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Tropomodulin protects α-catenin-dependent junctional actin networks under stress during epithelial morphogenesis

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Summary

α-catenin is central to recruitment of actin networks to the cadherin-catenin complex (CCC) [1, 2], but how such networks are subsequently stabilized against applied stress during morphogenesis is poorly understood. To identify proteins that functionally interact with α-catenin in this process, we performed enhancer screening using a weak allele of the *C. elegans* α-catenin, *hmp-1*, and identified UNC-94/tropomodulin. Tropomodulins (Tmods) cap the minus ends of F-actin in sarcomeres [3]. They also regulate lamellipodia [4], can promote actin nucleation [5], and are required for normal cardiovascular development [6, 7] and neuronal growth cone morphology [8]. Tmods regulate the morphology of cultured epithelial cells [9], but their role in epithelia *in vivo* remains unexplored. We find that UNC-94 is enriched within a HMP-1-dependent junctional actin network at epidermal adherens junctions subject to stress during morphogenesis. Loss of UNC-94 leads to discontinuity of this network, and high-speed filming of *hmp-1(fe4);unc-94(RNAi)* embryos reveals large junctional displacements that depend on the Rho pathway. In vitro, UNC-94 acts in combination with HMP-1, leading to longer actin bundles than with HMP-1 alone. Our data suggest Tmods protect actin filaments recruited by α-catenin from minus-end subunit loss, enabling them to withstand the stresses of morphogenesis.

Keywords

cadherin; α-catenin; *C. elegans*; morphogenesis; tropomodulin; actin

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Results and Discussion

C. elegans epidermal morphogenesis provides an excellent context in which to investigate the relationship between actin and α-catenin *in vivo*. The embryonic epidermis contains three types of cells: (1) dorsal cells, which eventually fuse into a syncytium; (2) lateral (seam) cells, arranged in a single row along the anterior-posterior axis on each side of the embryo; and (3) ventral cells A conserved CCC, including HMR-1/cadherin, HMP-2/β-catenin, HMP-1/α-catenin, and JAC-1/p120 catenin [10, 11] is crucial for epidermal morphogenesis [12, 13]. Actomyosin mediated contractile stresses are transmitted by circumferential actin filament bundles (CFBs) in dorsal and ventral epidermal cells. CFBs insert orthogonally at junctional boundaries between lateral (seam) epidermal cells and dorsal and ventral epidermal cells, and help to drive the four-fold elongation of the embryo. CFB anchorage at adherens junctions (AJs) requires the CCC [14–16].

Functional interactions between multiple pathways are important for both focal adhesions and hemidesmosomes [17, 18]. However, a systematic search for similar functional interactions has not been carried out for AJs. We performed such a search, using feeding RNAi against genes on Chromosome I to find lethal enhancers of a weak loss-of-function allele of *hmp-1*, *fe4*. *hmp-1(fe4)* mutants exhibit embryonic and early larval lethality; escapers develop into fertile adults with body shape defects [11]. The *fe4* lesion results in an amino acid substitution in the VH3 domain of HMP-1 [11], which slightly weakens F-actin binding (S. Maiden and J. Hardin, in preparation).

We identified several genes implicated in regulating cell-cell adhesion, including the AF6/Afadin homolog (*afd-1*) and an Exocyst component (*sec-8*) [19–21], validating our approach. A full analysis, including results for the other five chromosomes, will be published elsewhere (Lynch et al., in review). Among the enhancers was UNC-94, a tropomodulin family member.

To examine the functional relationship between HMP-1 and UNC-94 we first performed 4D Nomarski microscopy on *hmp-1(fe4); unc-94(RNAi)* embryos (Fig. 1A). Wild-type embryos elongate to approximately 4-fold their initial length before hatching. *unc-94(RNAi)* embryos appear superficially wild-type, even though *unc-94(RNAi)* lowers UNC-94 protein levels to virtually undetectable levels (Supp. Fig. S1A). *hmp-1(fe4)* embryos exhibit defective elongation (Fig. 1A) and approximately 80% die as embryos and L1 larvae (n=95). *hmp-1(fe4)* embryos that hatch typically elongate to only twice their original length with severe body shape defects. In contrast, 100% of *hmp-1(fe4);unc-94(RNAi)* embryos exhibit embryonic lethality (n=93). Of these, 95% fail to elongate past the 1.5 fold stage and then retract to their original body length (Fig. 1A), compared with only 13% of *hmp-1(fe4)* embryos (see Supplemental Movie S1 for movies of representative embryos). Thus HMP-1 and UNC-94 together are essential for epidermal elongation.

We next performed immunostaining with an antibody that we previously used to show that UNC-94 is found at body wall muscle cell:cell boundaries and the minus ends of sarcomeric thin filaments [22, 23]. UNC-94 is first detectable at the two-fold stage and is enriched at seam:ventral and seam:dorsal cell borders, the same borders where CFBs transmit stress during elongation (Fig. 1B; see Fig. 1C for the relationship between CFBs and junctional actin in a wild-type embryo). Although UNC-94 is near AJs, it is distal to them and extends into the cytoplasm, in the same location as the junctional actin band that runs parallel to AJs. Consistent with this localization pattern, UNC-94 does not coimmunoprecipitate with HMP-1 under conditions in which HMP-1 and HMP-2 do (Supp. Fig. S1B). Significantly, UNC-94 does not localize to epidermal junctions in *hmp-1(zu278)* homozygotes, which produce a truncated HMP-1 protein incapable of binding actin [16] (Fig. 1D), indicating that

HMP-1's actin-binding activity is required to mobilize actin filaments containing UNC-94 near epidermal cell borders. In contrast, UNC-94 localizes largely normally to epidermal cell borders in *hmp-1(fe4)* embryos (Fig. 1D), and localizes in pharyngeal cells in a HMP-1-independent manner (Supp. Fig. S1C). Thus HMP-1 acts upstream of UNC-94 at epidermal cell borders normally under tension, but this functional relationship is not mediated through direct physical binding.

To better understand why *hmp-1(fe4);unc-94(RNAi)* embryos fail to elongate, we covisualized AJs and actin during this process using phalloidin staining and JAC-1/p120 catenin::GFP. Strikingly, seam:dorsal and seam:ventral epidermal cell borders in *hmp-1(fe4);unc-94(RNAi)* embryos are highly disrupted, appearing ripped apart to yield a characteristic zig-zag pattern (Fig. 2A). Junctions between other epidermal cells are no more perturbed than those in *hmp-1(fe4)* homozygotes. In areas of perturbed JAC-1::GFP, only some faint actin filaments are visible. Despite this, CFBs are still present in *hmp-1(fe4);unc-94(RNAi)* embryos, interfacing with the edges of the mislocalized JAC-1::GFP (Fig. 2A). The junctional-actin belt is still present, but more diffuse in *hmp-1(fe4);unc-94(RNAi)* embryos compared to *hmp-1(fe4)* (Fig. 2A, black arrows). The perturbed AJs and actin organization at these epidermal cell borders in *hmp-1(fe4);unc-94(RNAi)* embryos likely accounts for their failed elongation.

Immunostaining experiments demonstrate that HMR-1 co-localizes with mislocalized JAC-1::GFP in *hmp-1(fe4);unc-94(RNAi)* embryos, indicating that the entire CCC is affected (Fig. 2B, top). In contrast, AJM-1, a component of the more basal AJM-1/DLG-1 complex, is unaffected in *hmp-1(fe4);unc-94(RNAi)* embryos (Fig. 2B, bottom). Thus UNC-94 specifically regulates the CCC and its associated actin.

To better characterize the range of defects in *unc-94(tm724)*, and *unc-94(RNAi)* embryos, we scored wild-type and *unc-94* loss of function embryos stained with phalloidin based on the extent of F-actin disruption (Supp. Fig. S2). We found defects in both junctional actin and CFBs, suggesting that UNC-94 has a role not only in maintaining proper junctional actin, but also in anchoring of CFBs to the junctional actin band. To examine junctional proximal actin defects in more detail, we measured the extent to which junctional actin was contiguous at seam:dorsal and seam:ventral boundaries in wild-type and *unc-94(tm724)* embryos using phalloidin staining (Fig. 2C,D). In wild-type embryos, 73.7 ± 2.7% (mean ± SEM; n = 11 cells) of the junctional perimeter contained signal, compared with 48.9 ± 4.2% in *unc-94(tm724)* embryos (n = 16 cells; significantly different, $p < 0.0002$, heteroscedastic T-test). Similarly, the mean length of contiguous regions of actin at junctions was significantly greater in wildtype (0.41 ± 0.11 μm, n = 11 cells) vs. *unc-94(tm724)* (0.13 ± 0.02 μm, n = 16 cells; significantly different, $p < 0.04$). Such defects may have a common cause: defects in the junctional actin band may affect proper anchoring/spacing of CFBs. Vertebrate Tmod3 may similarly stabilize F-actin at lateral cell membranes in immortalized epithelial cell lines [9].

Taken together, these data suggest an important role for HMP-1 and UNC-94 in regulation of AJs and junctional actin at cell borders under stress during morphogenesis. Next we observed CCC dynamics in living, pre-arrested embryos in *jac-1::gfp* expressing embryos using high-speed filming (Fig. 3A–C). In wild-type embryos, JAC-1::GFP is restricted to the apicolateral contact zones between epidermal cells (Fig. 3A). *unc-94(RNAi)* and *unc-94(tm724)* embryos exhibit JAC-1::GFP dynamics similar to wild-type, though rarely some mislocalization occurs (Supp. Fig. S3A,B). In *hmp-1(fe4)* homozygotes, the JAC-1::GFP distribution is slightly fragmented and some JAC-1::GFP is transiently pulled away from the main area of the junction (Fig. 3B). Strong zygotic loss of *hmp-1* function in *zu278* homozygotes yields a similar mild effect (Supp. Fig. S3C), consistent with our

previous report [24]. In contrast to single mutants, however, dislocation of JAC-1::GFP is greatly enhanced in *hmp-1(fe4);unc-94(RNAi)* embryos (Fig. 3C). Re-slicing images through the z-axis of these extended regions shows that they are linear and occur perpendicular to the AJ (data not shown). Their spacing and linearity is consistent with them being caused by pulling forces exerted by the CFBs. *hmp-1(fe4);unc-94(RNAi)* embryos form about twice as many JAC-1::GFP extensions (>= 0.5 μm long) as *hmp-1(fe4)* (Fig. 3D). Moreover, as demonstrated in Fig. 2C, the AJs of *hmp-1(fe4);unc-94(RNAi)* embryos become progressively more disrupted as time goes on, suggesting that applied stress results in dystrophic disruption of these junctions. Significantly, these regions extend into both seam and ventral/dorsal epidermal cells, with many more in the latter (Supp. Table S1). Tissue-specific rescue experiments further demonstrate that while UNC-94 plays a role in both seam cells and non-seam cells, there is a more stringent requirement in non-seam cells (Supp. Fig. S4A).

The phenotypes we observe are very similar to those described previously in *rga-2*/RhoGAP mutants [25], though they are less pervasive along the apicobasal axis. We therefore assessed whether reducing stress on epidermal junctions could ameliorate the JAC-1::GFP extensions observed in *hmp-1(fe4);unc-94(RNAi)* embryos, using RNAi against *let-502/Rho kinase*. *hmp-1(fe4);unc-94,let-502(RNAi)* embryos exhibit a significant decrease in the number of JAC-1::GFP extensions (Fig. 3D) and arrested embryos show less JAC-1::GFP mislocalization (cf. Supp. Fig. S3D vs. E). This suggests that the AJs in *hmp-1(fe4);unc-94(RNAi)* embryos are not able to withstand the stress transmitted by CFBs during elongation, and instead, become pulled in the direction of the exerted force. To investigate the effects of *let-502* loss of function on junctions further, we used a temperature-sensitive *let-502* mutant to assess whether LET-502 activity is required for recruitment of UNC-94 to cell borders, and found that this is not the case, although cell elongation along the anterior-posterior axis is required for compaction of the zone of UNC-94 expression along seam:non-seam borders (Supp. Fig. S4B).

To gain mechanistic insight into how HMP-1 and UNC-94 act together to modulate actin networks, we performed *in vitro* actin binding and bundling assays. We previously showed that UNC-94 alone can inhibit latrunculin A induced depolymerization of plus-end capped *C. elegans* F-actin, and that UNC-94 blocks minus-end F-actin depolymerization induced by UNC-60B/ADF-cofilin [23]. We also showed previously that full-length HMP-1 alone can cosediment with actin filaments in vitro. This activity appears to be regulated by intramolecular interactions within the full-length protein, since the full-length protein cosediments less avidly than C-terminal fragments [16]. We therefore examined the combined effects of HMP-1 and UNC-94 on actin filament morphology in vitro using fluorescently labeled actin filaments capped at plus ends by CapZ, to which HMP-1, UNC-94, or both were added (Fig. 4A). HMP-1 added alone induced actin bundles like other α-catenins [26–28]. However, actin bundles generated in the presence of both HMP-1 and UNC-94 (n = 603, average length = 6.1 μm ± 2.7; S.D.) were 42% longer than those resulting from HMP-1 alone (n= 663, average length = 4.3 μm ± 2.8; significantly different, $p<0.001$, Fig. 4B), and this increase is in part due to an increase in long bundles (Fig. 4B, bracket). Taken together, these data indicate that HMP-1 and UNC-94 likely act together to generate robust actin filaments in the junctional actin band, which in turn resist mechanical deformation due to Rho-mediated actomyosin contractility.

The actin cytoskeleton and AJs cooperate to drive numerous epithelial morphogenetic events [2]. AJs recruit actin via α-catenin; initial recruitment may be modulated by Arp2/3-mediated actin branching or by processive plus-end proteins that stimulate more linear networks [2]. Once actin networks form at AJs, however, they must withstand stress and

resist dissolution. Our results indicate a new role for Tmod at the minus ends of actin filaments in this process.

In the embryonic epidermis of *C. elegans*, UNC-94 is enriched at a subset of epidermal cell borders that interface with CFBs, within the dense network of junctional actin bundles that runs lateral to AJs at these cell borders. Our in vitro analysis indicates that UNC-94 can protect filaments bundled by HMP-1 from minus-end subunit loss, since this assay was performed under conditions that favor depolymerization from the minus end, rather than addition of monomers to plus ends. Since some Tmods promote actin nucleation [5], a non-mutually exclusive possibility is that UNC-94 also plays a supporting role in de novo formation of junctional actin filaments.

In *hmp-1(fe4);unc-94(RNAi)* embryos, inefficient actin recruitment by mutant α-catenin, coupled with minus-end subunit loss, may lead to a less robust junctional actin network, which in turn results in lateral instability of AJs. If CFBs are mechanically coupled to the junctional actin band near their tips (e.g., via actin crosslinking proteins, or through α-catenin itself), stress would tend to be distributed laterally throughout the junctional actin band, reducing stress at CFB insertion sites. We envision such reinforcement as functioning in much the same way that the roots of a tree protect them from being uprooted, by distributing stress laterally. This idea is supported by our observation that decreasing actomyosin contractility alleviates the junctional displacements observed in *hmp-1(fe4);unc-94(RNAi)* embryos. We propose that the retraction phenotype exhibited by *hmp-1(fe4);tmd-1(RNAi)* embryos (in which the embryos extend to ~1.5 fold and then retract to their original length) may be due to a combination of uneven CFB pulling forces from abnormally arranged CFBs and weakened ultrastructure of the junctional actin band. This could result in failure to translate the stress applied by CFBs into the epidermal cell shape changes that drive elongation.

Junctional actin bands are present in many epithelial cell types. The forces applied to them can be aligned predominantly along the junction or orthogonal to it, the latter being the case during *C. elegans* embryonic elongation (reviewed in [29]). Our work suggests that regulation of minus end actin dynamics via Tmod plays an important role in promoting stability of actin networks under such orthogonal stress. Future experiments aimed at teasing apart the ultrastructure and biochemical regulation of junctional actin bands should help to clarify their functions, and how multiple actin regulators contribute to the mechanical integrity of AJs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Tropomodulin (Tmod) has previously unrecognized roles in cell-cell adhesion
- Tmod accumulates at adherens junctions under tension in an α-catenin-dependent manner
- Tmod acts with α-catenin to stabilize junctions against Rho-mediated contractility
- Tmod and α-catenin together act to form more robust F-actin bundles

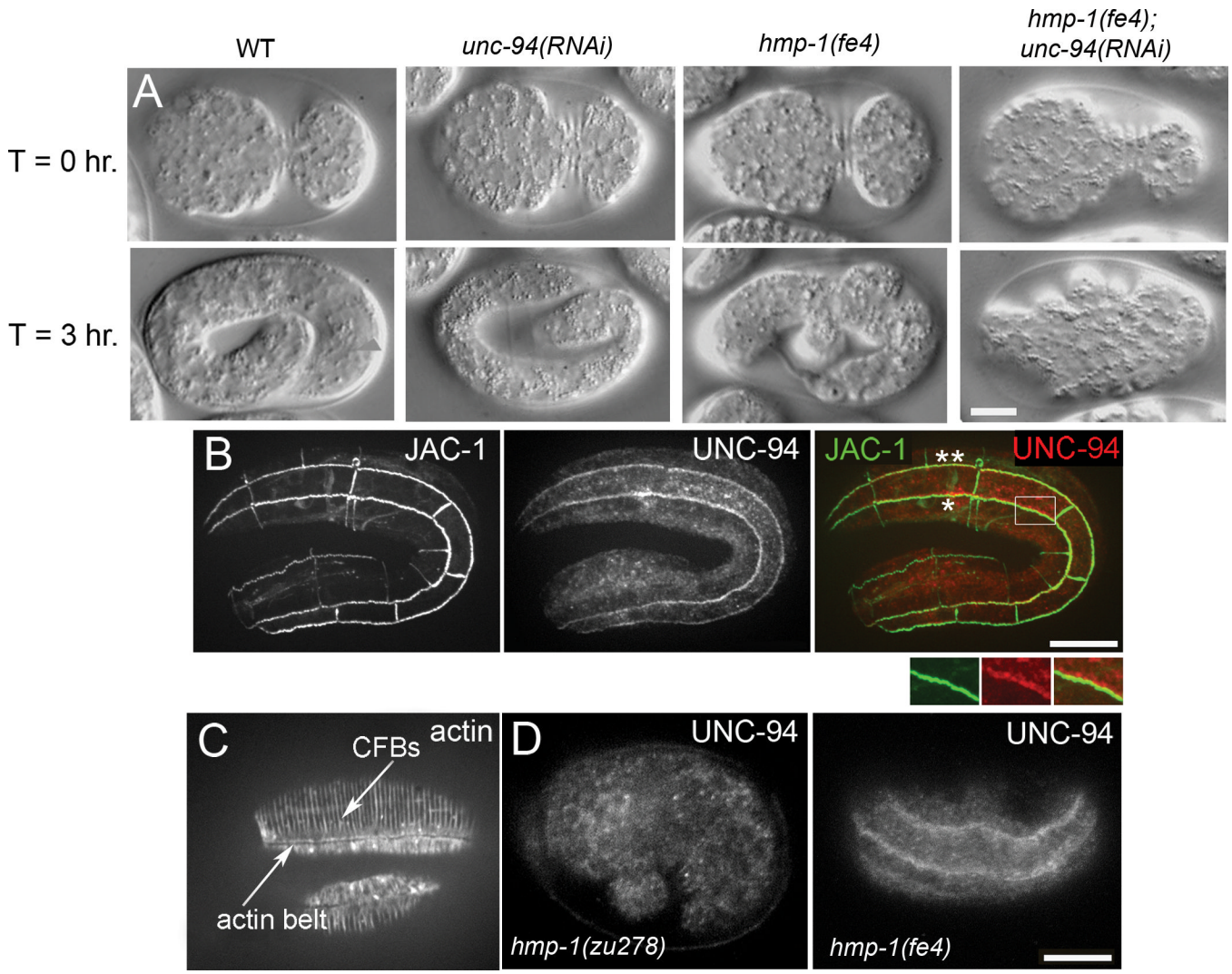

Figure 1. *hmp-1(fe4);unc-94(RNAi)* embryos arrest during embryonic elongation and epidermal UNC-94 localization to junctions requires *hmp-1* function

A. Nomarski images of representative embryos undergoing elongation are shown at the indicated time intervals. Embryos are initially oriented with anterior to the left and the ventral side up at T=0 hr., and lateral views are shown for all other time points. T=0 hr. shows embryos at the completion of enclosure. Wild-type (WT), *unc-94(RNAi)*, *hmp-1(fe4)*, and *hmp-1(fe4); unc-94(RNAi)* embryos are shown. No obvious defects in elongation are evident in the *unc-94(RNAi)* embryo. *hmp-1(fe4)* embryos develop mild body shape defects during elongation. 95% of *hmp-1(fe4);unc-94(RNAi*) embryos (n=93) elongate to the 1.5 fold stage or less, then retract to their original length. See Supplemental video S1, available online. B. Wild-type embryo expressing JAC-1::GFP to mark adherens junctions and stained with an affinity purified rabbit anti-UNC-94 antibody. Seam:ventral (*) and seam:dorsal (**) cell borders are indicated. UNC-94 is first detected at epidermal cell borders around the 2-fold stage of elongation. The color merge shows JAC-1::GFP in green and UNC-94 in red. Enlargement of the boxed region shows UNC-94 staining overlapping JAC-1::GFP. C. Wild-type stained with phalloidin. Circumferential filament bundles (CFBs) and the junctional actin belt are indicated. D. UNC-94 staining in wild-type and *hmp-1* mutants. In wild-type elongation-stage embryos, 23% have UNC-94 localization at epidermal cell borders (n = 79). In *hmp-1(zu278)* embryos, 0% have UNC-94 localization

at epidermal cell borders (n = 87). In *hmp-1(fe4)* embryos, 35% have UNC-94 localization at epidermal cell borders (n = 63). Note that this staining was done via freeze-cracking, which is known to disrupt actin, but preserves the UNC-94 epitope recognized by anti-UNC-94 antibodies. Disruption of junctional actin caused by this technique may account for the percentage of embryos exhibiting UNC-94 localization at epidermal cell borders. Bars = 10 μm.

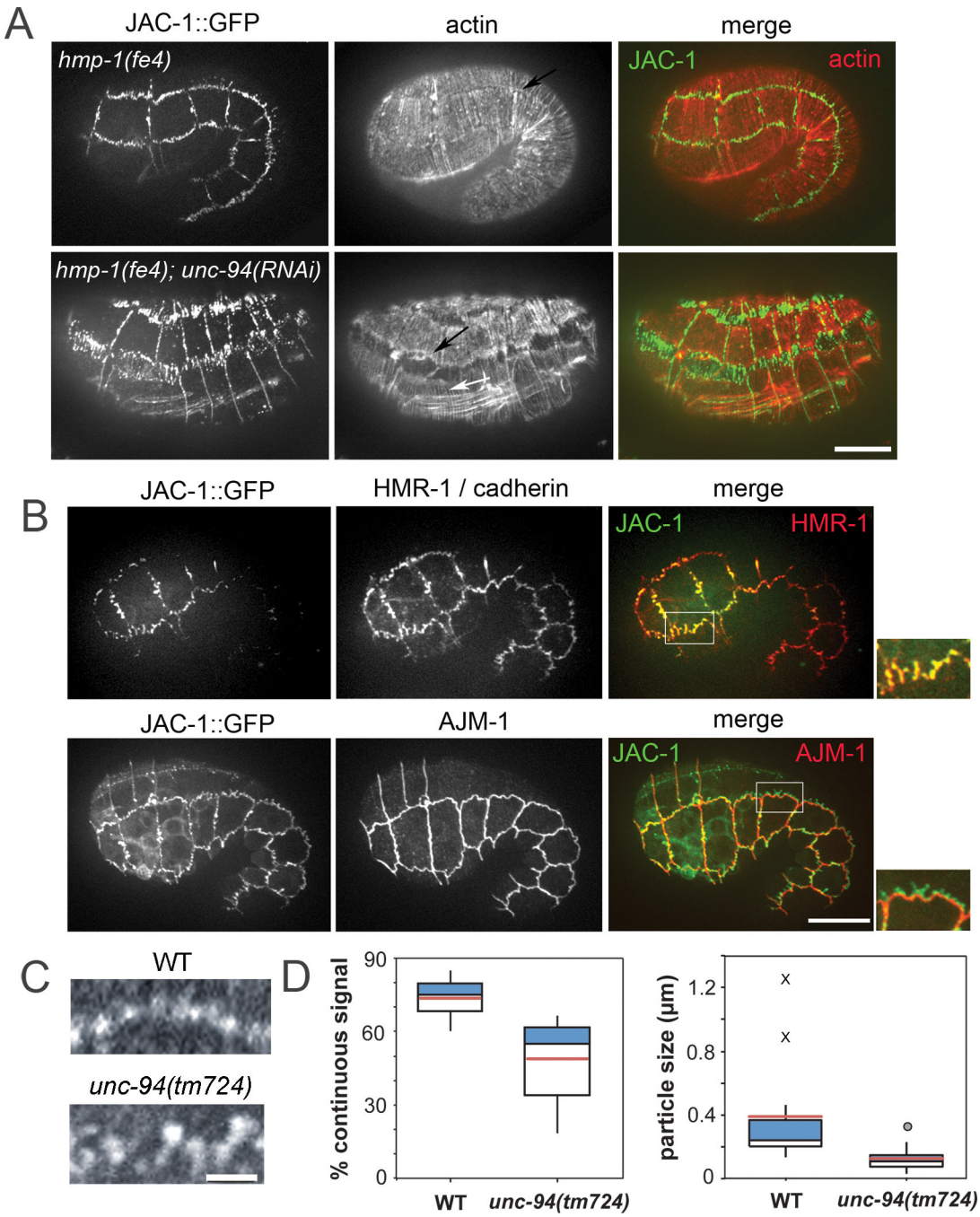

Figure 2. UNC-94 contributes to adherens junction stability

A. Representative *hmp-1(fe4)* and *hmp-1(fe4);unc-94(RNAi)* embryos. Embryos are of similar age (the *hmp-1(fe4);unc-94(RNAi)* embryo has retracted). Embryos express JAC-1/p120catenin::GFP and are stained with phalloidin to visualize actin. Color merges show JAC-1::GFP in green and actin in red. In *hmp-1(fe4);unc-94(RNAi)* embryos, JAC-1::GFP is fragmented and mislocalized at seam:dorsal and seam:ventral borders. Actin is depleted in areas of disrupted JAC-1::GFP; however, CFBs (white arrow) and diffuse junctional actin (black arrow) are still visible. Anterior is to the left in all panels. Bar=10 µm. B. In *hmp-1(fe4);unc-94(RNAi)* embryos the cadherin-catenin complex is selectively perturbed. Pre-arrest *hmp-1(fe4);unc-94(RNAi)* embryos expressing JAC-1::GFP were stained for

either HMR-1/cadherin or AJM-1. Color merges show that HMR-1 (red) co-localizes with JAC-1::GFP (green), and that AJM-1 is not perturbed in regions where JAC-1::GFP is mislocalized. For all images in A–D, anterior is to the left. C. Junctional proximal actin in representative wild-type (WT; top) and *unc-94(tm724)* embryos (bottom). Regular puncta of actin are connected along the junction in wild-type embryos, but gaps are present in junctions from *unc-94(tm724)* embryos. Bars=10 μm. D. Box plots of total percent junctional area in which actin signal is present (left) and average length of contiguous regions of actin (right). Blue: First quartile; white: third quartile; pink = mean. Circle = mild outlier; X = extreme outliers. In wild-type, there are occasional stretches of long, unbroken domains of positive signal along entire cells or multiple cells (X).

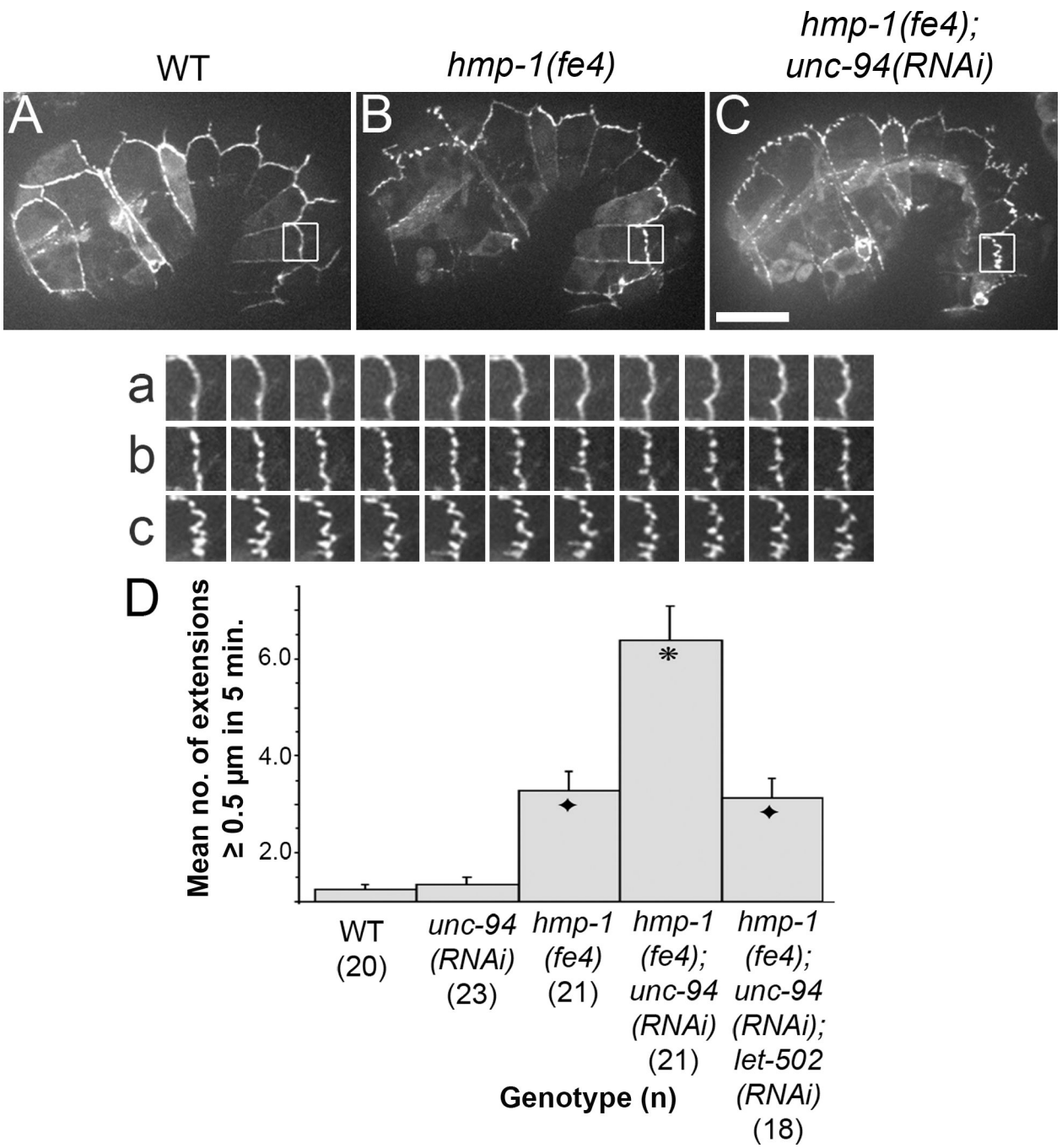

Figure 3. Adherens junctions of *hmp-1(fe4);unc-94(RNAi)* embryos exhibit abnormal dynamics

(A, B, C) Images from spinning disk confocal movies of embryos expressing JAC-1::GFP are shown. a–c show an enlargement of the boxed regions in A – C at 30 second intervals for 5 minutes. Supplemental video S2, corresponding to a–c, is available online. In wild-type embryos, JAC-1::GFP localizes to epidermal cell borders; in *hmp-1(fe4)* embryos, there are occasional areas in which JAC-1::GFP is transiently extended away from its normal position. In *hmp-1(fe4);unc-94(RNAi)* embryos this behavior is more pronounced. Bar=10 µm. (D) Bar graph showing quantification of the number (mean ± SEM; n indicated in parentheses) of JAC-1::GFP extension longer than 5 µm formed at either the seam:dorsal or seam:ventral cell border during 5 minutes of filming. Embryos at comma to 1.5 fold stage

were scored. Each extension was measured only once, at its longest length. Asterisk: significantly different from *hmp-1(fe4) and hmp-1(fe4);unc-94(RNAi);let-502(RNAi)* (Tukey test: $p < 0.01$). Black diamonds: not significantly different from *hmp-1(fe4)* ($p > 0.5$).

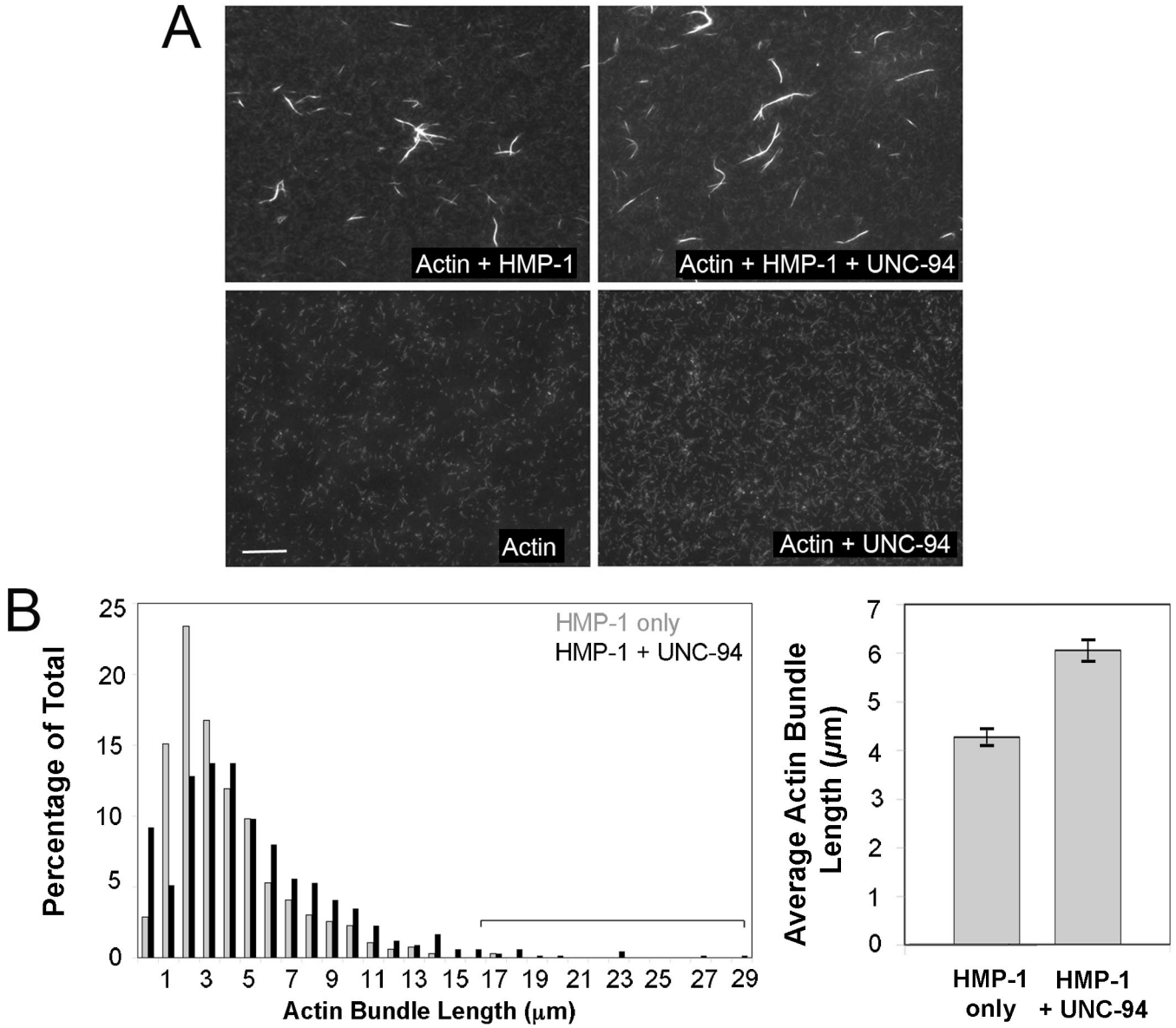

Figure 4. HMP-1 and UNC-94 synergistically regulate actin bundles in vitro

(A) Images showing fluorescently labeled, plus-end capped F-actin (5 μM) to which either HMP-1 (5 μM), UNC-94 (2.5 μM) or both have been added. Note that actin bundles form only when HMP-1 is present and that UNC-94 can lengthen HMP-1-generated actin bundles. Bar = 10 μm. (B) (Left) Histogram of actin bundle length when HMP-1 is added alone (n=663) or together with UNC-94 (n=603). Percentage of bundles exhibiting particular lengths (indicated in μm) is shown. The bracket indicates that when HMP-1 and UNC-94 are added together, there is an increase in the longest population of bundles filaments. (Right) Mean length of actin bundles formed by HMP-1 alone and HMP-1 plus UNC-94. Error bars, SEM. Actin bundles generated by HMP-1 plus UNC-94 are significantly longer than those generated by HMP-1 alone ($p < 0.001$, two tailed t-test).