Rho kinase-dependent actin turnover and actomyosin disassembly are necessary for mouse spinal neural tube closure

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ABSTRACT

The cytoskeleton is widely considered essential for neurulation, yet the mouse spinal neural tube can close despite genetic and non-genetic disruption of the cytoskeleton. To investigate this apparent contradiction, we applied cytoskeletal inhibitors to mouse embryos in culture. Preventing actomyosin cross-linking, F-actin assembly or myosin II contractile activity did not disrupt spinal closure. In contrast, inhibiting Rho kinase or blocking F-actin disassembly prevented closure, with apical F-actin accumulation and adherens junction disturbance in the neuroepithelium. Cofilin 1-null embryos yielded a similar phenotype, supporting a key role for actin turnover. Co-exposure to Blebbistatin rescued the neurulation defects caused by RhoA inhibition, whereas an inhibitor of myosin light chain kinase, ML-7, had no such effect. We conclude that regulation of RhoA/Rho kinase/LIM kinase/cofilin signalling is necessary for spinal neural tube closure through precise control of neuroepithelial actin turnover and actomyosin disassembly. In contrast, actomyosin assembly and myosin ATPase activity are not limiting for closure.
INTRODUCTION

Neurulation is the embryonic process by which the neural plate becomes converted into a closed neural tube, the developmental precursor of the brain and spinal cord. Bending of the neuroepithelium creates the neural folds whose elevation and fusion are required for neural tube formation. The role of the cytoskeleton has long been studied in neurulation. Actin microfilaments are located circumferentially in the apices of neuroepithelial cells (Sadler et al., 1982) together with non-muscle myosin II which is recruited to this site (Kinoshita et al., 2008; Nyholm et al., 2009). Regulated actomyosin contraction is often viewed as the principal ‘motor’ that induces apical constriction, thereby causing the neural plate to bend and close. This is analogous to proposed mechanisms of other epithelial invagination events (Sawyer et al., 2010).

Apical actomyosin contraction is regulated by proteins including RhoA and Shroom3 which localize to the apical actomyosin complex. They are linked to the function of apical junctional complexes: the sites of contractile microfilament insertion (Hildebrand, 2005; Nishimura and Takeichi, 2008). Interestingly, planar cell polarity (PCP) proteins, including Celsr1, have also been implicated in the regulation of actomyosin contractility in neural tube closure (Nishimura et al., 2012), extending the role of PCP that is known to function in early neural plate shaping (Wallingford and Harland, 2002; Ybot-Gonzalez et al., 2007b).

While actomyosin-driven apical constriction is widely accepted to drive neurulation, several considerations suggest this view may be overly simplistic. First, the neural plate of higher vertebrates bends focally, at median and dorsolateral hinge points (MHP; DLHPs), rather than through generalised invagination of the whole neuroepithelium. Actin does not localize specifically to hinge points, but is present in the apices of all neuroepithelial cells (Ybot-Gonzalez and Copp, 1999). While Shroom was reported to localize to bending regions in the Xenopus neural plate (Haigo et al., 2003), no specific protein localization has been described for higher vertebrate hinge points.

A second challenge is that neuroepithelial bending sites do not appear to be locations of active apical constriction. For example, the MHP of chick and mouse embryos is enriched for wedge-shaped cells (Schoenwolf and Franks, 1984; Smith et al., 1994), but this results from localized prolongation of the cell cycle that causes nuclei to become basally located within
the pseudostratified neuroepithelium (Smith and Schoenwolf, 1988). The apices of MHP cells appear to narrow passively as a secondary result of basal cell expansion.

A third line of evidence comes from experimental disruption of the cytoskeleton during closure. In the chick, the actin-microfilament inhibitor Cytochalasin D (CytD) blocks only the later stages of neurulation and considerable neural fold elevation occurs in its presence (Schoenwolf et al., 1988). In mammals, while cytochalasins are potent inhibitors of cranial neural tube closure, both in vivo (Austin et al., 1982; Wiley, 1980) and in cultured embryos (Morrisk Kay and Tuckett, 1985), spinal neurulation is resistant to CytD. Following actin microfilament disassembly in cultured mouse embryos, closure progresses and MHP and DLHPs continue to form (Ybot-Gonzalez and Copp, 1999). Consistent with this, cytochalasins fail to induce spina bifida when administered in vivo (Austin et al., 1982; Wiley, 1980).

Finally, the findings with cytoskeletal inhibitors are mirrored in studies of mice with null mutations in cytoskeletal genes. Cranial NTDs (exencephaly) are seen in mutants lacking a single cytoskeletal protein (e.g. palladin, vinculin, n-cofilin and Marcks), in double and triple mutants of actin regulatory proteins (e.g. Mena, Vasp and Evl), and in mice lacking protein kinases with cytoskeletal influence (e.g. Abl1/2 and Mapk8/9) (Copp and Greene, 2010). In contrast, spinal neurulation occurs normally in such embryos. Only knockouts of the cytoskeleton-associated proteins Shroom3 and MARCKS-related protein (Hildebrand and Soriano, 1999; Wu et al., 1996) yield both exencephaly and spina bifida and, even here, the frequency of exencephaly exceeds spina bifida. Hence, while the actomyosin cytoskeleton appears essential for cranial neurulation, its role in spinal closure is unclear.

Here, we examined in detail the role of the cytoskeleton in mouse spinal neural tube closure. Inhibitors were used to block specific cytoskeletal events in cultured embryos while closure was also examined in mice genetically null for the cytoskeletal protein Cofilin1. We show that actomyosin assembly and ATPase-dependent myosin contractile function are not limiting in spinal neural tube closure. In contrast, there is an essential role for F-actin turnover with the disassembly of actomyosin complexes being a crucial step that, when inhibited, leads to severe compromise of neurulation.
RESULTS

Inhibition of RhoA signalling disrupts the progression of spinal neural tube closure

Mouse embryos were explanted between embryonic days (E) 8.5 and 9.5, after neural tube closure had been initiated. Following culture for 5-6 h in the presence of cytoskeletal inhibitors, the length of spinal axis occupied by open neural folds (the posterior neuropore; PNP; Fig. 1A) was taken as a measure of neural tube closure progression. PNP enlargement indicates neurulation delay/cessation and predisposition to spina bifida (Copp, 1985).

Dose-response studies were used to select inhibitor concentration: we generally used the highest concentration at which general embryonic toxicity was not observed (Table S1). CytD, a drug preventing polymerisation of actin monomers (Flanagan and Lin, 1980), induced exencephaly at high frequency (not shown) but had no effect on spinal closure (Fig. 1B) consistent with previous findings (Wiley, 1980; Ybot-Gonzalez and Copp, 1999). Moreover, treatment with Blebbistatin which blocks myosin II in an actin-detached state, and prevents actomyosin cross-linking (Kovacs et al., 2004), also induced exencephaly but did not affect spinal closure (Fig. 1B), even when assessed over a 4-fold concentration range (Fig. S1A, D). In marked contrast, treatment with Y27632, a selective inhibitor of ROCK (Uehata et al., 1997), delayed spinal closure. After only 5-6 h exposure, Y27632-treated embryos had significantly enlarged PNPs compared with DMSO controls (Figs 1A, B; S1B), consistent with a report of neurulation disturbance by Y27632 in chick embryos (Wei et al., 2001).

To test whether Blebbistatin might require more prolonged exposure to be effective, or whether Y27632 had only a temporary effect, we cultured embryos for 18-20 h, to the 15-19 or 20-24 somite stage. All three inhibitors gave a similar result as in short-term exposure: CytD and Blebbistatin did not affect spinal closure whereas Y27632 produced enlarged PNPs (Figs 1C; S1B). Even longer culture, to the 25-36 somite stage when PNP closure is usually completed, yielded closed PNPs in embryos exposed to CytD (9/10 closed), Blebbistatin (7/7) and DMSO (12/12) but persistently open PNPs in Y27632-treated embryos (0/14 closed).

To replicate the effects of CytD and Y27632, we cultured embryos in additional inhibitors: Latrunculin B (LatB), an inhibitor of actin polymerisation (Spector et al., 1989), had no effect on PNP closure like CytD (Figs 1C; S1B), whereas hydroxyfasudil (HA-1100), another
ROCK inhibitor (Shimokawa et al., 1999), delayed PNP closure in a manner closely similar to Y27632 (Figs 1C; S1B). We conclude that ROCK inhibition (either by Y27632 or HA-1100) blocks the progression of mouse spinal neurulation, whereas CytD, LatB and Blebbistatin have no effect.

**Abnormal actomyosin accumulation in ROCK inhibitor-treated embryos**

Effectiveness and specificity of inhibitors was assessed by immuno-blotting and immuno-histochemistry. Y27632 inhibits ROCK-dependent phosphorylation of myosin light chain (MLC), LIM domain kinase (LIMK) and coflin (Maekawa et al., 1999), and we detected marked diminution of pMLC, pLIMK and p-cofilin in Y27632-treated embryos, but not in those exposed to Blebbistatin (Fig. 1D, E). Using a G-LISA assay, we detected a reduction of RhoA activity in Y27632-treated embryos (Fig. 1F), consistent with previous findings (Tang et al., 2012).

Phalloidin staining of neuroepithelial sections (Fig. 2A) revealed an organized actin network in DMSO-treated control embryos, with intense signal at the apical surface and aligned microfilaments within the neuroepithelium. Immunohistochemistry for non-muscle myosin II heavy chain B (MHCB; Fig. 2A) showed more generalized cellular staining, with marked co-localization of actin and myosin II at the apical neuroepithelial surface (merge; Fig. 2A). Both actin and myosin II distribution were severely disrupted in embryos treated with CytD or Blebbistatin: only a narrow domain of actomyosin remained visible in the most apical part of the neuroepithelium (Fig. 2A). We quantified actin and myosin II staining using a confocal-based scanning method to provide a basal-to-apical profile of staining intensity for each neuroepithelium (see Fig. S1E). This showed a striking diminution of both actin and myosin II staining in the Blebbistatin-treated neuroepithelium, particularly in the apical region, compared with DMSO (Fig. 2B, C). Combined exposure to Blebbistatin and CytD further diminished actin and myosin immunostaining (Fig. S2C, D), but these embryos also showed normal PNP closure (Fig. S1D). Hence, the lack of effect of CytD and Blebbistatin on spinal neural tube closure occurs despite a major disruption of actomyosin in the closing neuroepithelium.

Y27632 treatment led to dramatic enhancement of Phalloidin and anti-MHCB staining at the apical surface of the neuroepithelium (Fig. 2A). Quantitation showed an expansion of the average apical intensity peak of Phalloidin towards the basal neuroepithelial surface (Fig.
2B), which was highly reproducible between embryos (Fig. S2A). Dividing the apical portion of the neuroepithelium into three bins: 71-80, 81-90 and 91-100% of the normalized basal-to-apical distance (Fig. 2D), we found a significantly greater proportion of total actin in the more basal bins after Y27632 treatment (Fig. 2D, E). Biochemical fractionation showed that around 70% of total actin is filamentous in Y27632-treated embryos whereas only 25% is F-actin in DMSO controls (Figs 2F; S2B). In contrast, CytD-treated embryos contained extremely little F-actin, with the great majority being soluble G-actin (Figs 2F; S2B). Anti-MHCB staining intensity was also markedly redistributed to the apical neuroepithelial surface after Y27632 (Fig. 2A, C). We conclude that ROCK inhibition causes an abnormal actomyosin accumulation apically within the neuroepithelium.

**Increased F-actin stabilization is sufficient to delay spinal neural tube closure**

To test whether apical actomyosin accumulation, as seen after Y27632 treatment, may be responsible for defective spinal neurulation we used Jasplakinolide (Jasp), a drug that blocks actin depolymerisation and increases the F-actin pool (Cramer, 1999). Culture in Jasp for 18-21 h to the 15-19 and 20-24 somite stages caused significant closure delay compared with DMSO controls (Figs 3A; S2E). More than 70% of actin was filamentous in these embryos (Fig. 3B) with a 3-fold reduction in monomeric G-actin (Fig. S2F). Sections showed a massive accumulation of co-localized F-actin and MHCB at the apical surface as well as patches more basally within the neuroepithelium (Fig. 3C). Quantitation confirmed the mainly apical accumulation of actomyosin (Fig. 3D-F), which was reproducible between embryos (Fig. S2G). We considered the possibility that inhibition of ROCK might lead to a RhoA-dependent inhibition of embryonic cell proliferation or increase in cell death. However, measurement of the percentage of neuroepithelial cells positive for phospho-histone H3 staining and activated caspase 3 showed that neither cell proliferation nor programmed cell death was altered in Y27632- or Jasp-treated embryos (Fig. S2H, I). We conclude, therefore, that F-actin stabilization by Jasp is sufficient to cause apical actomyosin accumulation and to inhibit spinal neural tube closure.

**RhoA signalling requirement for neurulation is independent of myosin II contractility**

Downstream of RhoA, ROCK regulates phosphorylation of LIMK and MLC (Fig. 4A), with pMLC being a key regulatory component of myosin II ATPase activity in cellular constriction (Matsumura, 2005). To investigate this pathway, we used ML-7, a specific inhibitor of MLC kinase (Saitoh et al., 1987). Apical pMLC immunostaining was abolished
by culture for 5-6 h in 50 µM ML-7 (Fig. 4B) and only pMLC was reduced in immunoblots of ML-7 treated embryo extracts, whereas pLIMK and p-cofilin were unaffected (Fig. 4C). Despite these effects, ML-7 treatment was compatible with early spinal neural tube closure in embryos over a 5-fold concentration range (Figs 4D; S3A, B). Embryos treated with ML-7 had a reduced overall intensity of F-actin and MHCB immunostaining, whereas the apico-basal distribution was closely similar to DMSO controls (Fig. 4E-H). Embryos co-treated with ML-7 and Blebbistatin closed their PNPs normally (Fig. S1D), despite a dramatic reduction in Phalloidin and MHCB staining (Fig. S2C, D). Hence, the requirement for RhoA signalling in spinal neurulation is unlikely to be mediated through myosin II ATPase-dependent contractility.

Preventing actomyosin accumulation by Blebbistatin rescues Y27632-induced defects

We asked whether preventing the abnormal accumulation of actomyosin in Y27632-treated embryos might ameliorate their spinal neurulation defects. Simultaneous exposure to Y27632 and Blebbistatin, or 5-6 h in Y27632 followed by 13-15 h in Blebbistatin, produced embryos with PNP lengths closely similar to DMSO-treated controls (Figs 5A, B; S3C-E). Moreover, co-treatment with Y27632 plus Blebbistatin yielded a largely normal actomyosin distribution (Fig. 5C), which was confirmed by quantitation (Fig. 5D-F). The Jasp inhibitory effect on PNP closure also appeared to be abrogated by co-exposure to Blebbistatin (Fig. S3J, K). In contrast, embryos co-treated with Y27632 plus ML-7 showed delayed PNP closure like those exposed to Y27632 alone (Figs 5B; S3C) with abnormal actomyosin accumulation (Fig. 5C-F). These results argue that faulty neural tube closure in Y27632-exposed embryos resulted from abnormal actomyosin accumulation, and not elevated contractile myosin activity.

We explored in more detail the mechanism by which Blebbistatin can rescue embryos from the effects of ROCK inhibition. Short-term culture (STC; 5-6 h) in Y27632 followed by culture without inhibitor (‘washout’) yielded normal PNP lengths (Fig. S3F, H), suggesting the apparent rescue by Blebbistatin might be due simply to Y27632 removal. However, long-term culture (LTC; 13-15 h) in Y27632 had a persistent adverse effect of PNP closure, even when the inhibitor was removed, whereas culture in Blebbistatin following long-term exposure to Y27632 yielded a normal PNP length (Fig. S3F, H). Moreover, both STC and LTC in Y27632 followed by continued culture in Y27632 plus Blebbistatin rescued PNP closure at different somite stages (Fig. S3G, H). Embryos from this latter experiment had a normalised F/G actin ratio (Fig. S3I). Hence, Blebbistatin can rescue PNP closure both in the
absence and presence of Y27632, arguing that it may prevent ROCK-inhibitor-dependent actomyosin accumulation, and may favour actomyosin disassembly after it has accumulated.

A key role for cofilin in regulating actomyosin accumulation and spinal neurulation
Cofilin-mediated actin severing activity (Bamburg, 1999) is regulated by RhoA/ROCK, via LIMK-mediated phosphorylation of cofilin (Fig. 4A). We asked whether Cofilin 1 knockout embryos display reduced neuroepithelial F-actin turnover, similar to Y27632- and Jasp-treated embryos. Cofilin 1−/− embryos have cranial neurulation defects (Gurniak et al., 2005), as confirmed here (Fig. 6A). In spinal neurulation, PNP length did not differ between Cofilin 1−/− and wild-type at 15-19 somites whereas, by 20-24 somites, Cofilin 1−/− embryos had significantly longer PNPs than wild-type or heterozygotes (Figs 6A, B; S4A).

Cofilin 1−/− mutants with 20-24 somites exhibited abnormal actin accumulation and a marked increase in MHCB immunostaining at the apical neuroepithelium (Fig. 6C). Importantly, actomyosin accumulation was already present at 15-19 somites (Fig. S4B), before a difference in PNP length became evident. Quantitation of actin staining showed a basal expansion of the apical neuroepithelial intensity peak, at both 15-19 and 20-24 somites (Fig. 6D-E). Myosin II staining intensity was moderately increased in the apical neuroepithelium at 15-19 somites, and massively increased at 20-24 somites (Fig. 6F). Hence, Cofilin 1 mutants accumulate actomyosin at the neuroepithelial surface, similar to Y27632- and Jasp-treated embryos, before the onset of defective neural tube closure. Moreover, treatment of cultured Cofilin 1−/− embryos with Blebbistatin was able to partially restore normal actomyosin distribution (Fig. S4G) and to markedly reduced PNP length (mean = 0.43 mm; n = 2 compared with untreated mutants (mean = 0.66 mm; n = 5). This confirms the importance of regulating actomyosin accumulation for spinal neurulation.

Mean PNP length did not differ between Cofilin 1 heterozygotes and wild-type at 20-24 somites (Figs 6B; S4A). However, 4 out of 10 heterozygotes had PNP lengths in excess of 0.5 mm, overlapping with Cofilin 1−/− mutants (Fig. 6B). Strikingly, actomyosin was more intense and expanded at the apical neuroepithelium in heterozygotes with large PNPs, whereas actomyosin staining was similar to wild-type when the PNP was small (Figs 6G, H; S4C, D). In fact, actomyosin staining intensity in the sub-apical region of neuroepithelium was strongly correlated with PNP length (Fig. 6I). Hence, Cofilin 1 haploinsufficiency causes apical actomyosin accumulation and defective spinal neurulation in a proportion of embryos.
**Actin accumulation affects adherens junctions in a RhoA-dependent manner**

Actomyosin disassembly defects could affect neural tube closure through disturbance of the stereotypical pattern of neural plate bending, in which MHP and paired DLHPs are necessary for spinal closure (Greene et al., 1998; Shum and Copp, 1996; Ybot-Gonzalez et al., 2002). Inhibitor-treated and *Cofilin 1* mutant embryos with delayed PNP closure delay had relatively normal DHLPs (Figs 2A, 3C, 4B, 5C; Tables S2, S3). In contrast, we noted a reproducible lack of MHP bending in *Cofilin 1*--/-- embryos and in those treated with Y27632 or Jasp, specifically at the 20-24 somite stage but not at 15-19 somites (Tables S2, S3). Hence, a lack of focal midline neuroepithelial bending could mediate the effects of actomyosin accumulation at later stages of spinal neurulation.

Another possibility is that abnormal actomyosin accumulation may affect the structure and/or function of apical junctional complexes, which are implicated in neuroepithelial morphogenesis (Nishimura and Takeichi, 2009). Indeed, coflin plays a defined role in junctional complex remodelling (Chu et al., 2012). In wild-type embryos, β-catenin specifically localized to the lateral membranes of neuroepithelial cells with apical enrichment at adherens junctions (AJs), where it co-localized with Phalloidin (Fig. 7A, WT). In contrast, *Cofilin 1*--/-- embryos, and heterozygotes with large PNPs, exhibited markedly disorganized β-catenin staining, both apically and deeper within the epithelium, which co-localized with ectopic F-actin (Figs 7A; S4D). Exposure of *Cofilin 1*--/-- embryos to Blebbistatin in culture largely normalised β-catenin immunostaining (Fig. S4G).

ZO1 localizes to tight junctions (TJs) and showed precise immunolocalization on the apical surface of wild-type neuroepithelium (Fig. S4E). In contrast, ZO1 immunostaining was more intense apically in *Cofilin 1*--/-- embryos, with ectopic neuroepithelial patches that co-localized with Phalloidin staining (Fig. S4E). This ZO-1 staining defect was not normalised in *Cofilin 1*--/-- embryos treated in culture with Blebbistatin (Fig. S4H).

Immunostaining for β-catenin in Jasp- and Y27632-treated embryos revealed abnormally intense apical signal that was organised into clusters co-localizing with F-actin (Fig. 7A). In contrast, ML-7 or Blebbistatin exposure did not alter β-catenin immunolocalization apically, although staining was reduced on lateral membranes. Embryos cultured in Y27632 plus Blebbistatin, exhibited a β-catenin distribution resembling DMSO controls (Fig. 7A). ZO1
immunostaining was confined to the apical surface of the neuroepithelium in all cultured embryos, with no discernible abnormalities in inhibitor-treated embryos (Fig. S4F). Hence, AJs are dramatically disrupted in \textit{Cofilin 1}\textsuperscript{-/-} embryos and, less severely, when ROCK signalling and F-actin disassembly are inhibited. TJs are defective in \textit{Cofilin 1} mutants but not after inhibitor treatment. This argues that actin turnover is essential in enabling AJ remodelling as the spinal neuroepithelium undergoes morphogenesis.
DISCUSSION

We have examined the cytoskeletal requirements for mouse spinal neural tube closure, using inhibitor-treated and Cofilin1 mutant embryos. The RhoA/ROCK/LIMK/cofilin signalling pathway regulates F-actin turnover in the mouse neuroepithelium and has proven essential for closure. Surprisingly, prevention of actomyosin cross-linking (by Blebbistatin) or F-actin assembly (by CytD or LatB) does not halt mouse spinal neurulation. In contrast, inhibiting Rho kinase (by Y27632 or HA-1100) or blocking F-actin disassembly (by Jasp and in Cofilin 1 mutants) arrests closure. Actomyosin accumulation at the apical neuroepithelial surface correlated strongly with closure defects. Importantly, when the accumulated actomyosin was disassembled by Blebbistatin, neurulation disorders were rescued, wholly or partially, in Y27632- and Jasp-treated embryos and in Cofilin 1 mutants. In contrast, ML-7 that inhibits myosin II contractile activity was unable to rescue the closure defects. These findings suggest that apical actomyosin accumulation is causally related to failure of neural tube closure.

Requirement for precise regulation of RhoA/ROCK/LIMK/cofilin signalling

Apical actomyosin accumulation and closure failure resulted from either ROCK inhibition or Cofilin 1 mutation. Yet ROCK inhibition, by diminishing the sequential phosphorylation of LIMK and cofilin (Bamburg, 1999), produces abundant active cofilin whereas Cofilin 1 mutants lack cofilin activity. These findings suggest a model (Fig. 7B) in which actin turnover in the neuroepithelium, and spinal neural tube closure, depend on finely-tuned signalling through the RhoA/ROCK/LIMK/cofilin pathway. Cofilins sever actin filaments at low-cofilin binding density whereas, at high concentration, cofilin can nucleate actin assembly through monomer binding (Andrianantoandro and Pollard, 2006; Ghosh et al., 2004). Cofilin depletion leads to actomyosin accumulation through a mechanism in which cofilin competitively inhibits myosin II binding to F-actin (Wiggan et al., 2012). Hence, increased active cofilin in Y27632-treated embryos and its absence in Cofilin 1 mutants can both generate stable F-actin in the neuroepithelium. We conclude that mouse spinal neurulation requires precisely regulated ROCK signalling to maintain a balance between cofilin-mediated actin severing and nucleation, ensuring actin turnover and actomyosin disassembly at the apical neuroepithelium.
Mechanisms of faulty neurulation in embryos with apical actomyosin accumulation

The process of elevation and dorsal apposition of the spinal neural folds in mice involves the formation of focal bending sites, at the midline and dorsolaterally (Greene et al., 1998; Ybot-Gonzalez et al., 2002; Ybot-Gonzalez et al., 2007a). Between the sites of bending, the elevating neural folds appear strikingly straight (Shum and Copp, 1996), suggesting that the generation and maintenance of overall neuroepithelial tension is also important for normal neural fold morphology. The majority of embryos with faulty closure exhibited apparently normal DLHPs, although MHPs were consistently absent from embryos with delayed closure at the 20-24 somite stage. Hence, while faulty midline bending may contribute to the later stages of closure delay, it seems unlikely that accumulation of actomyosin initiates closure delay through interference with focal bending. More likely, disturbance of neuroepithelial tension may be the primary mechanism by which Y27632- and Jasp-treatment, and Cofilin1 mutation, leads to delay in PNP closure.

Is reduced or enhanced neuroepithelial tension likely to be more disruptive of neurulation? CytD- and Blebbistatin-treated embryos exhibit ‘floppy’ neural folds (Ybot-Gonzalez and Copp, 1999)(Table S2), and yet still progress in spinal neurulation. This suggests that diminished neuroepithelial tension is compatible with closure, provided MHP and DLHPs are formed. Interestingly, embryos exposed to ML-7 did not exhibit floppy neural folds (Table S2), suggesting that ATPase-dependent myosin II contractility is unlikely to be limiting for generation of neuroepithelial tension. Alternatively, ATPase-independent myosin contraction could play a role. In other systems, actomyosin ring contraction can result from dynamic de- and re-polymerization of actin filaments, independent of ATPase activity (Henson et al., 2002; Pelham and Chang, 2002; Sun et al., 2010). Hence, regulated assembly-disassembly of apical F-actin, mediated by ROCK/cofilin signalling, could be an important mechanism for generating neuroepithelial tension during spinal neurulation. We propose, therefore, that accumulation of actomyosin may generate excessive neuroepithelial tension, rendering the neural plate stiff and resistant to morphogenesis, despite the presence of MHP and DLHPs.

Adherens junctions and spinal neurulation

A further aspect of neuroepithelial tension maintenance likely involves AJs, which were consistently disturbed in neuroepithelia with actomyosin accumulation. Apical junctional complexes regulate epithelial integrity, polarity and dynamic intercellular movements during morphogenesis (Nishimura and Takeichi, 2009). In chick neurulation, PCP/ROCK-mediated
neuroepithelial cellular rearrangement depends on myosin cables that connect junctional complexes of adjacent cells (Nishimura et al., 2012), while AJ remodelling is also strongly dependent on RhoA/ROCK activity in other systems (Fang et al., 2008; Sahai and Marshall, 2002). In Drosophila amnioserosa cells, actin stabilization with Jasp inhibited the redistribution of AJJs and increased total AJ levels (Goldenberg and Harris, 2013). It seems likely, therefore, that the abnormal AJJs we observed in embryos with defective spinal neurulation resulted from disruption of the dynamic actomyosin assembly-disassembly cycle, and contributed to faulty neuroepithelial tension in embryos with faulty PNP closure.

Myosin dynamics and neural tube closure
Blebbistatin blocks myosin II in an actin-detached state (Kovacs et al., 2004), thereby inhibiting two of its key functions: ATP-binding required for motor activity and actin cross-linking required for development of tension, independent of MLC phosphorylation (Bresnick, 1999; Wang et al., 2011). Since spinal neurulation continued in the presence of Blebbistatin, we conclude that neither of these functions is limiting for closure. In fact, enhanced actin crosslinking by myosin II could also contribute to the postulated increase in neuroepithelial tension in embryos that fail in spinal closure. Of the three myosin II heavy chains, MHCB is the predominant isoform expressed in neuroepithelial cells (Wang et al., 2011) and fetal brain (Golomb et al., 2004), whereas MHCA and MHCC are present at only low levels. MHCB is characterized by a high ‘duty ratio’, with strong ADP-binding and propensity to exist in a rigor state, tightly bound to actin (Rosenfeld et al., 2003; Wang et al., 2003). The striking translocation of MHCB to the apical neuroepithelial surface in embryos with ROCK inhibition (and hence increased cofilin activity) suggests that apical actin may be stabilized by MHCB, further diminishing actomyosin disassembly. Interestingly, the opposite effect, in which myosin and F-actin were found to accumulate basally in the neuroepithelium, was recently described in the cranial region of Cofilin 1 mutants (Grego-Bessa et al., 2015).

How Blebbistatin acts to diminish actomyosin accumulation in the apical neuroepithelium, and rescue spinal neural tube closure, is unclear. As noted above, Blebbistatin inhibits actin cross-linking and this may help prevent actomyosin from accumulating. However, we also observed rescue when Blebbistatin was added to embryos that had been cultured for extended periods in ROCK inhibitor. This might suggest that Blebbistatin can enhance the disassembly of actomyosin, although the mechanism of this putative action is unknown.
Stage and species variations in cytoskeletal requirement for neurulation

The onset of neural tube closure is strongly PCP-dependent in mice (Copp and Greene, 2010) and it was shown recently that the severe PCP closure phenotype of homozygous loop-tail (Vangl2<sup>Lp</sup>) embryos is further exacerbated by co-inactivation of Cofilin 1 or by Jaspl treatment. This confirmed a role for F-actin turnover in PCP initiation at the onset of closure (Mahaffey et al., 2013). Once closure has been initiated, at the brain-spine boundary, the cranial neural plate undergoes major morphogenetic changes, starting with expansion of bi-convex neural folds, followed by dorsolateral bending which brings the neural fold tips together in the dorsal midline (Morriss-Kay, 1981). This latter event has been found to be exquisitely sensitive to disruption of apical actin microfilaments, with cytochalasins causing exencephaly both <i>in vivo</i> and <i>in vitro</i> (Austin et al., 1982; Morriss-Kay and Tuckett, 1985; Wiley, 1980). We observed cranial neural tube defects in embryos cultured in Blebbistatin and LatB (data not shown), suggesting a general requirement for contractile actomyosin in cranial neural folding. Following completion of cranial neurulation, the spinal neural tube ‘zips up’ along the body axis to complete closure of the primary neural tube, and this process has strikingly different cytoskeletal requirements from cranial closure: regulation of neuroepithelial tension is required with a key role for ATPase-independent actomyosin dynamics.

In contrast to mammals, lower vertebrates appear more completely reliant on ATPase-dependent actomyosin contraction for neural tube closure. Chick embryos exposed to Blebbistatin exhibit severe neural tube defects due to defective apical constriction (Kinoshita et al., 2008) while myosin II knockdown in <i>Xenopus laevis</i> impairs neural plate bending (Rolo et al., 2008). These differences between animal groups may reflect the somewhat ‘simpler’ pattern of neurulation events in birds and amphibians where closure of brain and spine are relatively homogeneous in timing and morphology compared with mammals where the events along the body axis cover a long developmental period and are morphologically extremely diverse (Morriss-Kay, 1981).
MATERIALS AND METHODS

Breeding and genotyping of mouse strains
Mouse procedures were performed under the UK Animals (Scientific Procedures) Act 1986 and ‘Responsibility in the Use of Animals for Medical Research’ (Medical Research Council, 1993). Cofilin1 (Cfl1) knockout mice were as bred and genotyped as described (Gurniak et al., 2005). Non-mutant embryos were from random-bred CD1 mice.

Embryo collection and culture
After overnight mating, pregnant females were killed at E8.5-E9.5 days of gestation (E0.5 is noon on the day of the copulation plug). Embryos were dissected in Dulbecco’s Modified Eagle's Medium (Invitrogen) containing 10% fetal bovine serum (Sigma). Following whole embryo culture (Copp et al., 2000), or immediately after dissection of non-cultured embryos, somites were counted and PNP length was measured using an eyepiece graticule on a Zeiss SV11 stereomicroscope. Embryos for immunostaining were fixed for 1 h in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), embedded in 7.5% gelatin in 15% sucrose/PBS and cryosectioned. Embryos for protein extraction were rinsed twice in ice-cold PBS, snap frozen on liquid N\textsubscript{2} and stored at -80°C. For Western blot, F/G actin and Rho activation assays, PNP and ‘body’ fragments were generated by cutting at the rostral border of the most recently formed somite pair. Both fragment types were assayed separately.

Chemical inhibitors
Inhibitors were prepared as stock solutions in DMSO and stored frozen: Cytochalasin D (C8273 Sigma, 100 mg/mL), Latrunculin B (#428020 Calbiochem, 25 mg/mL), Blebbistatin (#203390 Calbiochem, 75 mg/mL), ML-7 (#12764 Sigma,10 mM), Y27632 (#688000 Calbiochem, 5 mM), HA-1100 ((#390602 Calbiochem, 10 mM), Jasplakinolide (#420107 Calbiochem, 1 mM). Immediately prior to use, stocks were diluted with DMSO and added to embryo culture serum to a maximum of 0.1% (v/v). Cultures were performed in the dark to avoid photo-inactivation.

Protein extraction and western blotting
Proteins were extracted from frozen, unfixed tissue by sonication in RIPA buffer (1% Nonidet P-40,150 µM NaCl, 10 µM Tris-HCl, pH 8, 1× complete protease inhibitor cocktail,
in PBS). Tissue lysates were cleared of unbroken cells by centrifugation (2000 x g),
electrophoresed on 10% or 4-12% Bis-Tris gels (NuPage, Invitrogen) and transferred to
PVDF membrane (XCell II Blot Module, Invitrogen). Primary antibodies for
immunodetection are described in Table S4. Detection was by horseradish peroxidase-
conjugated secondary antibodies (DAKO), followed by development with ECL prime
Western blotting detection system (GE Healthcare). Autoradiographs were scanned using a
GS-800 Imaging Densitometer (BioRad) and analysed with Quantity One software (BioRad).

**F/G actin and Rho activation assays**

The ratio of free monomeric actin (G-actin) to filamentous actin (F-actin) was determined by
fractionation. Cells were lysed in ice-cold PHEM buffer (60 mM Pipes, 20 mM HEPES, 10
mM EGTA, 2 mM MgCl2, pH 7.0, 1% Triton-X100). Cleared lysates (2000 x g) were
centrifuged at 10,000 x g to separate Triton-X100-soluble G-actin from Triton-X100-
insoluble F-actin. Supernatant containing Gactin was collected and the F-actin pellet was
washed twice in cold PHEM buffer and suspended in SDS buffer. Fractions were
proportionally loaded onto SDS-polyacrylamide gels, electrophoresed, and transferred to
PVDF membrane for probing with anti-actin antibody (A5316, Sigma). The densitometric
quantification of the Western blot determined the G- versus F-actin content.

Activated RhoA was determined by RhoA G-LISA Activation Assay kit (# BK124,
Cytoskeleton Inc.) according to manufacturer's instructions. Active GTP-bound RhoA in
protein lysates was bound to RhoA-GTP-binding proteins linked to the wells of a 96-well
plate, while inactive GDP-bound forms were removed during wash steps. GTP-bound Rho
was detected by indirect immunofluorescence using anti-RhoA antibody. Absorbance was
measured at 490 nm using a microplate spectrophotometer reader (BioTek synergy HT).
Total RhoA was determined by ELISA assay (# BK150, Cytoskeleton Inc.). Assays were
performed with duplicate measures.

**Immunohistochemistry**

Gelatin was removed from cryosections by incubation in PBS for 30 min at 37°C. Samples
were blocked and permeabilized in 10% sheep serum, 0.1% Tween-20 in PBS, incubated
with primary antibody overnight at 4°C, rinsed in PBS, and incubated with secondary
antibody for 1 h at room temperature. For F-actin, sections were incubated in Phalloidin
(Alexa Fluor 568-Phalloidin, A12380, Life technologies) for 1 h at room temperature.
Samples were washed with DAPI (4’,6-diamidino-2-phenylindole) and mounted in Mowiol 4-88 mounting medium (Sigma-Aldrich, MO; prepared with glycerol and 0.2 M Tris pH 6.8). Primary antibodies are described in Table S4. Secondary antibody was Alexa Fluor 488 goat anti-rabbit IgG (A11070, Life technologies).

**Cell proliferation and cell death analysis**

Immunostaining was performed for phospho-histone H3 (Millipore) and cleaved-caspase 3 (Cell Signalling) on 12 μm transverse crysections at the axial level of the closing spinal neural tube (17–19 somites). Fluorescent images were collected using a Zeiss Axiophot microscope with Leica DC500 camera and FireCam software. Images were analysed using the Cell Counter plugin of the Image J software. Phospho-histone H3or caspase 3-positive cells were counted and expressed as a percentage of the total cell number visualized by DAPI staining. Three embryos per treatment were analysed and, for each embryo, cells from at least 3 sections were counted.

**MHP and DLHP scoring**

During spinal neural plate bending, the MHP is defined as an acute (focal) midline bend in the neural plate, present during the early stages (Modes 1 and 2) of mouse spinal neurulation. At the latest stage (Mode 3), an MHP is absent: the midline neural plate is not focally bent, but gradually or uniformly curved, or even flat (Shum and Copp, 1996). These criteria were used to score the MHP as either present or absent, after inspection of serial sections throughout the PNP. For examples of MHP-positive neural plates, see Figs 2A (DMSO) and 3C (DMSO), while MHP-negative neural plates are seen in Figs 2A (Blebb) and 3C (Jasp). DLHPs like the MHP are characterised by an acute, focal bend of the neural plate, present during late stages of spinal neurulation (Modes 2 and 3) but not at the earliest stage (Mode 1) (Shum and Copp, 1996). In transverse section, DLHPs are always centred at the point where the neural plate transitions from contact with surface ectoderm, dorsolaterally, to contact with paraxial mesoderm, ventromedially. These criteria were used to score DLHPs as either present or absent, after inspection of serial sections throughout the PNP. For examples of DHLPs, see Figs 3C, 5C and 6C.

**Confocal microscopy**

Labelled cells in immunohistochemistry or Phalloidin stained sections were examined and quantified by epifluorescence on an inverted LSM710 confocal system mounted on an Axio
Observer Z1 microscope (Carl Zeiss Ltd, UK). Images were acquired at room temperature using a 63× oil immersion objective. The thickness of optical sections was set at 0.2 to 0.9 μm. The Alexa Fluor 488 dye was excited by a 488 nm line of an Argon laser and Alexa Fluor 568 by a 561 nm diode laser. Z projections of confocal stacks were created in ImageJ. Images were further processed in Photoshop CS3 (Adobe).

**Image analysis and quantification**

Between three and five adjacent immunohistochemistry or Phalloidin stained sections were analysed per embryo. For each section and each side of the neural plate, a region of interest (ROI) was outlined, comprising the full thickness of the neural plate along approximately 30% of the dorsoventral extent of one hemi-plate. The fluorescence intensity within the ROI was measured using the Image function “Plot Profile” integrated in custom written ImageJ macro #1. In this macro, ImageJ plot profile function displays a ‘column average plot’, where the X-axis represents the basal-apical distance through the ROI, and the Y-axis represents the vertically averaged pixel intensity of the ROI. Each curve was normalized to a range of 0-100 along the basal-apical axis (X axis)). Once normalized, the intensity values (Y axis) were interpolated (integers between 0 and 100 for the X axis) using a custom written Matlab script (macro #2) to allow statistical analysis and comparison between samples. For some Phalloidin-stained sections, the intensity values (Y axis) were also normalized using macro #1. Macros are available upon request. See also Fig. S1E.

**Statistical analysis**

Statistical tests were performed using SigmaStat v3.5 (Systat Software Inc). At least three independent experiments were performed for each assay.
ACKNOWLEDGEMENTS

We thank Guillaume Charras and Ana Rolo for critical reading of the manuscript, and the Confocal Core Facility at the UCL Institute of Child Health for support. Funding was by grants from the Wellcome Trust (087525) and the Medical Research Council (G0801124).

AUTHOR CONTRIBUTIONS

SE contributed to the execution, and analysis of experiments. BV contributed to imaging analysis. DS, CBG and WW contributed to mouse genetic studies. SE, NDEG and AJC contributed to the concept, design and funding of experiments, and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.
REFERENCES


**FIGURES**

Figure 1. Inhibition of RhoA signalling disrupts spinal neural tube closure.

(A) Mouse embryos cultured for 18 h to 24 somite stage. Exposure to 5 μM Y27632 yields an enlarged PNP (between arrowheads), open forebrain (asterisks) and defective heart looping (h) compared with DMSO control. Inset: method of PNP length measurement, between the dashed lines. Bar: 0.5 mm.
(B) Progressive closure of PNP is arrested after the 13 somite stage in embryos cultured for 5-6 h in Y27632 (** p < 0.001 versus DMSO). Embryos exposed to 50 µM Blebbistatin or 0.05 µg/ml CytD close at the same rate as DMSO controls.

(C) PNP closure is significantly delayed after culture in ROCK inhibitor Y27632 or HA-1100, for 18-20 h to the 15-19 or 20-24 somite stage (** p < 0.001 versus DMSO). Embryos cultured in Blebbistatin, CytD or LatB resemble DMSO controls.

(D) Immunohistochemistry (green: anti-pMLC; nuclei: DAPI) shows apically localized pMLC (arrowheads) in DMSO- and Blebbistatin-treated embryos with 19 somites. Signal is abolished by 5-6 h culture in Y27632. Grayscale insets: pMLC staining only. Bars: 30 µm.

(E) Western blots for pLIMK/LIMK, p-Cofilin/Cofilin and pMLC/MLC. Phospho-forms are quantitated as a proportion of total, after normalizing to GAPDH. Culture for 5-6 h in Y27632 significantly reduces relative amount of pMLC, pLIMK and p-cofilin compared with DMSO controls, whereas Blebbistatin has no effect (n = 3, ** p < 0.001).

(F) G-LISA assay of active GTP-bound RhoA (top) relative to total RhoA (below), normalized to DMSO (mean ± s.d.). Y27632-treated embryos have significantly reduced RhoA activation whereas total RhoA does not differ between treatments (n = 3, * p < 0.001).
Figure 2. ROCK inhibition leads to actomyosin accumulation in apical neuroepithelium.

(A) Immunohistochemistry for F-actin (Phalloidin, red) and myosin II (anti-MHCB, green).
Culture for 5-6 h in CytD or Blebbistatin strongly reduces apical (arrowheads) and non-apical
(single asterisks) staining. In contrast, actomyosin appears abnormally extensive after Y27632 treatment (multiple asterisks). Note presence of MHP (white dot) and DLHPs (red arrowheads) in both Y27632-treated and DMSO control embryos. Right: enlarged boxed areas. Yellow box in top left image: typical area of neuroepithelium in which basal-to-apical staining intensity was measured. Embryos shown have 17-19 somites. Bars: 30 μm.

(B, C) Intensity profile scans of Phalloidin (B) and anti-MHCB (C) staining along neuroepithelial basal-to-apical axis. Basal surface = 0; apical surface = 100 (a.u. = arbitrary units). F-actin is enhanced apico-basally and MHCB is redistributed to the apical surface in Y27632-treated embryos compared with DMSO. Blebbistatin reduces both F-actin and MHCB.

(D, E) With maximum intensity normalized to 100%, the apical domain of Phalloidin staining extends farther basally in Y27632-treated embryos than DMSO controls (arrows in D). Quantitation in the three apical neuroepithelial bins (71-80, 81-90, 91-100) shows that Phalloidin extends significantly more basally in Y27632-treated embryos compared with DMSO (** p < 0.001 in E).

(F) Biochemical fractionation of F- and G-actin. Stable F-actin represents 70% of actin in Y27632-treated embryos, only 25% in DMSO controls, and less than 3% after CytD treatment (** p < 0.001).
Figure 3. Stabilization of F-actin delays spinal neural tube closure.

(A) PNP length is significantly increased at 15-19 and 20-24 somites after 18-20 h culture in 10 nM jasplakinolide (Jasp), compared with DMSO controls (* p < 0.05).
(B) Biochemical fractionation shows proportionately increased F-actin and reduced G-actin in Jasp-treated embryos relative to DMSO controls (** p < 0.001).

(C) Immunohistochemistry (Phalloidin, red; anti-MHCB, green) reveals actomyosin accumulation at the apical neuroepithelial surface (asterisks) and on some lateral cell surfaces (arrowheads) after Jasp treatment (embryos have 20-21 somites). Right: enlarged boxed areas. Bars: 30 µm.

(D-F) Intensity profile scans along the neuroepithelial basal-to-apical axis. Jasp-treated embryos show extension of Phalloidin staining (intensity normalized to 100%) towards the basal surface (arrows in D), which is confirmed by quantitation in the most apical 30% of the neuroepithelium (E; ** p < 0.001). MHCB staining intensity (non-normalized) is greater apically in Jasp-treated embryos than DMSO controls (F).
Figure 4. Spinal neurulation proceeds despite inhibition of myosin II contractility.

(A) Downstream effectors of ROCK and inhibitory action of Y27632 and ML-7.

(B) Immunohistochemistry shows absence of pMLC (green; nuclei: DAPI) from apical neuroepithelium of ML-7 treated embryos, compared with DMSO controls (embryos have 18-19 somites). Grayscale insets: pMLC staining only. Bar: 30 µm.

(C) Western blots for pLIMK/LIMK, pCofilin/Cofilin and pMLC/MLC. Embryos cultured for 5-6 h in ML-7 have significantly reduced pMLC compared with DMSO controls, whereas pLIMK and pCofilin are unaffected (n = 3, ** p < 0.001).
(D) Culture for 5-6 h in 50 µM ML-7 does not significantly affect closure compared with DMSO at the 15-19 somite stage (n.s: p > 0.05).

(E) Immunohistochemistry (Phalloidin, red; anti-MHCB, green) of ML-7 treated embryos shows apical actomyosin closely resembling DMSO controls (arrowheads; see insets on right), whereas actomyosin is reduced more basally (asterisks). Embryos have 18 somites. Bars: 30 µm.

(F-H) Intensity profile scans show unchanged apical Phalloidin staining after ML-7 treatment (F), as confirmed by quantitation (G; n.s: p > 0.05 versus DMSO). More basal Phalloidin (F) and MHCB (H; non-normalized) staining is reduced by ML-7.
Figure 5. Prevention of Y27632-related closure defects by Blebbistatin but not ML-7

(A, B) The delayed PNP closure seen after culture in 5 µM Y27632 (B) is rescued by co-exposure to 50 µM Blebbistatin (A, B; ** p < 0.001) but not by ML-7 (B; n.s. p > 0.05). Cultures were for 5-6 h (A) or 15-18 h (B), ending at the somite stages indicated.
(C) Immunohistochemistry (Phalloidin, red; anti-MHCB, green) reveals a normal actomyosin distribution in embryos co-exposed to Y27632 + Blebbistatin (upper panels; compare with DMSO in Figs 2A, 3C). In contrast, embryos co-exposed to Y27632 + ML-7 (lower panels) show actomyosin accumulation apically (asterisks), as in those treated with Y27632 alone (see Fig. 2A). Embryos have 21 somites.

(D-F) Intensity profile scans confirm expanded apical Phalloidin staining in embryos treated with Y27632 alone, and Y27632 + ML-7, whereas those exposed to Y27632 + Blebbistatin resemble DMSO controls (D). This is confirmed by quantitation (E; comparisons with DMSO: ** p < 0.001; * p < 0.05; n.s. p > 0.05). MHCB staining profile is closely similar in Y27632 + Blebbistatin and DMSO controls, but markedly abnormal in those treated with Y27632 alone and Y27632 + ML-7 (F).
Figure 6. Requirement for RhoA signalling is via the LIMK/cofilin pathway.

(A) E9.5 embryos from Cofilin 1\textsuperscript{+/+} x Cofilin 1\textsuperscript{+/-} mating. Homozygotes (top right) are smaller than WT, and exhibit exencephaly (arrow) and enlarged PNP (arrowheads). Heterozygotes (lower panels) appear normal, except some have an enlarged PNP. Bar: 0.5 mm.
(B) PNP length is significantly increased at 20-24 somites, but not at 15-19 somites, in Cofilin 1−/− mutants compared with WT and heterozygotes (** p < 0.001).

(C) Immunohistochemistry (Phalloidin, red; anti-MHCB, green) shows apical actomyosin accumulation in Cofilin 1−/− mutants (asterisks) but not in WT, at 21-22 somites. Right: enlarged boxed regions. Bars: 30 µm.

(D-F) Intensity profile scans show that Phalloidin staining extends more basally in Cofilin 1−/− embryos than WT (arrows in D; intensity normalized to 100%), which is confirmed by quantitation (E; ** p < 0.001). MHCB is redistributed to the apical surface in Cofilin 1−/− embryos at both 15-19 and 20-24 somites (F; non-normalized).

(G, H) Phalloidin staining extends more basally (G) and MHCB staining is more intense apically (H) in Cofilin 1 heterozygous embryos with enlarged PNPs, whereas those with small PNPs resemble WT.

(I) Phalloidin staining intensity in the 71-80% basal-to-apical bin of neuroepithelium (see D) correlates with PNP length in individual Cofilin 1+/− (green and blue symbols) and WT embryos.
Figure 7. Contribution of AJ disruption and summary of cytoskeletal signalling requirements for spinal neural tube closure.

(A) Immunohistochemistry (Phalloidin: red; anti-β-catenin: green) reveals precise co-localization of β-catenin and Phalloidin at neuroepithelial AJs of E9.5 WT embryos, which
severely disrupted in Cofilin 1−/− littermates. Culture in Y27632 or Jasp disrupts co-localization of β-catenin and Phalloidin at AJs of 15-19 somite embryos compared with DMSO controls, whereas ML-7 or Blebbistatin have no adverse effects. Culture in Y27632 + Blebbistatin restores a normal staining pattern. Arrowheads: normal staining; asterisks: disrupted staining pattern. Boxed regions are enlarged for β-catenin alone and β-catenin/Phalloidin merged views. Embryos have 18-21 somites. Bars: 30 µm.

(B) Summary of cytoskeletal signalling requirements for spinal neural tube closure, based on findings in the study. Normal closure (B1) requires signalling through RhoA/ROCK/LIMK/Cofilin to regulate actin turnover and actomyosin disassembly. We suggest this is a pre-requisite for both actomyosin-based neuroepithelial tension and ATPase-independent myosin contraction, the latter regulating remodelling of AJs without affecting TJs. Myosin ATPase-dependent contractile activity regulated by MLC phosphorylation is not limiting for spinal NT closure, as closure is not inhibited by ML-7. Failure of closure (B2) results when RhoA/ROCK signalling is inhibited by Y27632 (left arrows in B2), with apical actomyosin accumulation probably because of enhanced Cofilin activity, leading to F-actin-assembly via increased actin nucleation. Closure also fails when F-actin is stabilized (right arrows in B2), in Jasp-treated embryos or following diminution of actin severing activity in Cofilin 1 mutants, similarly leading to actomyosin accumulation. Abnormal AJs and disruption of neuroepithelial tension are suggested to result from actomyosin accumulation, and prevent spinal closure.
Supplementary figure legends

**Figure S1. Effect of cytoskeletal inhibitors on neural tube closure, and method of intensity profile analysis**

(A–B) Mean PNP length (mm ± SEM) after treatment with different inhibitors for 5-6 h (A) or 18-20 h (B). P-values are from pairwise t-tests compared with DMSO controls, following 1-way ANOVA (p < 0.05).

(C) Embryos exposed to 12.5, 20 or 50 μM Blebbistatin for 5-6 h undergo PNP closure at the same rate as DMSO controls (best fit linear regression lines plotted).

(D) Graph (top) and table (below) showing PNP length at 20-24 somites following 16-17 h culture is not significantly different from DMSO control after exposure to 60 μM Blebbistatin (a higher concentration than the 50 μM used elsewhere in the study). Similarly, a combination of 50 μM Blebbistatin + 0.05 μg/mL CytD, or 50 μM Blebbistatin + 50 μM ML-7, does not yield PNP lengths significantly different from DMSO controls (P-values in table = 0.694 for 1-way ANOVA; p > 0.05).

(E) Method of analysing fluorescence intensity of Phalloidin and anti-MHCB staining on sections through the neural plate. (E1) Fluorescence intensity was measured along the basal-to-apical (X) axis of each region of interest. Image J function “Plot Profile” and custom written ImageJ macro #1 were used to provide a ‘column average profile’ for the region of interest. (E2) Average profile curves were normalised to a range of 0-100 along the basal-to-apical axis. (E3) Intensity values were interpolated (integers between 0 and 100 for the X axis) using a custom written Matlab script (macro #2), enabling statistical analysis and comparison between samples.

**Figure S2. Effect of cytoskeletal inhibitors on Phalloidin intensity, F/G actin fractions and neural tube closure**

(A) Intensity profile scans of Phalloidin staining along the basal-to-apical axis of the neuroepithelium. Basal surface = 0; apical surface = 100 (arbitrary units, a.u.). The maximum intensity is normalised to 100%. Note the reproducibility of the basally extended Phalloidin staining in individual Y27632-treated embryos compared with DMSO controls.

(B) Biochemical fractionation shows increased F-actin and reduced G-actin in Y27632-treated embryos, but dramatically reduced F-actin and increased G-actin in CytD-treated embryos (**p < 0.001 compared with DMSO control).
(C, D) Intensity profile scans of Phalloidin staining (C) and MHCB (D) along the basal-to-apical axis of the neuroepithelium. Note the very low intensity of Phalloidin and MHCB in embryos treated with Blebbistatin + CytD or Blebbistatin + ML-7, consistent with severe reduction of neuroepithelial actomyosin by these inhibitors.

(E) PNP length (mm ± SEM) is significantly increased after treatment with Jasp for 18-20 h to the 15-19 or 20-24 somite stage. P-values are from t-tests compared with DMSO controls.

(F) Biochemical fractionation shows increased F-actin and reduced G-actin in Jasp-treated embryos compared with DMSO control embryos (** p < 0.001).

(G) Intensity profile scans of Phalloidin staining along the basal-to-apical axis of the neuroepithelium in embryos treated with Jasp or DMSO. The maximum intensity is normalised to 100%. Note the reproducibility of the basally extended Phalloidin staining in individual Jasp-treated embryos, compared with DMSO controls.

(H, I) Quantitation of cell proliferation by % phospho-histone H3 positive nuclei (H) and programmed cell death by % cleaved caspase 3 positive cells (I) in embryos cultured in Y27632 or Jasp for 18-20 h. There is no statistical difference between either treatment group and DMSO control embryos.

Figure S3. Effects of cytoskeletal inhibitors on neural tube closure and F/G actin fractions, and rescue of Y27632-induced and Jasp-induced defects by Blebbistatin

(A) Mean PNP length (mm ± SEM) after treatment with 50 μM ML-7 or DMSO for 5-6 h. There is no significant difference between ML-7 and DMSO-treated embryos at either 15-19 or 20-24 somites. P-values are from t-tests compared with DMSO controls.

(B) Embryos exposed to varying concentrations of ML-7 for 5-6 h undergo PNP closure at a similar rate as DMSO-treated controls (best fit linear regression lines plotted).

(C) Mean PNP length (mm ± SEM) after treatment with DMSO, Y27632, Y27632 + Blebbistatin, Y27632 + ML-7 for 15-18h. Note the rescue of PNP closure delay in Y27632-treated embryos by Blebbistatin, but not by ML-7. P-values are from pairwise t-tests compared with DMSO controls, following 1-way ANOVA (p < 0.05)

(D, E) Mean PNP length (mm ± SEM) after Y27632 treatment for 5-6 h followed by Blebbistatin for 13-15 h (D) or treatment with Y27632 + Blebbistatin for 5-6 h to the 15-19 or 20-24 somite stage (E). In both cases, PNP length does not differ from DMSO controls. P-values are from t-tests compared with DMSO controls.
(F, H) Mean PNP length (mm ± SEM) after culture for 5-6 h (Short Term Culture, STC) or 15-18 h (Long Term Culture, LTC), shown graphically (F) and in table form (H, lower). Y27632 produces enlarged PNPs (pink symbols) but this effect is reversed if embryos are cultured in rat serum alone after removal (‘washout’) of Y27632 (STC Y27632 then RS). However washout with rat serum after longer exposure to Y27632 does not restore PNP length (LTC Y27632 then RS) whereas subsequent culture in Blebbistatin rescues closure (LTC Y27632 then Blebb). P-values are from t-tests compared with DMSO controls.

(G, H) Mean PNP length (mm ± SEM) after culture in Y27632 followed by Blebbistatin, shown graphically (G) and in table form (H, upper). The effect of Y27632 in producing enlarged PNPs (pink symbols) is reversed when Blebbistatin is added to either short-term (STC Y27632 then Blebb) or long-term cultures (LTC Y27632 then Blebb). The same result is observed in embryos reaching three different somite stages.

(I) Biochemical fractionation shows similar a F/G ratio in embryos treated with a mixture of Y27632 and Blebbistatin after STC or LTC in Y27632 (as in G). There are no statistical differences in F/G ratio from DMSO control embryos.

(J, K) Exposure to a combination of 10 nM Jasplakinolide + 50 μM Blebbistatin for 15-18h does not significantly affect PNP closure compared with DMSO controls (p > 0.05). Compare with the marked PNP closure-delaying effect of Jasp alone in Fig. 3A and Fig. S2E.

Figure S4. Actomyosin accumulation, apical junction formation, and PNP closure in Cofilin mutant and inhibitor-treated embryos

(A) Mean PNP length (mm ± SEM) for wild-type (WT), Cofilin 1+/− and Cofilin 1−/− littermate embryos. Note the enlarged PNPs in Cofilin 1 mutant embryos at 20-24 somites. P-values are from pairwise t-tests compared with WT following 1-way ANOVA (p < 0.05).

(B) Immunohistochemistry (Phalloidin, red; anti-MHCB, green) in WT and Cofilin 1+/− mutants at 16 somites, preceding onset of PNP closure delay. Note apical accumulation of actomyosin (asterisks) in the mutant neuroepithelium. Scale bar: 30 μm in all panels

(C, D) Apical accumulation of actomyosin and disruption of adherens junctions (Phalloidin, red; anti-MHCB, green in B; β-catenin, green in C) in Cofilin 1+/− embryos with a large PNP (lower panels) compared with those with a small PNP (upper panels). The latter exhibit relatively normal actomyosin and β-catenin immunostaining.

(E) Immunohistochemistry (Phalloidin: red; anti-ZO1: green) in the neuroepithelium of E9.5 WT (upper panel) and Cofilin 1+/− (lower panel) embryos. ZO1 immunostaining is disrupted in
Cofilin 1 mutants compared with WT. Arrowheads: normal staining; asterisks: disrupted staining pattern. Right panels show enlargements of boxed regions. Scale bar: 30 μm in all panels.

(F) Immunohistochemistry (anti-ZO1: green) in the neuroepithelium of E9.5 embryos following culture with inhibitors for 6 or 18 h. ZO1 immunostaining is unaffected in inhibitor-treated embryos compared with DMSO controls. Embryos have 18-21 somites. Arrowheads: normal staining. Scale bar: 30 μm in all panels.

(G) Immunohistochemistry (Phalloidin, red; anti-MHCB, green, middle; anti-β-catenin, green, right) reveals a less severely disrupted actomyosin distribution in Cofilin 1 mutant embryos exposed to Blebbistatin (G) compared with untreated Cofilin 1 mutants (compare with Figs 6C, S4B). Blebbistatin treatment is also able to restore normal β-catenin staining (compare with Fig. 7A).

(H) In contrast, Cofilin 1 embryos exposed to Blebbistatin show abnormal ZO1 staining, indicating that TJ structure is not rescued (compare with Fig. S4E). Asterisks: abnormal immunostaining.
Figure S1
Figure S2
Figure S3
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**Figure S4**
### Table S1.

**Dose-response experiments used to select inhibitor concentrations for embryo culture**

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<tr>
<td></td>
<td></td>
<td>5 µM</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>10 µM</strong></td>
<td>11/13</td>
<td>11/13</td>
<td>11/13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 µM</td>
<td>1/4</td>
<td>2/4</td>
<td>2/4</td>
</tr>
<tr>
<td><strong>Blebbistatin</strong></td>
<td>Inhibits myosin ATPase activity</td>
<td>12.5 µM</td>
<td>8/9</td>
<td>9/9</td>
<td>9/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 µM</td>
<td>24/26</td>
<td>24/26</td>
<td>24/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>50 µM</strong></td>
<td>28/32</td>
<td>28/32</td>
<td>29/32</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75 µM</td>
<td>1/3</td>
<td>2/3</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>100 µM</strong></td>
<td>0/4</td>
<td>0/4</td>
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<tr>
<td><strong>Y27632</strong></td>
<td>Inhibits Rho kinase ROCK</td>
<td>5 µM</td>
<td>42/43</td>
<td>43/43</td>
<td>43/43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 µM</td>
<td>2/6</td>
<td>4/6</td>
<td>2/6</td>
</tr>
<tr>
<td><strong>HA-1100</strong></td>
<td>Inhibits Rho kinase ROCK</td>
<td>10 µM</td>
<td>4/4</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>20 µM</strong></td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 µM</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50 µM</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td><strong>ML-7</strong></td>
<td>Inhibits myosin light chain kinase MLCK</td>
<td>5 µM</td>
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<tr>
<td></td>
<td></td>
<td>10 µM</td>
<td>3/3</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 µM</td>
<td>5/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 µM</td>
<td>10/10</td>
<td>10/10</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>50 µM</strong></td>
<td>17/18</td>
<td>17/18</td>
<td>18/18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>75 µM</td>
<td>7/7</td>
<td>7/7</td>
<td>7/7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 µM</td>
<td>8/9</td>
<td>8/9</td>
<td>8/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>125 µM</strong></td>
<td>1/5</td>
<td>2/5</td>
<td>1/5</td>
</tr>
<tr>
<td><strong>Jasplakinolide</strong></td>
<td>Blocks F-actin depolymerisation</td>
<td><strong>10 nM</strong></td>
<td>31/32</td>
<td>32/32</td>
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<tr>
<td></td>
<td></td>
<td>50 nM</td>
<td>0/2</td>
<td>1/2</td>
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<tr>
<td></td>
<td></td>
<td><strong>100 nM</strong></td>
<td>0/3</td>
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</tr>
</tbody>
</table>

*a Embryos were cultured for 18-20h to the 15-24 somite stage, after which presence of yolk sac circulation and heart beat were evaluated as measures of viability.

*b Concentrations in red are those used in the experimental studies.

*c Overall morphology was scored with the following defects considered ‘abnormal’: non-smooth and round yolk sac, non-well formed branchial arches and maxillary, abnormal shaped somites.

*d The heart was bigger and the beating slower in Blebbistatin-treated embryos at all concentrations tested.

*e Some embryos treated with Y27632 or Jasplakinolide did not complete axial rotation.
Table S2.

Number of embryos that exhibited MHP and DLHP bending in the PNP following culture in inhibitors

<table>
<thead>
<tr>
<th>Bending regions</th>
<th>Non-bending regions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHP (^a)</td>
</tr>
<tr>
<td><strong>Short cultures (5-6 h)</strong></td>
<td></td>
</tr>
<tr>
<td>15-19 somites</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>20/20</td>
</tr>
<tr>
<td>CytD</td>
<td>6/6</td>
</tr>
<tr>
<td>Blebbistatin</td>
<td>13/13</td>
</tr>
<tr>
<td>Y27632</td>
<td>14/14</td>
</tr>
<tr>
<td>Y27632 + Blebbistatin</td>
<td>7/7</td>
</tr>
<tr>
<td>ML-7</td>
<td>6/6</td>
</tr>
<tr>
<td><strong>20-24 somites</strong></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>21/26</td>
</tr>
<tr>
<td>CytD</td>
<td>10/10</td>
</tr>
<tr>
<td>Blebbistatin</td>
<td>8/9</td>
</tr>
<tr>
<td>Y27632</td>
<td>3/9</td>
</tr>
<tr>
<td>Y27632 + Blebbistatin</td>
<td>8/9</td>
</tr>
<tr>
<td>ML-7</td>
<td>11/12</td>
</tr>
<tr>
<td><strong>Long cultures (18-20 h)</strong></td>
<td></td>
</tr>
<tr>
<td>15-19 somites</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>17/19</td>
</tr>
<tr>
<td>CytD</td>
<td>4/4</td>
</tr>
<tr>
<td>Blebbistatin</td>
<td>3/3</td>
</tr>
<tr>
<td>Y27632</td>
<td>20/21</td>
</tr>
<tr>
<td>Jasplakinolide</td>
<td>16/16</td>
</tr>
<tr>
<td><strong>20-24 somites</strong></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>39/45</td>
</tr>
<tr>
<td>CytD</td>
<td>3/3</td>
</tr>
<tr>
<td>Blebbistatin</td>
<td>6/7</td>
</tr>
<tr>
<td>Y27632</td>
<td>3/22</td>
</tr>
<tr>
<td>Jasplakinolide</td>
<td>2/16</td>
</tr>
<tr>
<td>Y27632 + Blebbistatin</td>
<td>9/10</td>
</tr>
<tr>
<td>Y27632 + ML-7</td>
<td>4/14</td>
</tr>
</tbody>
</table>

\(^a\) Values are number with MHP or DLHP/total number of embryos analysed.
\(^b\) Morphological assessment of non-bending regions of neural plate which are normally straight.
\(^c\) Note that MHP bending was absent from the majority of Y327632- and Jasp-treated embryos at 20-24 somites, but not at 15-19 somites.
**Table S3.**

**Number of Cofilin 1 mutant embryos that exhibited MHP and DLHP bending in the PNP**

<table>
<thead>
<tr>
<th></th>
<th>Bending regions</th>
<th>Non-bending regions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHP *</td>
<td>DLHP *</td>
</tr>
<tr>
<td><strong>15-19 somites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>9/9</td>
<td>9/9</td>
</tr>
<tr>
<td>Cofilin 1 +/-</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Cofilin 1 -/-</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td><strong>20-24 somites</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>13/14</td>
<td>14/14</td>
</tr>
<tr>
<td>Cofilin 1 +/-</td>
<td>9/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Cofilin 1 -/-</td>
<td>2/5 *</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* Values are number with MHP or DLHP/total number of embryos analysed.

* Morphological assessment of non-bending regions of neural plate which are normally straight.

* Note that MHP bending was absent from the majority of cofilin 1 homozygotes at 20-24 somites.
Table S4.

Primary antibodies

| Protein specificity | Supplier | Usage *
<table>
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<tbody>
<tr>
<td>Cofilin</td>
<td>sc-8441, Santa Cruz</td>
<td>WB</td>
</tr>
<tr>
<td>pCofilin</td>
<td>#3313, Cell Signaling</td>
<td>WB</td>
</tr>
<tr>
<td>LIMK</td>
<td>sc-8389, Santa Cruz</td>
<td>WB</td>
</tr>
<tr>
<td>pLIMK</td>
<td>#3841, Cell Signaling</td>
<td>WB</td>
</tr>
<tr>
<td>MLC</td>
<td>#3672, Cell Signaling</td>
<td>WB</td>
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<tr>
<td>pMLC</td>
<td>#3671, Cell Signaling</td>
<td>WB</td>
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<td>GAPDH</td>
<td>MAB374, Millipore</td>
<td>WB, IHC</td>
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<tr>
<td>β-catenin</td>
<td>ab16051, abcam</td>
<td>IHC</td>
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<tr>
<td>MHCIIIB</td>
<td>PRB-445P, Covance</td>
<td>IHC</td>
</tr>
<tr>
<td>ZO1</td>
<td>#40-2200, Life technologies</td>
<td>IHC</td>
</tr>
<tr>
<td>PHH3</td>
<td>06-570, Millipore</td>
<td>IHC</td>
</tr>
<tr>
<td>Cleaved-Caspase 3</td>
<td>#9661, Cell signaling</td>
<td>IHC</td>
</tr>
</tbody>
</table>

* WB, Western blot; IHC, immunohistochemistry