

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 [1, 2], but how such networks are subsequently stabilized against stress applied 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*, thereby identifying 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 that Tmods protect actin filaments recruited by α -catenin from minus-end subunit loss, enabling them to withstand the stresses of morphogenesis.

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 cadherin-catenin complex (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 epidermal cells and dorsal and ventral epidermal cells and help to drive the 4-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 RNA interference (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 that have 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.H., unpublished data).

We identified several genes implicated in regulating cell-cell adhesion, including the AF6/Afadin ortholog (*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 (A.M.L. et al., unpublished data). Among the enhancers was UNC-94, a tropomodulin (Tmod) family member.

To examine the functional relationship between HMP-1 and UNC-94, we first performed four-dimensional Nomarski microscopy on *hmp-1(fe4);unc-94(RNAi)* embryos (Figure 1A). Wild-type embryos elongated to approximately 4-fold their initial length before hatching. *unc-94(RNAi)* embryos appeared superficially wild-type, even though *unc-94(RNAi)* lowered UNC-94 protein to virtually undetectable levels (Figure S1A available online). *hmp-1(fe4)* embryos exhibited defective elongation (Figure 1A) and approximately 80% died as embryos and L1 larvae ($n = 95$). *hmp-1(fe4)* embryos that hatched typically elongated to only twice their original length and had severe body-shape defects. In contrast, 100% of *hmp-1(fe4);unc-94(RNAi)* embryos exhibited embryonic lethality ($n = 93$). Of these, 95% failed to elongate past the 1.5-fold stage, and they then retracted to their original body length (Figure 1A), compared with only 13% of *hmp-1(fe4)* embryos (see Movie S1 for movies of representative embryos). Thus, HMP-1 and UNC-94 together are essential for epidermal elongation.

Second, we performed immunostaining with an antibody that we previously used to show that UNC-94 is found at body wall-muscle cell-cell boundaries and at the minus ends of sarcomeric thin filaments [22, 23]. UNC-94 was first detectable at the 2-fold stage and was enriched at seam-ventral and seam-dorsal cell borders, the same borders where CFBs transmit stress during elongation (Figure 1B; see Figure 1C for the relationship between CFBs and junctional actin in a wild-type embryo). Although UNC-94 is near AJs, it is distal

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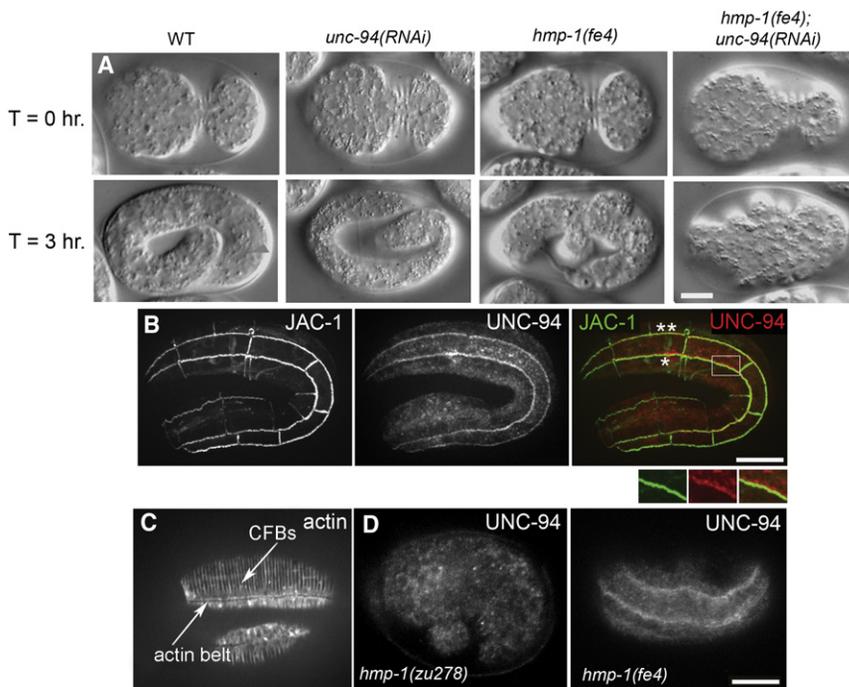


Figure 1. *hmp-1(fe4);unc-94(RNAi)* Embryos Arrest during Embryonic Elongation, and Epidermal UNC-94 Localization to Junctions Requires *hmp-1* Function

(A) Nomarski images of representative embryos undergoing elongation are shown at the indicated time intervals. Embryos are initially oriented with the anterior to the left and the ventral side up at time (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 [Movie S1](#).

(B) Wild-type embryo expressing JAC-1::GFP to mark AJs and stained with an affinity-purified rabbit 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. 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 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 represent 10 μ m.

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 did not coimmunoprecipitate with HMP-1 under conditions in which HMP-1 and HMP-2 did ([Figure S1B](#)). Significantly, UNC-94 did not localize to epidermal junctions in *hmp-1(zu278)* homozygotes, which produce a truncated HMP-1 protein incapable of binding actin [16] ([Figure 1D](#)), indicating that HMP-1's actin-binding activity is required for mobilization of actin filaments containing UNC-94 near epidermal-cell borders. In contrast, UNC-94 largely localized normally to epidermal-cell borders in *hmp-1(fe4)* embryos ([Figure 1D](#)) and localized in pharyngeal cells in a HMP-1-independent manner ([Figure 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 were highly disrupted, appearing ripped apart and yielding a characteristic zigzag pattern ([Figure 2A](#)). Junctions between other epidermal cells were no more perturbed than those in *hmp-1(fe4)* homozygotes. In areas of perturbed JAC-1::GFP, only some faint actin filaments were visible. Despite this, CFBs were still present in *hmp-1(fe4);unc-94(RNAi)* embryos, interfacing with the edges of the mislocalized JAC-1::GFP ([Figure 2A](#)). The junctional-actin belt was still present but was more diffuse in *hmp-1(fe4);unc-94(RNAi)* embryos compared to *hmp-1(fe4)* ([Figure 2A](#), black arrows). The perturbed AJs

and actin organization at these epidermal-cell borders in *hmp-1(fe4);unc-94(RNAi)* embryos probably account for their failed elongation.

Immunostaining experiments demonstrated that HMR-1 colocalized with mislocalized JAC-1::GFP in *hmp-1(fe4);unc-94(RNAi)* embryos, indicating that the entire CCC was affected ([Figure 2B](#), top). In contrast, AJM-1, a component of the more basal AJM-1/DLG-1 complex, was unaffected in *hmp-1(fe4);unc-94(RNAi)* embryos ([Figure 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 ([Figure 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 the 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 ([Figures 2C](#) and [2D](#)). In wild-type embryos, $73.7 \pm 2.7\%$ (mean \pm SEM; n = 11 cells) of the junctional perimeter contained signal, compared with $48.9 \pm 4.2\%$ 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 wild-type embryos ($0.41 \pm 0.11 \mu$ m, n = 11 cells) versus *unc-94(tm724)* embryos ($0.13 \pm 0.02 \mu$ m, 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 and/or spacing

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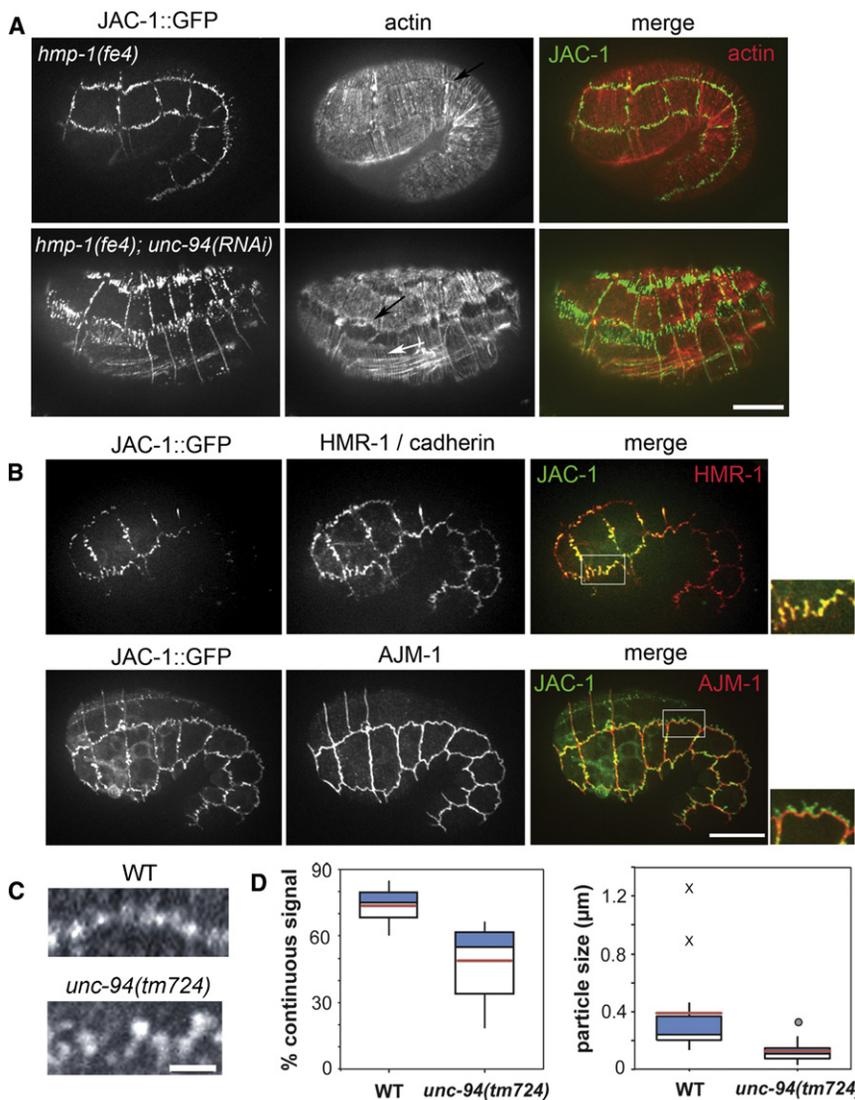


Figure 2. UNC-94 Contributes to AJ Stability

(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 for visualization of 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. The anterior is to the left in all panels. Bar represents 10 μ m.

(B) In *hmp-1(fe4);unc-94(RNAi)* embryos, the CCC is selectively perturbed. Prearrest *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) colocalizes 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 represent 10 μ m.

(D) Box plots of the total percentage of junctional area in which actin signal is present (left) and the average length of contiguous regions of actin (right). Blue: First quartile; white: third quartile; pink: mean. The circle represents a mild outlier; X represents extreme outliers. In wild-type, there are occasional stretches of long, unbroken domains of positive signal along entire cells or multiple cells (X).

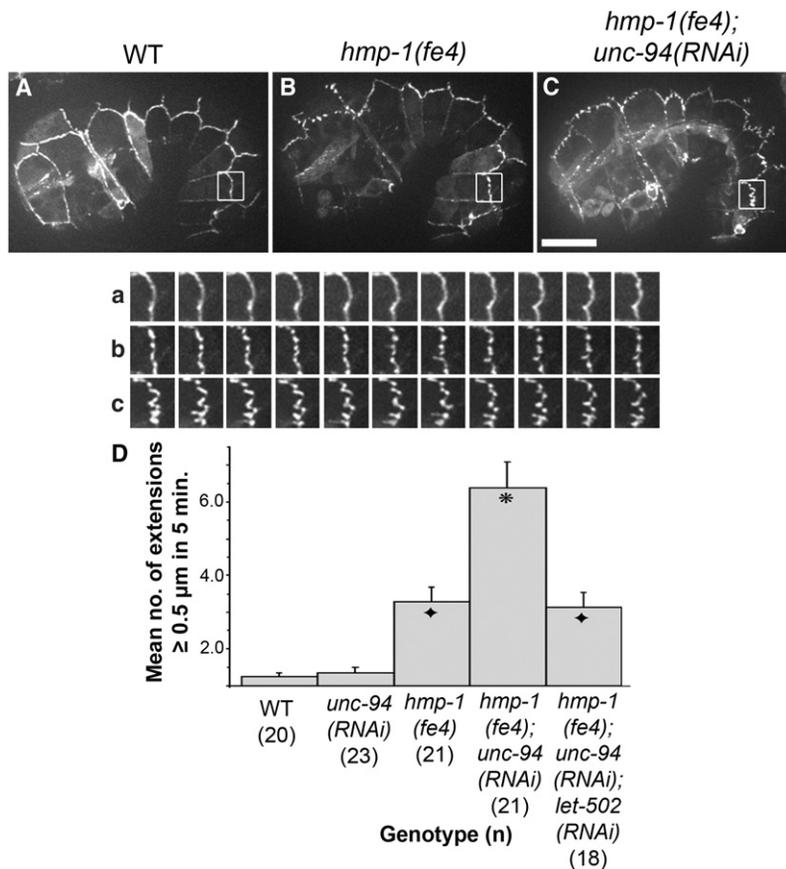
(data not shown). Their spacing and linearity is consistent with them being caused by pulling forces exerted by the CFBs. *hmp-1(fe4);unc-94(RNAi)* embryos formed about twice as many JAC-

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, prearrested embryos in *jac-1::gfp*-expressing embryos using high-speed filming (Figures 3A–3C). In wild-type embryos, JAC-1::GFP was restricted to the apicolateral contact zones between epidermal cells (Figure 3A). *unc-94(RNAi)* and *unc-94(tm724)* embryos exhibited JAC-1::GFP dynamics similar to wild-type, though rarely some mislocalization occurred (Figures S3A and S3B). In *hmp-1(fe4)* homozygotes, the JAC-1::GFP distribution was slightly fragmented, and some JAC-1::GFP was transiently pulled away from the main area of the junction (Figure 3B). Strong zygotic loss of *hmp-1* function in *zu278* homozygotes yielded a similar mild effect (Figure S3C), consistent with our previous report [24]. In contrast to single mutants, however, dislocation of JAC-1::GFP was greatly enhanced in *hmp-1(fe4);unc-94(RNAi)* embryos (Figure 3C). Reslicing images through the z axis of these extended regions showed that they are linear and occur perpendicular to the AJ

1::GFP extensions ($\geq 0.5 \mu$ m long) as *hmp-1(fe4)* embryos (Figure 3D). Moreover, as demonstrated in Figure 2C, the AJs of *hmp-1(fe4);unc-94(RNAi)* embryos became progressively more disrupted as time goes on, suggesting that applied stress results in dystrophic disruption of these junctions. Significantly, these regions extended into both seam and ventral or dorsal epidermal cells, with many more in the latter (Table S1). Tissue-specific rescue experiments further demonstrated that although UNC-94 plays a role in both seam cells and non-seam cells (Figure S4A).

The phenotypes we observed 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 exhibited a significant decrease in the number of JAC-1::GFP extensions (Figure 3D), and arrested embryos showed less JAC-1::GFP mislocalization (cf. Figure S3D versus Figure S3E). 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 further investigate the effects of *let-502* loss of function on junctions, 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-nonseam borders (Figure 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 showed previously 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, given that 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 the plus ends by CapZ, to which HMP-1, UNC-94, or both were added (Figure 4A). Added alone, HMP-1 induced actin bundles, as did other α -catenins [26–28]. However, actin bundles generated in the presence of both HMP-1 and UNC-94 ($n = 603$, average length = $6.1 \mu\text{m} \pm 2.7$ SD) were 42% longer than those resulting from HMP-1 alone ($n = 663$, average length = $4.3 \mu\text{m} \pm 2.8$; significantly different, $p < 0.001$,

Figure 3. AJs of *hmp-1(fe4);unc-94(RNAi)* Embryos Exhibit Abnormal Dynamics

(A)–(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 s intervals for 5 min. **Movie S2** corresponds to (a)–(c). In wild-type embryos (A), JAC-1::GFP localizes to epidermal-cell borders; in *hmp-1(fe4)* embryos (B), 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 (C), this behavior is more pronounced. Bar represents $10 \mu\text{m}$.

(D) Bar graph showing quantification of the number (mean \pm SEM; n indicated in parentheses) of JAC-1::GFP extensions longer than $0.5 \mu\text{m}$ formed at either the seam-dorsal or seam-ventral cell border during 5 min 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 4B), and this increase is due in part to an increase in long bundles (Figure 4B, bracket). Taken together, these data indicate that HMP-1 and UNC-94 probably 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, because this assay was performed under conditions that favor depolymerization from the minus end rather than addition of monomers to plus ends. Because 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 will 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 it from being uprooted: by distributing stress laterally. This idea is supported by our observation that decreasing actomyosin contractility alleviates the junctional displacements

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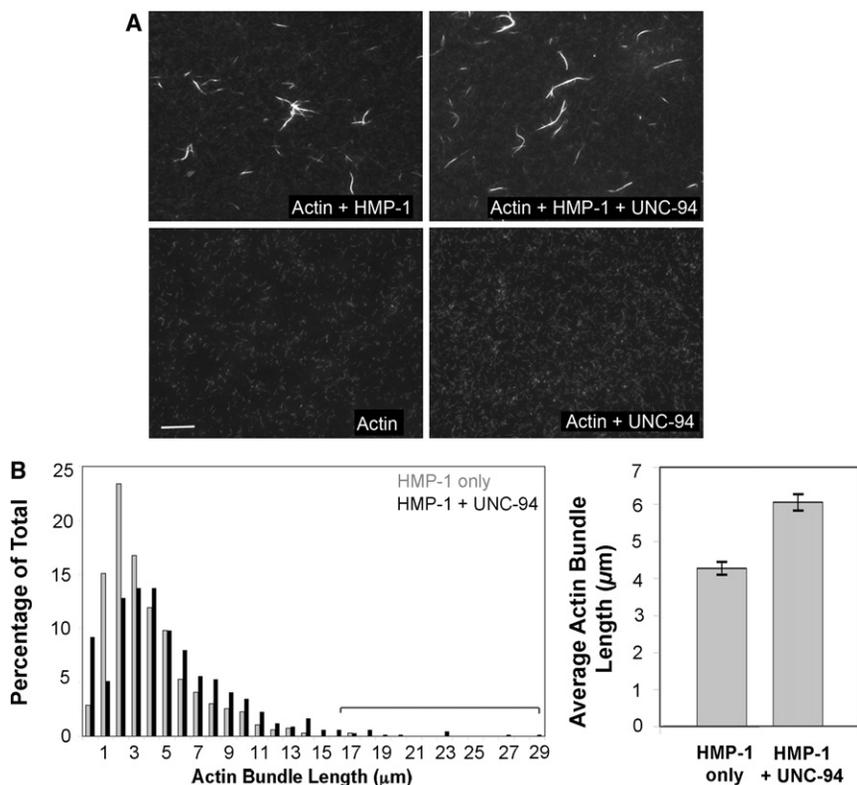


Figure 4. HMP-1 and UNC-94 Synergistically Regulate Actin Bundles In Vitro

(A) Images show fluorescently labeled, plus end-capped F-actin (5 μ M), to which either HMP-1 (5 μ M), UNC-94 (2.5 μ M), 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 represents 10 μ m.

(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 μ m) is shown. The bracket indicates that when HMP-1 and UNC-94 are added together, there is an increase in the longest population of bundled filaments. (Right) Mean length of actin bundles formed by HMP-1 alone and HMP-1 plus UNC-94. Error bars represent 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).

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 this orthogonal stress. Future experiments aimed at teasing apart the ultrastructure and biochemical regulation of junctional-actin bands should help to clarify their function, as well as how multiple actin regulators contribute to the mechanical integrity of AJs.

Supplemental Information

Supplemental Information includes four figures, one table, two movies, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2012.06.025>.

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