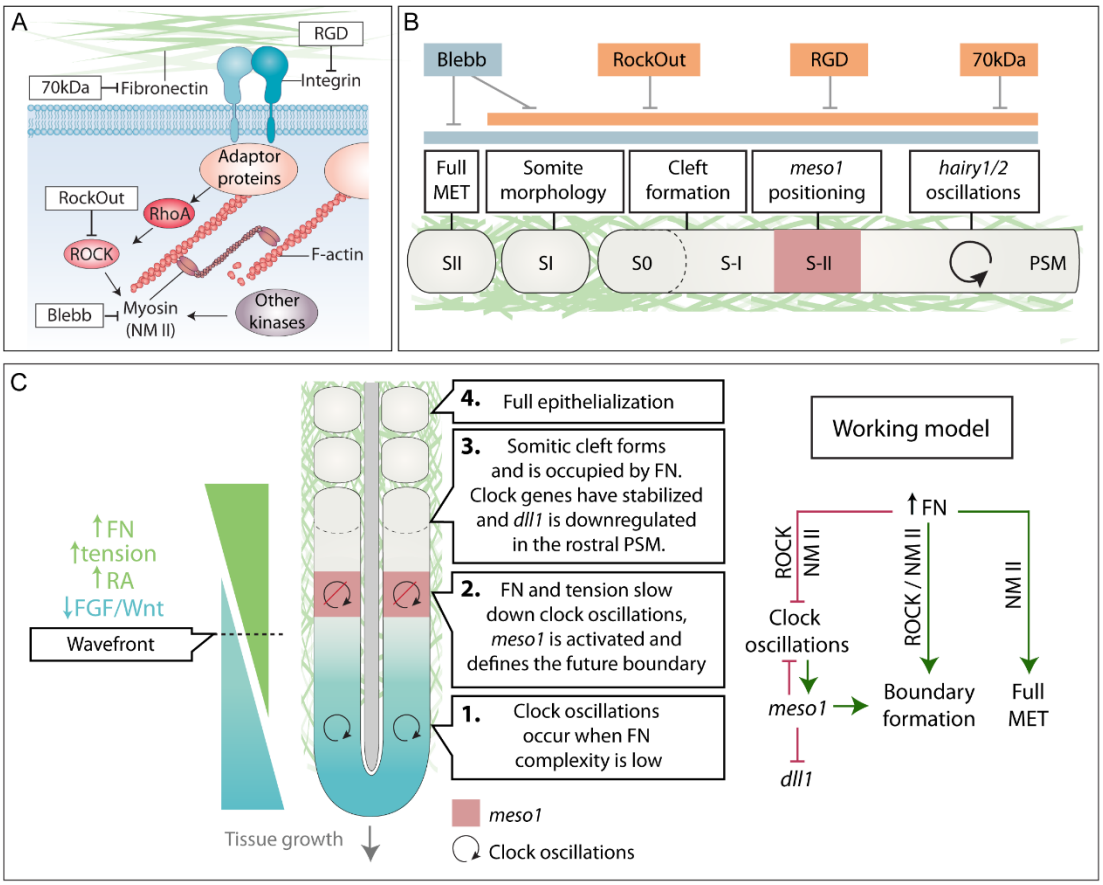

Figure 8. Summary of the results and working model.

(A) Schematic representation of the fibronectin – integrin – ROCK – NM II axis and targets of experimental inhibition. **(B)** Effect of the different treatments on somitogenesis. Interfering with fibronectin matrix assembly (70kDa), integrin-fibronectin binding (RGD), ROCK I/II (RockOut) or NM II (Blebb) activity, leads to altered segmentation clock dynamics, misregulated *meso1* expression, impaired or deficient cleft formation and alterations in somite morphology. Interfering directly with NM II further disrupts full somite epithelialization. **(C)** Working model proposing that an increase of fibronectin matrix complexity in the PSM, from caudal to rostral, is sensed by cells as a tensional gradient, with anterior PSM cells being in the stiffest environment. This increased tensional gradient occurs concomitantly with a decrease in Fgf/Wnt levels and an increase in RA. As cells reach the wavefront and sense a threshold of fibronectin-induced stiffness, there is an increase in fibronectin-integrin engagement which activates ROCK and NM II, leading to increased actomyosin contractility and slower clock oscillations. As Notch signaling becomes stabilized, it activates *meso1* expression in the S-II/S-I region, setting the stage for cleft formation and *dll1* downregulation in the rostral-most PSM later on. As the clefts form, somites bud off as SI. Finally, ROCK-independent NM II activity is required for full somite epithelialization into SII.

480 **Materials and Methods:**

481 Embryos

482 Fertile eggs were obtained from commercial sources (Sociedade Agrícola Quinta da Freiria
483 or Pintobar Exploração Avícola, Lda, Portugal) and incubated at 37.5ºC in a humidified chamber
484 until the desired HH stage (Hamburger and Hamilton, 1992). Somite nomenclature is according to
485 Pourquié and Tam (2001).

486

487 Embryo explant culture and chemical treatments

488 Explant tissues of HH11-14 embryos were collected and cultured as previously described
489 (Palmeirim et al., 1997; Rifes et al., 2007). Embryos were bisected along the midline and then cut
490 transversally rostral to somites IV and Hensen's node. The two contralateral halves thus retained
491 half of the neural tube and notochord as well as the first four somites and the PSM, with all
492 remaining neighboring tissues intact. Explants were placed on top of a polycarbonate filter floating
493 on M199 medium supplemented with 10% chick serum, 5% fetal calf serum and 100 U/ml of
494 penicillin and streptomycin (Palmeirim et al., 1997). Explants were then cultured at 37ºC with 5%
495 CO_2 from 6 to 12 hours.

496 InSolution™Blebbistatin (Calbiochem) and RockOut (Calbiochem) diluted in DMSO were
497 used at a final concentration of 50 µM in culture medium. Equal volumes of DMSO (Sigma-Aldrich)
498 were used as control for both drugs. The RGD peptide (Sigma) was diluted in culture medium and
499 used at 0.9 mM, while control explants were cultured in medium only. RGD peptide efficiency was
500 confirmed in a cell adhesion assay (Danen et al., 2002; Pierschbacher and Ruoslahti, 1984) before
501 using it on explants.

502

503 Embryo electroporation and *ex ovo* culture

504 HH4-5 embryos were electroporated on one (randomly selected) side of the primitive
505 streak in the presumptive PSM and/or ectoderm and cultured *ex ovo* using the Early Chick culture

506 method (Chapman et al., 2001). The electroporation mixture contained plasmid DNA at 0.5-1 µg/µl
507 mixed with 0.4% Fast Green for visualization. Embryos were submerged in an electroporation
508 chamber filled with Tyrode's saline and three pulses of 6-9 V, 50 ms each, at 350 ms intervals were
509 applied. Control embryos were electroporated with pCAGGs containing a GFP reporter (pCAGGs-
510 GFP; abbreviated pCAGGs). pCAGGs-70kDa qFN1 was kindly provided by Yuki Sato (Sato et al.,
511 2017) and was co-electroporated with the pCAGGs-GFP plasmid in experimental embryos
512 (treatment abbreviated 70kDa). Electroporated embryos were screened for GFP after fixation to
513 select embryos with an intense signal on only one side to process for whole mount morphological
514 analysis, *in situ* hybridization experiments and transverse sectioning. For morphological analysis in
515 sagittal sections, the embryo side electroporated with pCAAGGs was compared to the 70kDa-
516 electroporated side of other same stage embryos.
517
518 Cryosectioning and immunohistochemistry
519 Cryosectioning was performed on whole embryos and embryo explants fixed in 4%
520 paraformaldehyde in 0.12 M phosphate buffer containing 4% sucrose and processed for
521 cryoembedding as previously described. Briefly, fixed samples were embedded in 7.5% gelatin in
522 0.12 M phosphate buffer containing 15% sucrose, frozen on dry ice-chilled isopentane and stored
523 at -80ºC until sectioning. Cryostat sections (10-30 µm) were processed for immunofluorescence as
524 previously described (Gomes de Almeida et al., 2016). Permeabilization of sections was performed
525 with 0.2% Triton-X100 in phosphate buffered saline (PBS). 5% bovine serum albumen (BSA) or a
526 combination of 1% BSA and 10% Normal Goat Serum (NGS) in PBS were used for blocking
527 depending on the presence or absence of anti-fibronectin antibodies, respectively. Primary and
528 secondary antibodies were diluted in 1% BSA in PBS. Sections were incubated with primary
529 antibodies overnight at 4ºC and with secondary antibodies for 1 hour at room temperature. For
530 whole-mount immunodetection, explants were fixed in 4% paraformaldehyde in PBS and
531 processed as previously described (Martins et al., 2009; Rifes and Thorsteinsdóttir, 2012). 1%

532 Triton-X100 in PBS was used for permeabilization and 1% BSA in PBS was used for blocking and
533 antibody dilution. Antibody incubation was performed overnight at 4ºC.
534 The following primary antibodies were used: anti-ZO-1 (Zymed, 1:100 or Invitrogen,
535 1:100); anti-N-cadherin (BD Biosciences, 1:100); anti-fibronectin (Sigma, 1:400), anti-activated
536 caspase3 (Cell Signaling, 1:1000) and anti-GFP (Invitrogen, 1:100). For F-actin staining we used
537 Alexa 488-conjugated phalloidin (Invitrogen, 1:40) and for staining DNA we used ToPro3
538 (Invitrogen, 1:500) in conjunction with ribonuclease A (Sigma, 10 µg/ml), 4% Methyl Green (Sigma,
539 diluted 1:250; Prieto et al., 2015) or 4',6-diamidino-2-phenylindole (DAPI, 5µg/ml in PBS with 0.1%
540 Triton-X100). For detection of the primary antibodies the adequate secondary goat anti-mouse
541 and anti-rabbit Alexa 488-, Alexa 568- or Alexa 546-conjugated F'ab fragments from Invitrogen
542 were used (1:1000).
543
544 *In situ* hybridization
545 *In situ* hybridization using DIG-labeled RNA probes was performed as described previously
546 (Henrique et al., 1995) with minor alterations (Gomes de Almeida et al., 2016). RNA probes were
547 synthetized from linearized plasmids: *dll1* (Henrique et al., 1995), *meso1* (Buchberger et al., 1998),
548 *hairy1* (Palmeirim et al., 1997) and *hairy2* (Jouve et al., 2000).
549
550 Statistical analysis
551 Paired Student's t-tests were performed to assess for differences in the number of somites
552 formed in Blebbistatin-, RockOut- and RGD-treated explants relative to the respective controls,
553 and in embryos electroporated with pCAGGs only and pCAGGs + 70kDa. Differences in the
554 frequency of morphological and gene expression phenotypes found in 70kDa-electroporated
555 embryos compared to pCAGGs-electroporated control embryos was tested through a Chi-square
556 test. Differences in somite size between pCAGGs- or 70kDa-electroporated sides compared to the
557 control (non-electroporated) sides of embryos was tested through a nested ANOVA. The side in

558 which the embryo was electroporated (left or right) was nested in the treatment (non-
559 electroporated, pCAGGs-electroporated or 70kDa-electroporated) to account for a potential
560 variability between the two sides. Statistical significance was set at $p<0.05$. Statistical analyses
561 were performed in Statistica 10, Graphpad Prism 5 and RStudio.
562
563 Sample preparation and imaging
564 Whole mount explants were gradually dehydrated in methanol and cleared in
565 methylsalicylate (Sigma-Aldrich) as described previously (Martins et al., 2009; Rifes and
566 Thorsteinsdóttir, 2012), except for phalloidin-labelled embryos and explants, where a shorter
567 series of ethanol dehydration series was used. Cryostat sections were mounted in Vectashield
568 (Vector Laboratories) or in 5mg/ml propyl gallate in glycerol/PBS (9:1) with 0.01% azide.
569 Immunofluorescence images were taken on a confocal Leica SPE microscope, following imaging
570 acquisition steps described previously (Rifes and Thorsteinsdóttir, 2012). Imaging of
571 electroporated embryos and explants processed for *in situ* hybridization was performed using a
572 Zeiss LUMAR V12 Stereoscope coupled to a Zeiss Axiocam 503 color 3MP camera. Image analysis
573 was performed using Fiji v. 1.49 and Amira V.5.3.3 (Visage Imaging Inc.) software. Image histogram
574 corrections were performed in Fiji and exported as TIFF files. When applicable, contiguous images
575 were stitched together into a single image using the pairwise stitching Fiji plugin (Preibisch et al.,
576 2009). For the analysis of *in situ* hybridization patterns along the PSM explants, the Fiji plugin
577 Straighten (Kocsis et al., 1991) was used.
578
579
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589

590 **Competing interests:**

591 The authors declare no competing interests.

592

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806 **Supplementary Figures**

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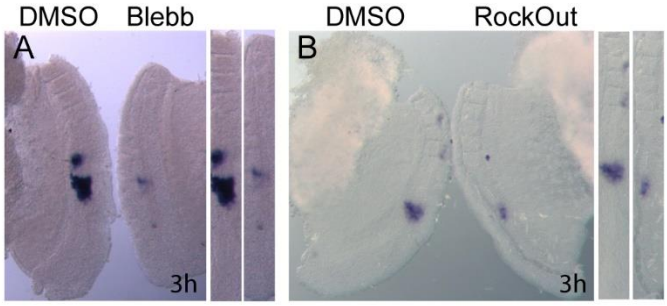

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811 **Supplementary Figure 1. Blocking NM II or ROCK I/II activity for 3 hours leads to altered *meso1* expression.**
812 **(A, B)** m*eso1* expression evaluated by *in situ* hybridization in explants treated for 3 hours with Blebbistatin
813 (**A**) or RockOut (**B**) and in the respective contralateral controls, shows that *meso1* expression is already
814 altered two clock cycles after adding the drugs. Straightened images of the respective explant pairs (right)
815 were aligned by SIV. Rostral is on top.
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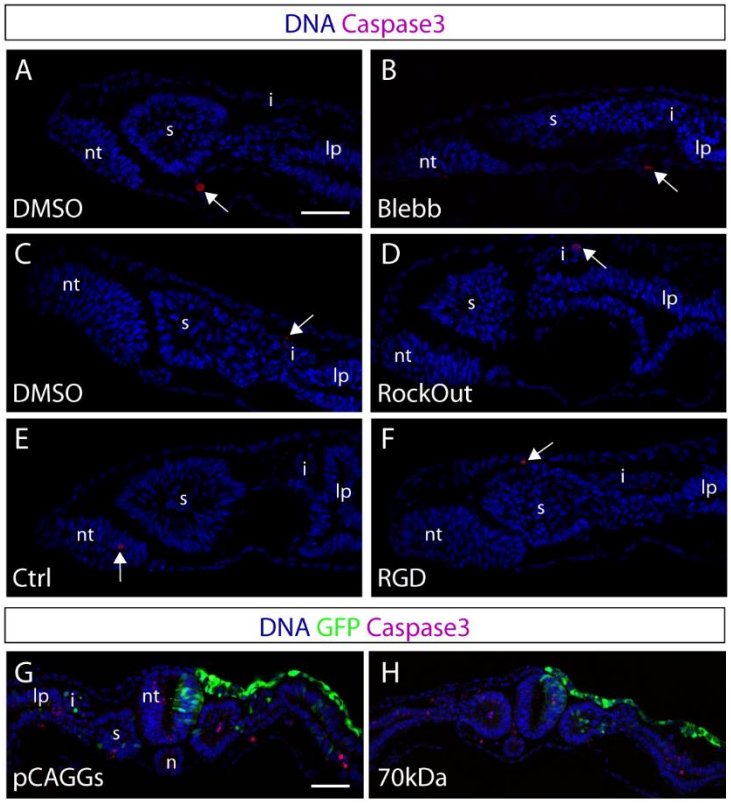
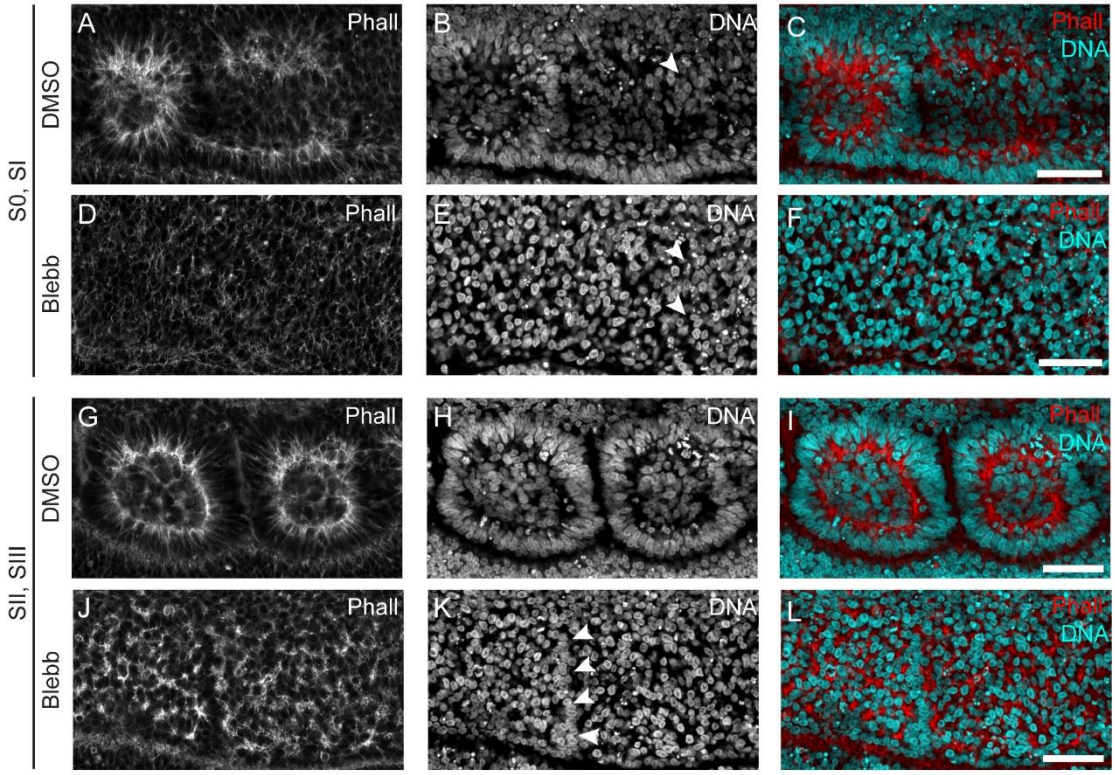
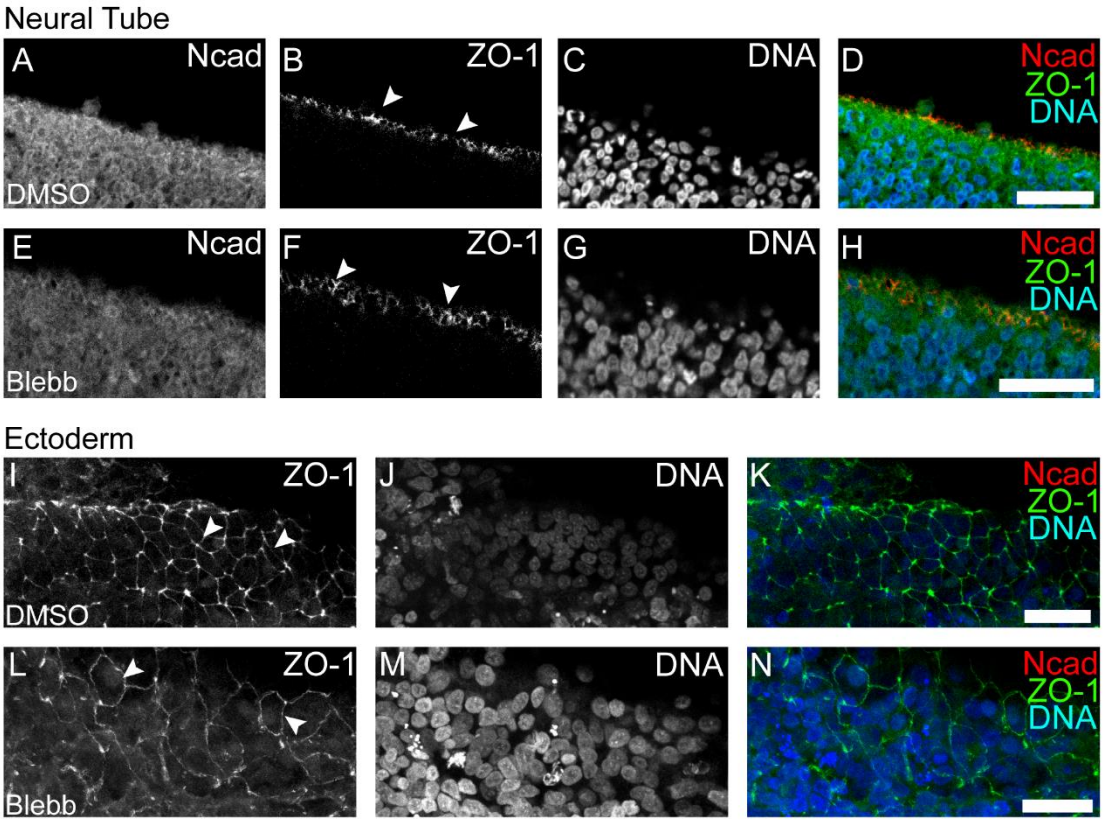


Supplementary Figure 2. Apoptosis is not enhanced in the experimental culture conditions used. **(A, H)** Immunostaining for activated Caspase3 in transverse sections of control and contralateral explants treated with either Blebbistatin (**A, B**), RockOut (**C, D**), or RGD (**E, F**) for 6 hours, and in pCAGGs- and 70kDa-electroporated embryos (**G, H**). DNA (blue), activated Caspase3 (magenta) and GFP (green). Arrows indicate Caspase3-positive cells. Apoptosis levels are not increased by any of the treatments. Blebb – Blebbistatin; nt – neural tube; s – somite; i – intermediate mesoderm; lp – lateral plate mesoderm. Dorsal is on top. Scale bars: 50 µm.



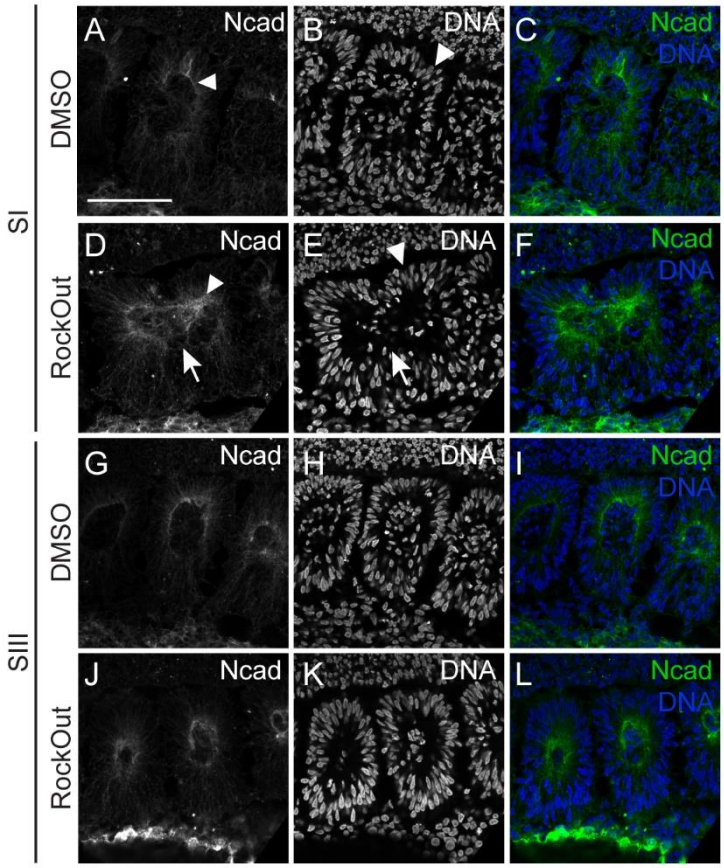
Supplementary Figure 3. NM II inhibition impairs F-actin apical enrichment and nuclear alignment in somites formed during culture.

(A, L) Sagittal sections of DMSO-treated explants (A-C, G-I) and contralateral Blebbistatin-treated explants (D-F, J-L) stained for F-actin and DNA. Rostral PSM epithelialization of DMSO-treated explants occurs normally, with F-actin apical enrichment (A) and nuclear alignment (B) in S0 and SI. At the same axial level in the contralateral Blebbistatin-treated explant (D-F), F-actin staining is dispersed (D) and nuclei do not align (E). SII and SIII of DMSO-treated explants are epithelial (G-I), composed of an outer cell layer with aligned nuclei (H) and elongated cells with apically enriched F-actin (G). At the equivalent axial level in the Blebbistatin-treated explant (J-L), the somitic segments were severely affected. There is only a slight nuclear alignment at the prospective inter-somitic border (K, arrowheads) and F-actin aggregates into dispersed and separate foci (J, L). Rostral to the left and dorsal on top. Blebb – Blebbistatin; Phall – phalloidin F-actin staining. Scale bars: 50 µm.



Supplementary Figure 4. Epithelial structure of the neural tube and ectoderm are unaffected by NM II inhibition.

(A, N) Control explants (A-D and I-K) showed strong apically located ZO-1 labeling (B, I, arrowheads) both in the neural tube (A-D; transverse optical section, medial on top) and in the overlying surface ectoderm (I-K; dorsal view of ectoderm). In the presence of Blebbistatin (E-H, L-N), ZO-1 labeling remained restricted to the apical end of neural tube cells (F, arrowheads) and of surface ectoderm cells (L, arrowheads). Blebb – Blebbistatin; Ncad – N-cadherin. ZO-1 – *Zonula occludens* protein 1. Arrowheads point to ZO-1 labeling. Scale bars: 20 µm.



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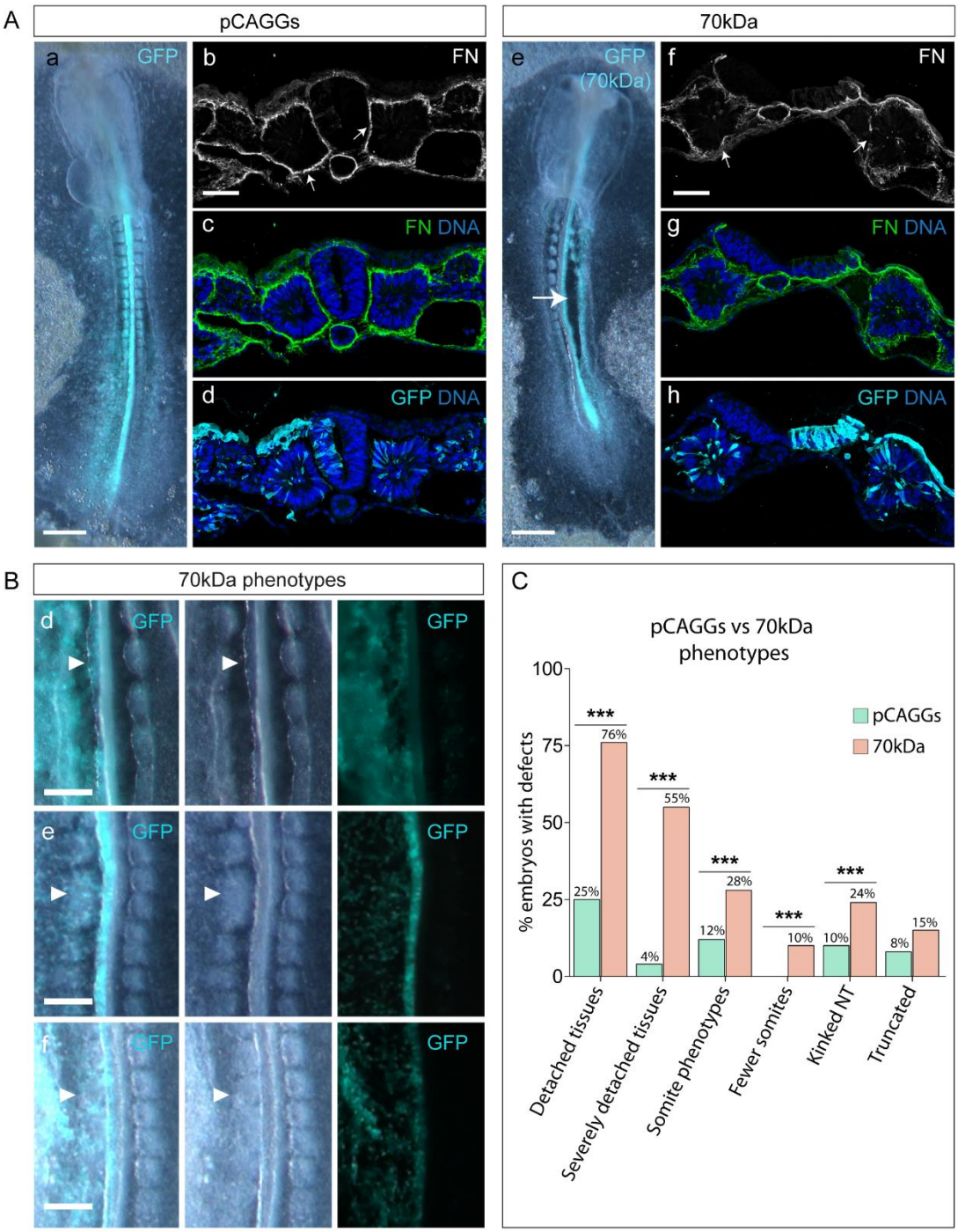
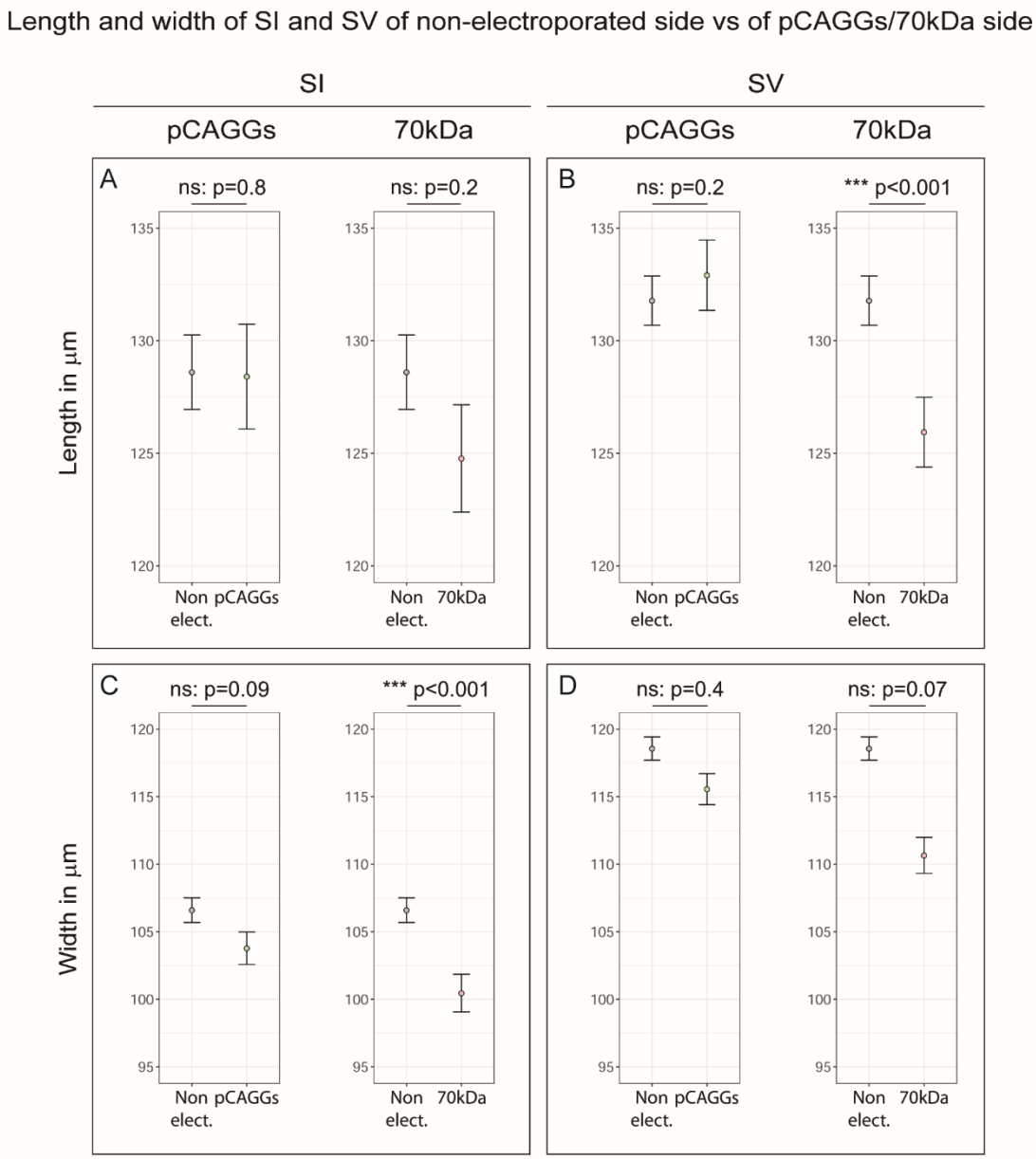
849 **Supplementary Figure 5. ROCK I/II inhibition impairs apical polarization of N-cadherin in nascent somites.**
850 **(A, L)** Longitudinal views of explants cultured for 6 hours in control (DMSO) medium (A-C, G-I) and their
851 contralateral RockOut-treated halves (D-F, J-L) at SI (A-F) and SIII (G-L) levels, immunostained for N-cadherin
852 (first column) and stained for DNA (second column). Third column shows the respective merge of all
853 stainings. SI in control explants shows normal accumulation of N-cadherin (A, arrowhead) and nuclear
854 alignment (B, arrowhead). In contrast, SI in contralateral RockOut-treated explants fail to form a clear cleft
855 (D-F, arrows) leading to partially fused somites. N-cadherin is, however, partially polarized (D, arrowhead)
856 and nuclei are aligned (E, arrowhead). At SIII level, both explants show normal N-cadherin polarization (G, J)
857 and nuclear alignment (H, L). Rostral on the left and midline on top. Ncad - N-cadherin. Scale bars: 50 µm.



Supplementary Figure 6. Embryos electroporated with the 70kDa construct exhibit numerous morphological defects.

(A) Representative images of the morphology of embryos electroporated with either pCAGGs only (a) or 70kDa, the latter being a severe phenotype (e). Severe phenotypes included severely detached tissues (e, arrow) and a truncated A-P axis. Transverse sections of pCAGGs- (b-d) and 70kDa-electroporared embryos (f-h) immunostained for fibronectin (b-c, f-g), GFP (d, h) and stained for DNA (c-d, g-h). Electroporated side is on left (a-d) or right (e-h). Detachment of tissues is clearly visible in 70kDa-electroporated embryos (e), which is accompanied by a severe disruption in the fibronectin matrix (compare b-c with f-g, arrows). **(B)** Close up of embryos electroporated with 70kDa showing kinked neural tube (d, arrowheads), fused somites (e, arrowheads) and fewer somites on the electroporated side (f, arrowheads). Electroporated sides are on left. Ventral view and rostral on top. **(C)** Percentage of pCAGGS- (green bars) and 70kDa-electroporated (pink

870 bars) embryos with morphological defects, including detached (pCAGGs: 39/154, 70kDa: 97/144) and
871 severely detached tissues (pCAGGs: 6/154, 70kDa: 79/144), kinked neural tube (pCAGGs: 15/154, 70kDa:
872 34/144), truncated A-P axis (pCAGGs: 13/154, 70kDa: 21/144), abnormal somite morphology (pCAGGs:
873 18/154, 70kDa: 41/144), and fewer somites on the electroporated side compared to the control non-
874 electroporated side (pCAGGs: 0/154, 70kDa: 15/144). ***$p<0.001$. FN – fibronectin. Scale bars: (A, a, e; C,
875 a-c) 500 µm, (C, d-f) 200 µm, (A, b-d, f-h) 50 µm.



Supplementary Figure 7. Quantification of somite length and width in pCAGGs- and 70kDa-electroporated embryos.

(A-D) Length of SI (A) and SV (B) and width of SI (C) and SV (D) from pCAGGS- and 70kDa-electroporated embryos compared to the control non-electroporated side. The measurements were made on images from whole mount embryos. Somites from pCAGGs-electroporated embryos did not show a significant difference in either length or width between electroporated vs non-electroporated sides (n=151), but the width of SI and length of SV of the electroporated side of 70kDa-treated embryos were significantly smaller than that of the contralateral non-electroporated control side (n=143). Bars represent the standard error of the mean. ns – not significant; *** p<0.001.