Formin-mediated actin polymerization at cell–cell junctions stabilizes E-cadherin and maintains monolayer integrity during wound repair

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ABSTRACT Cadherin-mediated cell–cell adhesion is required for epithelial tissue integrity in homeostasis, during development, and in tissue repair. E-cadherin stability depends on F-actin, but the mechanisms regulating actin polymerization at cell–cell junctions remain poorly understood. Here we investigated a role for formin-mediated actin polymerization at cell–cell junctions. We identify mDia1 and Fmnl3 as major factors enhancing actin polymerization and stabilizing E-cadherin at epithe"al junctions. Fmnl3 localizes to adherens junctions downstream of Src and Cdc42 and its depletion leads to a reduction in F-actin and E-cadherin at junctions and a weakening of cell–cell adhesion. Of importance, Fmnl3 expression is upregulated and junctional localization increases during collective cell migration. Depletion of Fmnl3 or mDia1 in migrating monolayers results in dissociation of leader cells and impaired wound repair. In summary, our results show that formin activity at epithelial cell–cell junctions is important for adhesion and the maintenance of epithelial cohesion during dynamic processes, such as wound repair.

INTRODUCTION

Cell-to-cell adhesion, a characteristic feature of an epithelium, is facilitated by the transmembrane calcium-dependent epithelial-cadherin (E-cadherin) receptor (van Roy and Berx, 2008). Clusters of E-cadherin receptors are organized into structures called adherens junctions (AJs; Harris and Tepass, 2010), where the extracellular domains of E-cadherin engage in cis and trans interactions to promote cell–cell adhesion (Harrison et al., 2011). In addition, links between the cytoplasmic tails of E-cadherin and the actomyosin cytoskeleton are vital for the stability of cadherin clusters (Hong et al., 2013) and confer on the AJ force-sensing and force-generation capabilities that allow it to participate in the complex tissue rearrangements underlying morphogenesis and wound repair (Collinet and Lecuit, 2013). At the diffraction-limited light microscopy level, the AJ can be spatially segregated into an apical densely packed band of cadherin termed the zonula adherens (ZA), and sparsely distributed clusters along the lateral membranes called lateral junctions (Takeichi, 2014; Wu et al., 2014). More recently, superresolution microscopy analysis revealed that the ZA and lateral junctions comprise qualitatively and quantitatively similar E-cadherin clusters, except for increased spacing between clusters at the lateral junctions (Wu et al., 2015). Moreover, at nanoscale resolution, F-actin was found to “fence” and delimit cadherin clusters, and bundles of F-actin were also evident at the apical ZA (Wu et al., 2015).

Given the close association between E-cadherin receptors and the actomyosin cytoskeleton, it is not surprising that the AJ is a major hub for the activity of actin nucleators, nucleation-promoting factors (NPFs), and their regulators, such as Rho GTPases, kinases, and phosphatases (Zaidel-Bar, 2013; Guo et al., 2014; Van Itallie et al., 2014). By far the best-studied nucleator that is involved in regulation of actin assembly at the AJ is the Arp2/3 complex. The Arp2/3

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complex localizes to the AJ and coimmunoprecipitates with E-cadherin, and inhibition of Arp2/3 severely affects cell–cell junction formation and steady-state F-actin turnover at a mature AJ (Verma et al., 2004; 2012; Kovacs et al., 2011). Furthermore, due to the poor intrinsic nucleation activity of Arp2/3, several NPFs for the Arp2/3 complex also localize to and regulate actin polymerization at the AJ (Yamazaki et al., 2007; Kovacs et al., 2011).

In contrast to the Arp2/3 complex, roles at the AJ for members of the formin family of actin nucleators and elongation-promoting factors are less clearly defined. Formins are large (120–200 kDa), multidomain proteins categorized into seven subfamilies comprising 15 members in mammals (Chesarone et al., 2010). Functioning as a dimer, the catalytic formin homology 2 (FH2) domain carries out actin polymerization, acting as a processive barbed-end capper, and the adjacent FH1 domain recruits profilin–G-actin complexes that serve as the substrate for polymerizing linear F-actin filaments (Pruyne et al., 2002; Sagot et al., 2002). Several classes of formins exist in an autoinhibited state via the intramolecular interaction of the C-terminal Diaphanous autoregulatory domain (DAD) with the N-terminal Diaphanous-inhibitory domain (DID; Chesarone et al., 2010). Rho-GTPase signaling and posttranslational modifications, including phosphorylation and myristoylation, are known to regulate the activation and localization of formins (Takeya et al., 2008; Chesarone et al., 2010; Cheng et al., 2011; Kuhn and Geyer, 2014). Furthermore, application of piconewton pulling forces to actin filaments polymerized by Diaphanous-related formin 1 (mDia1) significantly enhances the elongation rate, indicating the mechanosensitive nature of formins (Jegou et al., 2013).

Studies have begun to shed light on roles for formins in polymerizing actin at the AJ to support junctional architecture and function. Studies in mammalian cell culture, Drosophila, and mouse models demonstrate a role for mDia1 in augmenting F-actin and E-cadherin at the AJ in a RhoA-dependent manner to preserve epithelial integrity and polarity (Carramusa et al., 2007; Homem and Peifer, 2008; Ryu et al., 2009; Thumkeo et al., 2011). Formin-like 2, a member of the formin-like (FMNL) family, was shown to localize to the AJ in a Rac1-dependent manner in three-dimensional (3D) cultures of Madin–Darby canine kidney cells, in which its depletion resulted in defective lumen formation in 3D spheroids (Grikscheit et al., 2015). Similarly, actin polymerization by a related family member, Fmn13, was essential at endothelial cell junctions in zebrafish to facilitate proper blood vessel lumenization (Phng et al., 2015). The molecular mechanisms underlying the effect of formins on cell–cell adhesion are still poorly understood. Moreover, the role of formins in dynamic multicellular processes such as collective cell migration and wound closure that involve increased tension and turnover at the AJ has not been addressed.

Here we identify mDia1 and Fmn13 as major formins functioning at the AJ in epithelial cells. By measuring the dynamics of E-cadherin and F-actin at the AJ, we show that actin polymerization by these formins decreases the mobility of E-cadherin at AJs, stabilizes the junction, and increases cell–cell adhesion strength. Using small interfering RNA (siRNA)–mediated knockdown, we show that depletion of mDia1 or Fmn13 also triggers increased protrusive activity and cell spreading, resulting in the loss of lateral junctions. Furthermore, we elucidate a Src kinase and Cdc42–dependent signaling pathway responsible for recruitment and activation of Fmn13 at the AJ and demonstrate that Fmn13 recruitment is tension dependent. Finally, we provide evidence that Fmn13 expression and junctional localization is up-regulated during wound healing to augment AJ and monolayer integrity and facilitate coherent cell sheet migration.

RESULTS
Formin activity affects AJ organization and columnar cell morphology

In polarized Eph4 mammary epithelial monolayers, junctional F-actin and E-cadherin are organized in two distinct forms: a condensed band called the ZA located at the apicalmost portion of the membrane, and sparsely distributed F-actin and E-cadherin along the lateral membranes of contacting cells, termed lateral junctions (Figure 1A). To assess the contribution of formin-dependent actin polymerization to AJ organization, we inhibited endogenous formin activity using the pan-formin small-molecule inhibitor SMIFH2 (Rizvi et al., 2009; Isogai et al., 2015). Here we applied low concentrations of SMIFH2 (20 μM) to minimize cytotoxicity and reduce artifacts induced by Golgi reorganization and loss of p53 transcriptional activity associated with higher doses of SMIFH2 (≥25 μM; Isogai et al., 2015). Suppression of formin activity resulted in several striking phenotypes, including reduction in F-actin and E-cadherin intensity at the AJ, disappearance of lateral junctions, and flattening of cells, resulting in a marked increase in cell-spread area (Figure 1A). Using an automated algorithm for quantification of junctional and cellular parameters on summed intensity Z-stack projections, we identified 30 and 21% decreases in fluorescence intensity of F-actin and E-cadherin, respectively, at cell–cell junctions (Figure 1, B and C) as a result of the reduction in lateral junctions. Concurrently, the cell-spread area in SMIFH2-treated monolayers increased significantly in comparison to nontreated monolayers (Figure 1D). Whereas 87% of control cells had an area of ≤800 μm², SMIFH2 treatment resulted in 55% of cells with an area of ≥1200 μm² (Figure 1D). The increase in cell area was specific to Eph4 monolayers, as formin inhibition in isolated cells did not induce a change in cell spreading (Supplemental Figure S1, A and B). Furthermore, cell spreading was an active process dependent on Arp2/3 complex activity, as double inhibition of formin and Arp2/3 activity, using a mixture of SMIFH2 and CK666 inhibitors, did not result in any change of cell-spread area in treated monolayers (Supplemental Figure S1C).

The loss of lateral junctions after formin inhibition could, in theory, be a direct result of reduced formin activity at the AJ or the indirect consequence of cell spreading. To distinguish between these two possibilities, we plated cells on micropatterned fibronectin islands of defined diameter (100 μm) to limit cell spreading (Figure 1E). We then subjected these islands of cells to SMIFH2 treatment and observed little or no spreading beyond the boundaries of the fibronectin pattern (Figure 1, E and F). Under these conditions, lateral junctions were preserved (Figure 1E), suggesting that loss of lateral junctions is secondary to an active process of cell spreading and loss of typical epithelial columnar cell morphology. Nevertheless, even when cells were forced to remain columnar on the restricting pattern, the SMIFH2-treated islands displayed a small but significant reduction in E-cadherin levels at the AJ (Figure 1G). Taken together, these data suggest a role for F-actin polymerization by formins in stabilizing E-cadherin at the AJ. In addition, inhibition of formin activity triggers Arp2/3-dependent cell spreading, which in turn leads to cell flattening and loss of lateral junctions.

Formin activity is essential for AJ stability

Despite the observed reduction in F-actin and E-cadherin levels at the AJ upon formin inhibition (Figure 1, B and C), Eph4 monolayers remained intact even after 24 h of SMIFH2 treatment. We therefore performed live-cell imaging to examine whether cell–cell junction dynamics was affected by formin inhibition. Short-term imaging (40 min) of E-cadherin–enhanced green fluorescent protein (EGFP) at apical and basolateral planes revealed significant differences
Figure 2: B and E, and Supplemental Movie S2). On the other hand, dial extensions that originated from and collapsed back into the AJ layers exhibited numerous E-cadherin–containing transient filopodial regions of cell–cell contacts, nearly 52% junctions in control monolayers revealed a significantly greater variation in SMIFH2-treated monolayer remodeling (either extension or shortening of the junction) during 40 min also obtained using an algorithm for endogenous mDia1 revealed diffuse localization in Eph4 cells for mDia1 that was previously shown to stimulate endogenous mDia1 (Alberts, 2001; Rao et al., 2008). Antibody staining in monolayers pretreated with SMIFH2 before imaging, significantly lower filopodial activity was detected at basolateral junctions (15% junctions); instead, prominent junction-associated lamellipodial protrusions (65% cells) were observed that often terminated at tricellular cell–cell contacts (Figure 2, B and E, and Supplemental Movie S2).

Formins mDia1 and Fmnl3 regulate AJ organization in epithelial cells

To identify specific formins involved in actin regulation and thereby cadherin stability at the AJ, we first used semiquantitative reverse transcription PCR (RT-PCR) analysis to generate a formin expression profile in Eph4 cells for formins belonging to the Diaphanous-related family of formins, namely, Dia, DAAM, FMIN, and FHOD classes. RT-PCR analysis revealed mDia1 as the most abundant formin, followed by formin-like 3 (Fmnl3), and lower expression levels of other formins, including Daam1, Fmnl1/2, and Fhod1/3 (Supplemental Figure S2A). Next we used siRNA-based knockdown (KD) to systematically deplete each of these formins and screened for those that produced phenotypes similar to SMIFH2 treatment (Supplemental Figure S2, B–E). Of interest, KD of the two most abundant formins in Eph4 cells, mDia1 and Fmnl3, recapitulated the phenotype we observed after SMIFH2 treatment (Figure 3A and Supplemental Figure S2, B–E). Furthermore, double KD of both mDia1 and Fmnl3 led to exaggerated defects, resulting in the formation of a sparse monolayer with punctate AJ (Figure 3A). Quantification of junctions in mDia1 KD or Fmnl3 KD monolayers showed 29–31% reduction in F-actin and ~30% reduction in E-cadherin fluorescence intensity at junctions (Figure 3B). mDia1 was previously shown to regulate F-actin and E-cadherin at the AJ downstream of RhoA (Carramusa et al., 2007; Homem and Peifer, 2008). Antibody staining between control monolayers and monolayers treated with SMIFH2 for 2 h (Figure 2, A and B). In comparison to the more stable apical ZA in control monolayers, apical junctions in SMIFH2-treated monolayers exhibited greater plasticity and a higher degree of junctional remodeling (either extension or shortening of the junction) during the imaging period (Figure 2A and Supplemental Movie S1). This trend was also reflected in quantitative assessment of the change in junction length over 40 min (Figure 2C) obtained using an algorithm to track the length of junctions at each time point. Analyses of the maximum increase or decrease in junction length during 40 min also revealed a significantly greater variation in SMIFH2-treated monolayers compared with control junctions (Figure 2D). On the basolateral regions of cell–cell contacts, nearly 52% junctions in control monolayers exhibited numerous E-cadherin–containing transient filopodial extensions that originated from and collapsed back into the AJ (Figure 2, B and E, and Supplemental Movie S2). On the other hand,
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Staining for FMNL3 (Supplemental Figure S3B). Taken together, immunolabeling to detect endogenous formin localization in MCF10A cells revealed diffuse staining for DIA1 and junctional spreading (Supplemental Figure S3A; KD shown in Supplemental Figure S3, G and H), similar to phenotypes observed in Eph4 cells. Further, immunolabeling for junctions also demonstrated elevated levels (33%) of E-cadherin (Figure 3, G and H). Along with the increase in F-actin, DAD-expressing cells also demonstrated an initial fast recovery phase \( t_{1/2} = 112 \, \text{s} \) and increased mobile fraction of 65 ± 2.63% (Figure 4, B and C). Conversely, expression of the DAD to stimulate F-actin polymerization by mDia1 led to significantly slower recovery rates \( t_{1/2} = 173 \, \text{s} \) and lower mobile fractions (43 ± 1.89%; Figure 4, B and C). Recent FRAP analysis of E-cadherin at cell–cell junctions demonstrated that a significant proportion of E-cadherin molecules were immobilized via specific binding interactions mediated either by the extracellular domain (EC1) and/or interaction with actin via the cytoplasmic domain (Erami et al., 2015). Disruption of cis or trans interactions in the EC1 domain or actin binding via the cytoplasmic domain of E-cadherin was found to perturb the immobilization of cadherin at the AJ, resulting in increased mobile fractions (Erami et al., 2015). Here we show that modulating F-actin levels also affects the fraction of cadherin immobilized at junctions, highlighting the importance of formin–polymerized F-actin in the stabilization of E-cadherin at the AJ.

To understand at the molecular level the observed correlation between formin activity and E-cadherin level at AJ, we performed fluorescence recovery after photobleaching (FRAP) experiments on cell–cell junctions in Eph4 monolayers stably expressing GFP-tagged E-cadherin (Figure 4A). From recovery curves obtained for control monolayers, the half-life \( t_{1/2} \) of recovery for E-cadherin was determined to be 151 s, with a mobile fraction of 53 ± 1.43% (Figure 4, B and C). Pretreatment of monolayers with SMIFH2 to inhibit both mDia1 and Fmnl3 before FRAP resulted in an accelerated recovery rate with \( t_{1/2} = 112 \, \text{s} \) and increased mobile fraction of 65 ± 2.63% (Figure 4, B and C). Given the distinct localization of Fmnl3 at the AJ, we next focused our efforts on specifically characterizing actin dynamics upon KD or overexpression of Fmnl3 (Figure 4, D–F). The recovery curve for tdTomato-actin at control junctions demonstrated an initial fast recovery phase \( t_{1/2} = 19.95 \, \text{s} \) (Figure 4, E and F), followed by a slower phase that likely represents G-actin polymerization by mDia1. Perturbation of formin activity by mDia1 or Fmnl3 depletion was determined to be 112 ± 1.83 s, with a mobile fraction of 94 ± 1.43% (Figure 4, B and C). Conversely, expression of the DAD to stimulate F-actin polymerization by mDia1 led to significantly slower recovery rates \( t_{1/2} = 173 \, \text{s} \) and lower mobile fractions (43 ± 1.89%; Figure 4, B and C). Recent FRAP analysis of E-cadherin at cell–cell junctions demonstrated that a significant proportion of E-cadherin molecules were immobilized via specific binding interactions mediated either by the extracellular domain (EC1) and/or interaction with actin via the cytoplasmic domain (Erami et al., 2015). Disruption of cis or trans interactions in the EC1 domain or actin binding via the cytoplasmic domain of E-cadherin was found to perturb the immobilization of cadherin at the AJ, resulting in increased mobile fractions (Erami et al., 2015). Here we show that modulating F-actin levels also affects the fraction of cadherin immobilized at junctions, highlighting the importance of formin–polymerized F-actin in the stabilization of E-cadherin at the AJ.

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Fmnl3 and mDia1 are essential for monolayer cohesion during collective cell migration

Next we examined the effects of Fmnl3 KD on monolayer dynamics using an in vitro scratch assay to assess collective cell migration. As shown in Figure 4, G and H, all control monolayers (transfected with a nontargeting siRNA) successfully closed the gap within 9–12 h, with cells from both edges migrating coherently and perpendicular to the scratch axis (Figure 4, G and H, and Supplemental Movie S3). In contrast, in Fmnl3 KD monolayers, on average only 62% of the gap was closed after 12 h (Figure 4, G and H, and Supplemental Movie S4). Closer analysis revealed that cells in Fmnl3 KD monolayers failed to maintain a coherent migrating front, with increased incidence of cell detachment (24.5 ± 2.25% in Fmnl3 KD conditions vs. 4.9 ± 1.23% in control), particularly at the leading edge (Figure 4I and Supplemental Movie S4). Consistently, we also observed the aforementioned phenotypes and associated migration defects in MCF10A subjected to a scratch assay (Supplemental Figure S3, C–E, and Supplemental Movies S5 and S6). Next we investigated the effects on collective migration of silencing both Fmnl3 and mDia1. As illustrated in Figure 3A, combined KD of mDia1 and Fmnl3 in Eph4 cells resulted in the formation of a sparse monolayer, rendering this condition unsuitable for a scratch assay. Hence we chose to introduce siRNAs against these formins on preformed monolayers and subject them to the in vitro scratch assay. Of interest, depletion of both mDia1 and Fmnl3 in Eph4 (Figure 4G, bottom, and Supplemental Movie S7) resulted in complete abrogation of migration (Figure 4, G and H), striking absence of lamellipodium formation at the leading edge, and increased incidence of cytokinesis failure, resulting in numerous rounded cells.

Combining our previous FRAP observations and analyses of collective migration, we hypothesized that weaker cell–cell adhesion was the cause of increased detachment of cells from migrating monolayers. To confirm this, we used a dispase-based dissociation assay (Calautti et al., 1998). In unperturbed monolayers, after treatment with the dispase enzyme, which cleaves the cell–extracellular matrix attachment without affecting cell–cell linkage, only 16.2 ± 2.23% of cells dissociated from the monolayer (Figure 4J). Aggressive perturbation of cell–cell adhesion by ethylene glycol tetraacetic acid (EGTA)–based calcium chelation (Kartenbeck et al., 1982) resulted in 93.2% (±2.4%) of cells being released as single cells into suspension (Figure 4J). Fmnl3 KD before the dispase assay resulted in a dissociation rate of 42.5 ± 3.47%; Figure 4J). This phenotype was the cause of increased detachment of numerous rounded cells.
an intramolecular interaction between the N-terminal DID and C-terminal DAD (Chesarone et al., 2010). Autoinhibition is released upon binding of an activated Rho-GTPase to the N-terminal GTPase-binding domain (Chesarone et al., 2010). The AJ is a hub for signaling activity involving several classes of Rho-GTPases, as well as serine/threonine and tyrosine kinases (Bertocchi et al., 2012; Zaidel-Bar, 2013).

In an siRNA screen of kinases associated with the AJ (unpublished results), we found that KD of the nonreceptor tyrosine kinase Src resulted in a phenotype similar to mDia1 or Fmnl3 KD, with reduced E-cadherin at the AJ and increased cell spread area. Using the chemical inhibitor of Src (PP2) or siRNA-mediated KD, we confirmed that Src inhibition in Eph4 monolayers leads to loss of lateral junctions and increased cell spreading (Figure 5, A and B, and Supplemental Figure S4A). In addition, Src kinase inhibition also led to weaker cell-cell adhesion strength, as tested via the dispase-based dissociation assay (Figure 5C).

Owing to the similarities in phenotype produced by Src and formin inhibition, we sought to discern whether Src kinase acted upstream of or in parallel with formin activity at the AJ. To this end, we expressed the DAD in Eph4 monolayers and subjected them to Src kinase inhibition (Figure 5D). We found that activation of mDia1 by the exogenous DAD was sufficient to rescue the effects of Src inhibition on E-cadherin levels at AJs and prevent the loss of lateral junctions (Figure 5, D and E). This suggested that formin activation was downstream of Src kinase activity.

**FIGURE 4:** Formin activity affects molecular dynamics at the AJ and is essential for monolayer cohesion during collective cell migration. (A) Eph4 monolayer expressing E-cadherin–GFP; junctional E-cadherin–GFP was photobleached (white rectangle) in FRAP experiments. Magnifications illustrate recovery of E-cadherin fluorescence. (B) FRAP recovery curves comparing the effects of SMIFH2 treatment and DAD expression on E-cadherin dynamics at the AJ. Data are shown as an average for 15 junctions tested for each condition. (C) Mobile fractions corresponding to conditions tested in B; 15 junctions for every condition tested, with mean ± SEM. (D) Eph4 cells transfected with tdTomato-actin and plated on fibronectin islands; junctional actin was photobleached (white rectangle) in FRAP experiments. Magnifications illustrate recovery of actin fluorescence. (E) FRAP recovery curves comparing the effects of Fmnl3 KD and Fmnl3 overexpression (Fmnl3-GFP) on actin dynamics at the AJ. Data are shown as an average for 18 junctions tested for Neg siRNA and 17 junctions in Fmnl3 KD and Fmnl3-GFP conditions from three independent experiments. (F) Mobile fractions corresponding to conditions tested in E; 18 (Neg siRNA) or 17 (Fmnl3 KD and Fmnl3-GFP) junctions tested, with mean ± SD. (G) Montage from a movie of an in vitro scratch assay in control (Neg siRNA), Fmnl3 KD, or double KD (mDia1 and Fmnl3) conditions. Images are pseudocolored in yellow to indicate cohesive regions of the cell sheet and in blue (middle) to highlight cells that have detached from the migrating front. See Supplemental Movies S3, S4, and S7. (H) Quantification of wound closure. Eight (Neg siRNA and Fmnl3 KD) and nine (mDia1 + Fmnl3 KD) movies imaged in G, with mean ± SEM. (I) Quantification of cells detaching from migrating front (%). Eight movies for each condition, with mean ± SD. (J) Dispase assay to determine adhesion strength for conditions tested in G. EGTA treatment served as a low-adhesion strength control. Individual dots represent a single experiment (n = 3), with mean ± SD. Statistical significance was assessed using one-way ANOVA in C, F, and J; Student’s t test in I. Scale bars, 10 μm (A, D), 5 μm (magnification in A, D), 50 μm (G).
To further test the role of Src kinase as an upstream regulator of formins, we coexpressed in cells full-length GFP-tagged Src kinase together with a nontargeting control siRNA. As illustrated in Figure 5, F–H, Src-GFP robustly localized at the AJ, with a concomitant increase in F-actin and E-cadherin levels at the junction. Next we cotransfected Src-GFP together with siRNAs targeting Fmnl3. Whereas Src-GFP retained its prominent AJ localization, in this case, we did not observe a significant increase in junctional F-actin or E-cadherin (Figure 5, F–H), suggesting that Fmnl3 is an essential downstream effector of Src kinase at the AJ responsible for the increase in F-actin and E-cadherin.

Previous studies established a role for Src kinase in regulating Rho-GTPase activity at the AJ (Fukuhara et al., 2004). Furthermore, Rho-GTPases are immediate upstream regulators of formin activity. We therefore hypothesized that a Rho-GTPase intermediate existed in the signaling cascade between Src kinase and formin activation. To test this hypothesis, we attempted to rescue the effects of Src kinase inhibition through the expression of constitutively active mutants of the Rho GTPases RhoA, Rac1, and Cdc42. As shown in Figure 5, I and J, expression of constitutively active Cdc42 (Q61L mutation) was sufficient to rescue the phenotype observed with PP2 treatment. Cells expressing active Cdc42 not only had increased E-cadherin levels at the AJ, but also demonstrated the presence of lateral junctions, unlike their nontransfected counterparts (Figure 5, I and J). In contrast, constitutively active mutants of RhoA (V14) or Rac1 (L61) were inadequate to rescue the effects of Src kinase inhibition, with no visible changes in AJ organization detected in comparison to PP2-treated monolayers (Supplemental Figure S4, B and C). These results suggest a role for the Rho-GTPase Cdc42 as a signaling intermediate between Src kinase and formin activation. More recently, Fmnl3 was demonstrated to play a role in filopodia extension during angiogenesis in zebrafish in a Cdc42-dependent manner (Wakayama et al., 2015). Hence we asked whether this was also true at epithelial cell–cell junctions. To test this, we expressed in cells Fmnl3-GFP containing a point mutation (I111D) that abolishes binding to Cdc42 (Wakayama et al., 2015). Close analysis of this mutant revealed no specific cellular localization (Figure 5K) and no qualitative (Figure 5K) or quantitative (Figure 5, L and M) change in F-actin or E-cadherin organization at the AJ, suggesting that Fmnl3 activation and localization at the AJ depended on...
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Fmnl3 expression is elevated in response to epithelial wounding and is lost in cells that underwent epithelial–mesenchymal transition

As shown in Figure 3C, Fmnl3 localized to the AJ in nonmigrating monolayers, and its absence led to weak cell–cell adhesion and cell detachment in migrating monolayers (Figure 4, G and J). Therefore we hypothesized that epithelial cells would up-regulate their expression of Fmnl3 in response to wounding in order to maintain cell–cell junction integrity in the face of increased forces during collective cell migration (Tambe et al., 2011).

To test our hypothesis, we used semi-quantitative RT-PCR analysis to assess changes in Fmnl3 expression concurrent with the time course of migration in an in vitro scratch assay. As shown in Figure 6A, the two migrating fronts generated during the scratch assay began to establish contact within ~6 h. Subsequently, between 8 and 10 h from the start of migration, the migrating fronts established numerous points of contact, and within 12 h, the wound was completely sealed, and long-range collective cell movements ceased. For every time point captured in Figure 6A, we analyzed the mRNA expression level for Fmnl3. Of interest, our data revealed gradual but steady up-regulation of Fmnl3 levels during the course of migration (Figure 6, B and C, and Supplemental Figure S5E), with a peak in increase of 1.4-fold between 8 and 10 h, corresponding to the time when the two migrating fronts had established multiple cell–cell contacts. After gap closure was completed between 12 and 14 h, we observed a moderate decrease in Fmnl3 expression levels.

In addition to changes at the RNA level, we also investigated changes in endogenous Fmnl3 localization in cells that were positioned four or five cell rows behind the scratch edge (Supplemental Figure S5, A and B). In good agreement with the aforementioned expression data, we observed a steady increase in the level of Fmnl3 at cell–cell junctions between 6 and 10 h from the

Cdc42. Taken together, these results confirm the existence in Eph4 epithelial cells of a Src-Cdc42-Fmnl3 signaling axis that regulates F-actin and E-cadherin at the AJ.
start of migration. After a nearly twofold increase in Fmnl3 levels at the AJ between 6 and 8 h, we observed a steady reduction at 12 and 14 h when collective migration had halted (Supplemental Figure S5, A and B). These data support the notion that actin polymerization by Fmnl3 is of particular importance during collective cell migration, when cell–cell junctions experience greater forces and require reinforcement of the AJ to support coherent migration as a continuous epithelial sheet.

Next we probed directly whether Fmnl3 recruitment to cell–cell junctions was indeed force sensitive, taking advantage of the α18 antibody, which binds to α-catenin at the AJ in a force-dependent manner (Yonemura et al., 2010). We first confirmed that cell junctions in Eph4 were tension sensitive by exposing monolayers to nocodazole or blebbistatin to increase or decrease cellular contractility, respectively. Consistent with previous studies (Yonemura et al., 2010) and as shown in Supplemental Figure S5, C and D, nocodazole treatment resulted in markedly higher α18 labeling at the AJ, indicating that a higher proportion of α-catenin existed in a stretched conformation. Conversely, reduction of contractility after blebbistatin treatment resulted in near-disassembly of junctions, with little to no detectable α18 or α-catenin labeling at the AJ (Supplemental Figure S5C, bottom). We then compared the localization of α18 and Fmnl3 under similar conditions of increased or decreased contractility. As shown in Figure 6, D and E, recruitment of Fmnl3 at the AJ was significantly enhanced after nocodazole treatment and lost upon blebbistatin treatment. These data further suggest that Fmnl3 is recruited at cell–cell junctions under conditions of increased force application, such as during collective migration.

The phenotype we observed of cell detachment from monolayers upon Fmnl3 KD (Figure 4, G and I) was reminiscent of cells undergoing epithelial-mesenchymal transition (EMT), which often promotes cancer metastasis. Although EMT is a highly complex process, it is commonly associated with loss of cell–cell adhesion and acquisition of mesenchymal features with increased migratory potential (Cannito et al., 2010). Therefore, using semiquantitative RT-PCR analysis, we investigated FMLN3 expression levels in a range of human ovarian cancer cell lines with nontransformed (IOSE-523), epithelial (PEO-1), mesenchymal (HEY-A8), or intermediate/transitioning phenotypes (SKOV-3 and OVCA429WT; Figure 6F and Supplemental Figure S5F). Our analysis revealed highest expression of FMLN3 in nontransformed and epithelial ovarian cancer cells (Figure 6, F and G), with no detectable FMLN3 expression as the cells tended toward more mesenchymal types. Overall these results corroborate a role for Fmnl3 in supporting the epithelial phenotype.

**DISCUSSION**

Our findings provide evidence of the requirement of formin-dependent actin polymerization in stabilizing E-cadherin at AJs, contributing to cell–cell adhesion strength, preserving columnar cell morphology, and maintaining cohesion during collective migration. Although previous studies identified several formins, including mDia1, FMNL2, and FMNL3, at the AJ (Carramusa et al., 2007; Homem and Peifer, 2008; Gauvin et al., 2015; Grikseit et al., 2015; Phng et al., 2015), this is the first to show an essential role for formin activity in preventing leader cell dispersion and demonstrate up-regulation of the formin Fmnl3 after epithelial monolayer wounding. Previous studies using wound-healing assays implicated the formin mDia1 in facilitating directed cell migration through its ability to stabilize microtubules in a RhoA-dependent manner (Wen et al., 2004; Goulimir et al., 2005). Although we were unable to specifically localize mDia1 at the AJ or migrating cell fronts in our study, we do not rule out the possibility that the migration defects we observed could also be due to the microtubule-stabilizing property of mDia1.

The identification of Src and Cdc42 as upstream activators of formin activity raises the possibility that formins are mechanoregulated and respond to increased tension at cell–cell and/or cell–matrix adhesions, since both Src and Cdc42 have been shown to be activated in response to mechanical stimulation (Tzima et al., 2003; Wang et al., 2005; Niediek et al., 2012). Indeed, we show here, by varying cellular contractility, that Fmnl3 is recruited to cell–cell adhesions in a force-dependent manner. It is also worth noting that single-molecule tracking studies have demonstrated processive actin polymerization by mDia1 in response to forces applied on cell surfaces (Higashida et al., 2013), further highlighting the mechanosensitive nature of formins. Of interest, in vivo studies in rabbit corneal epithelial wound healing demonstrate an increase in Cdc42 expression correlated with migration, with expression levels tapering off when migration is complete (Pothula et al., 2013), which is highly reminiscent of the trend we observe with Fmnl3. In addition, up-regulation of the actin regulatory protein annexin-2 in a Rho-dependent manner was shown to be crucial for collective migration in intestinal epithelial wound-healing models (Babbin et al., 2007). Hence it is likely that the up-regulation of Rho-GTPases, actin regulators, and actin nucleators, as we demonstrate here, may be a widespread mechanism to regulate actin turnover at cell–cell/matrix adhesions during collective cell migration.

Both literature and proteomic analyses of E-cadherin–based junctions have clearly established the AJ as a focal point of signaling by Rho-GTPases, kinases, and phosphatases (Bertocchi et al., 2012; Zaidel-Bar, 2013; Guo et al., 2014; Van Italie et al., 2014). Several lines of evidence indicate that cadherin-activated Src kinase signaling positively influences cadherin function at the AJ via various downstream effectors, including phosphoinositide 3-kinase (Pang et al., 2005; McLachlan et al., 2007) and cortactin (Ren et al., 2009). In addition, Src kinase signaling is also known to act upstream of the Rho-GTPase Cdc42 in several cellular contexts, such as establishment of nectin-based cell–cell adhesions (Fukuhara et al., 2004), formation of the cortical actin cap (Kuga et al., 2008), and podosome assembly in endothelial cells (Tatin et al., 2006). Here we describe an additional role for Src kinase at the AJ, which is to support AJ organization and dynamics by promoting the function of Fmnl3 in a Cdc42-dependent manner.

The increase in lamellipodial protrusion and cell spreading we observed upon depletion of mDia1 or Fmnl3 is likely explained by the tilting of balance between Arp2/3 and formin activity, as it was recently demonstrated in both yeast and mammalian cells that the two factors compete for a limited pool of G-actin (Burke et al., 2014; Rotty et al., 2015; Suarez et al., 2015). Lomakin et al. (2015) showed that inhibiting myosin-II activity can switch cells from an epithelial to a protrusive, Arp2/3-dependent migratory phenotype, with a key step being the disassembly of the cortical acto-myosin bundles found in the ZA of epithelial tissues (Lomakin et al., 2015). Thus it would not be unreasonable to assume that inhibition of formin activity in our system would induce excessive activity of other cellular nucleators (e.g., the Arp2/3 complex) due to the sudden availability of polymerization-competent G-actin in the cell. Hence it is worthwhile to remember in the interpretation of our data that the effects of formin inhibition could be twofold: a direct effect on actin polymerization and E-cadherin stabilization at the AJ, and an indirect effect via stimulation of Arp2/3 complex activity.

Consistent with having an important role in maintaining AJ integrity, we found a correlation between loss of FMNL3 expression and
EMT in ovarian cancer cells. Whether down-regulation of FMNL3 is directly involved in EMT will be the subject of future studies. Overall our results identify roles for formins mDia1 and Fmnl3 at cell–cell junctions and delineate a novel Src-Cdc42-Fmnl3 signaling pathway that regulates F-actin and E-cadherin at the epithelial AJ. We demonstrate that this pathway is vital for the maintenance of epithelial cohesion during collective cell migration, making it essential for dynamic processes such as wound repair.

MATERIALS AND METHODS

Cell lines, plasmids, and chemical inhibitors

Eph4 (mouse mammary epithelial origin) was cultured at 37°C and 5% CO2 atmosphere in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin–streptomycin (Invitrogen). Eph4 cells with stable expression of GFP-tagged E-cadherin were kindly shared by Jean-Paul Thiery (Institute of Molecular and Cellular Biology, Singapore) and maintained under similar conditions as wild-type Eph4 cells. MCF10A cultures (human mammary epithelial origin) were maintained at 37°C and 5% CO2 atmosphere in growth medium composed of DMEM/F12 (Invitrogen) supplemented with 5% horse serum (Invitrogen), 20 ng/ml epidermal growth factor (Peptech, Rocky Hill, NJ), 0.5 mg/ml hydrocortisone (Sigma-Aldrich, St. Louis, MO), 100 ng/ml chola toxin (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), and 1% penicillin-streptomycin. DMEM/F12 supplemented with 20% horse serum (resuspension medium) was used for trypsinizing and resuspending MCF10A cells during routine passages and sample preparation. Ovarian cancer cell lines of varying phenotypes were obtained from Ruby Huang (Cancer Science Institute, Singapore) and were previously characterized (Huang et al., 2013). IOSE-523, PEO-1, and Hey-A8 were cultured in RPMI 1640 (Invitrogen), and SKOV-3 and OVCA429WT cell lines were grown in DMEM, both supplemented with 10% FBS (without antibiotic).

Plasmid encoding the DAD from formin mDia1 (amino acids 1177–1222) fused C-terminally to an mVenus fluorescent tag (mVenus-DAD) was a gift from Klaus M. Hahn (University of North Carolina, Chapel Hill, NC). Plasmids for expression of GFP-tagged full-length human FMNL3 and FMNL3-I111D were generously shared by Shigetomo Fukuhara (C3M University of Nice Sophia Antipolis, Nice, France) and were previously characterized (Huang et al., 2013). IOSE-523, PEO-1, and Hey-A8 were cultured in RPMI 1640 (Invitrogen), and SKOV-3 and OVCA429WT cell lines were grown in DMEM, both supplemented with 10% FBS (without antibiotic).

Plasmid for expression of RhoA and Cdc42 with the activating mutations V14 and L61, respectively, were provided by Alexander Bershadsky (Mechanobiology Institute, Singapore). Constitutively active yellow fluorescent protein–tagged Rac1 (L61 mutation) was provided by the Lemichez group (C3M University of Nice Sophia Antipolis, Nice, France).

For inhibition of cellular formin activity, confluent Eph4 monolayers were incubated for up to 12 h with 20 μM SMIFH2 (Tocris Biosciences, Bristol, UK), fixed with 4% paraformaldehyde posttreatment, and processed for immunofluorescence staining as described later. Arp2/3 complex or Src kinase inhibition was achieved by incubating confluent monolayers with 100 μM CK666 (Calbiochem, San Diego, CA) or 20 μM PP2 (Sigma-Aldrich), respectively, for 1 h. Nocodazole and blebbistatin treatments were performed for 1 h at concentrations of 10 and 50 μM, respectively. Dimethyl sulfoxide (DMSO) controls were used for all experiments, and CK689 (100 μM/1 h) served as an inactive control for the Arp2/3 complex inhibitor CK666.

Plasmid transfection and siRNA knockdown

All plasmid transfections were performed on Eph4 monolayers plated at 60–70% confluence, using 0.5–1 μg of purified plasmid and Lipofectamine 2000 (Invitrogen) as per the manufacturer’s instructions.

Gene silencing experiments were performed by transfecting Eph4 or MCF10A cells with species-specific ON-TARGET plus siRNA oligonucleotides (GE Dharmacon, Lafayette, CO) or a nontargeting siRNA (Neg siRNA; GE Dharmacon) at a final concentration of 50 nM using Lipofectamine 2000 as per the manufacturer’s instructions. Cells were fixed and immunolabeled for analysis 24–26 h posttransfection. Concurrently, cell lysates were also prepared from duplicate samples to assess gene knockdown efficiency using semiquantitative PCR analysis. siRNA oligonucleotides used in this study are listed in Supplemental Table S1.

RNA extraction and semiquantitative PCR

Total RNA was extracted from confluent monolayers of cells using TRIzol (Ambion, Carlsbad, CA) as per the manufacturer’s instructions. cDNA was synthesized via oligo-dT–primed reverse transcription from 1 μg of total RNA and reagents supplied in the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Equal volumes of cDNA were then used in a subsequent PCR as template to amplify genes of interest and quantitate expression levels. Gene-specific primers used are listed in Supplemental Table S2. Samples were then analyzed via agarose gel electrophoresis, and images were captured using the ChemidocMP imaging system (Bio-Rad, Hercules, CA) and quantified using the Gel Analyzer plug-in available in ImageJ/Fiji (National Institutes of Health, Bethesda, MD).

Fibronectin patterning

The protocol for stamping circular fibronectin patterns for cell confinement experiments was adapted from Doxzen et al. (2013). Briefly, PDMS stamps bearing circular features of 100 μm diameter were prepared using a silanized wafer generated using soft lithography techniques. Stamps were then incubated with a mixture of fibronectin (Sigma-Aldrich) and cyanine 5 (Cy5)-conjugated fibronectin for 1 h, followed by three washes with phosphate-buffered saline (PBS) and air drying. Dried and labeled fibronectin stamps were then gently pressed against a glass-bottom dish (ibidi, Martinsried, Germany 81151) to replicate the patterns on the dish. The area outside the patterns was then blocked by treatment with 0.2% Pluronic F-127 (Sigma-Aldrich) for 1 h, followed by two washes with PBS before cells were seeded.

In vitro scratch assay

For the in vitro scratch assay, Eph4 or MCF10A cells were plated until confluent on glass-bottom dishes (Iwaki, Newport, UK). The dishes were incubated at 37°C to allow complete cell spreading and formation of a monolayer. Before imaging, a “scratch” was created in the monolayer using a p10 pipette tip. The dishes were rinsed three times with sterile PBS to remove cell debris, imaging medium was added, and cell migration was captured via live-cell imaging as described later (image acquisition was performed for 12–13 h for Eph4 and 24 h for MCF10A).

Dispase-based dissociation assay

Confluent 60-mm dishes of epithelial cells were washed twice in PBS, treated with 2 ml of dispase solution (Life Technologies, Carlsbad, CA), and incubated at 37°C. Samples were monitored using an inverted transmission microscope at 5- to 10-min intervals for ~40–45 min. Postincubation, single cells or clumps of cells
released from each sample were collected and washed twice with PBS. Samples were then centrifuged, resuspended in 500 μl of PBS, and subjected to mechanical disruption by vortexing. The single cells released into suspension were counted using a Countess automated cell counter (Invitrogen). The whole samples were then centrifuged and incubated for 3–4 min at 37°C in TrypLE Express (Life Technologies) to release all cells into suspension. Cell count posttrypsinization was determined as before and used for assay normalization. For a low–adhesion strength control, confluent monolayers were treated with EGTA (4 mM) for 2 h before the disperse assay was performed.

**Immunofluorescence and phalloidin staining**

Samples for immunofluorescence and/or phalloidin staining were prepared by seeding cells on glass coverslips or 35-mm glass-bottom dishes (Iwaki) in 2 ml of growth medium at a density of $2 \times 10^5$ cells/ml, followed by transfection with the plasmids of interest using Lipofectamine 2000 as per the manufacturer’s instructions (Invitrogen). Immunofluorescence staining was performed 20–22 h posttransfection. Briefly, cells were fixed by incubation in prewarmed 4% paraformaldehyde for 20 min, followed by permeabilization with 0.2% Triton X-100 for 3 min. Immunostaining was performed at room temperature with the appropriate dilution of desired primary antibody for 1 h, followed by secondary antibody and/or Alexa Fluor–conjugated phalloidin (Life Technologies) for 30 min in dark conditions before mounting of coverslips on glass slides using FluorSave mounting medium (Merck Millipore, Darmstadt, Germany). Primary antibodies used are as follows: E-cadherin (for Eph4 cells, 1:1600, U3254; Sigma-Aldrich), E-cadherin (for MCF10A, 1:100, 610181; BD Biosciences, San Jose, CA), Fmn3 (1:100, sc-66770; Santa Cruz Biotechnology, Dallas, TX), mDia1 (1:100, 610848; BD Biosciences), α-catenin (1:1000, C2081, Sigma-Aldrich), and α18 (1:200; gift from Shigenobu Yonemura, RIKEN Center for Life Science Technologies, Kobe, Japan). Species-specific secondary antibodies (anti-rat/goat/mouse/rabbit) conjugated to Alexa Fluor 488/568/647 dyes (Life Technologies) were all used at 1:500 dilution, and Alexa Fluor 488/568/647–conjugated phalloidin was used at a dilution of 1:1000.

**Image acquisition, live-cell imaging, and FRAP**

Images were acquired using a confocal microscope (Ti; Nikon, Tokyo, Japan) fitted with a spinning-disk head (CSU-X1; Yokogawa, Tokyo, Japan), laser launch unit (Ls2; Roper Scientific, Trenton, NJ) and charge-coupled device camera (Evolve Rapid-Cal; Photometrics, Tucson, AZ). A 60×/1.40 numerical aperture (NA) Plan-Apo (Nikon) was used for image acquisition, with z-axis movement controlled by a Perfect Focus System (Nikon). MetaMorph software (Molecular Devices, Sunnyvale, CA) was used for image acquisition. Phase-contrast imaging for the in vitro scratch assay was performed with the same microscope setup but at lower magnification using a 20×/0.75 NA Plan-Apo objective (Nikon). All live-cell imaging experiments were carried out in a heated chamber at 37°C and 5% CO2 atmosphere in DMEM without phenol red, supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Invitrogen) for Eph4 or in growth medium for MCF10A cells.

FRAP experiments were performed on stable E-cadherin–GFP–expressing Eph4 cells or Eph4 transfected with tdTomato–actin in combination with Fmn3-GFP or anti-Fmn3 siRNA. Photobleaching experiments were carried out using the foregoing microscope setup. Prebleach and postbleach (recovery phase) images were acquired using the appropriate laser (20% 491-nm laser power with 100-ms exposure time for E-cadherin–GFP and 15% 561-nm laser with 100-ms exposure for tdTomato–actin). For bleaching, a rectangular region of interest along the junctions (10 μm2) was bleached using a 405-nm laser pulse at maximum laser power (100%), with an exposure time of 600 (GFP) or 700 ms (tdTomato) for one frame. Images were captured every 1 s for 5 min, followed by every 3 s for an additional 5 min. Data for individual recovery curves were extracted using MetaMorph software and exported to Excel (Microsoft, Redmond, WA) and Prism 6 (GraphPad, La Jolla, CA) for exponential curve fitting. The exponential function used for fitting was $Y = Y_0 + a(1 - \exp(-bx))$. The values of a and b derived from the exponential curve fit were used to calculate half-time of recovery $t_{1/2} = (\ln 2)/b$ and immobile fraction $F_0 = 100(1 - a/(1 - Y_0))$. Student’s t test was then used to verify statistical significance between different experimental groups being assessed.

**Image analysis and statistics**

Image segmentation and analysis of junctional and cellular parameters were performed using an in-house MATLAB algorithm as follows.

Briefly, the algorithm performs segmentation and analyzes the shape properties of each cell and its junctions using the E-cadherin image, whereas intensity measurement is performed on both E-cadherin and F-actin images. First, each z-projected image from the E-cadherin channel is background subtracted and thresholded to generate a binary mask to segment the image into junction region and cell region. Next the junction region is partitioned into junction segments, where each junction segment is the joining boundary of two cell segments. Normal lines are then drawn across every junction segment with a fixed length of 50 pixels. Each normal line, when overlaid on the binary mask, gives us the subset of normal line pixels that belong to the junction segment. The set of intensity values for each junction segment is then obtained by overlying these pixels on the background-subtracted image. The computation of junction intensity is straightforward by averaging the intensity of all the pixels that belong to each junction segment. The area for each cell segment is estimated from the skeleton image. For analysis of junction lengths from time-lapse movies, a similar algorithm was used that identifies junction segments as the boundaries joining two cell segments. The length of each junction was calculated from the number of pixels of each junction skeleton segment scaled by the pixel size. For each time point, a junction length map was generated and used for tracking length changes over time in Fiji. MATLAB algorithms used in this study for analysis of junctional and cellular parameters are available upon request.

All data generated were exported as Excel files and subsequently to Prism 6 for preparation of graphs and statistical analysis. Quantitative data sets were subjected to Student’s t test (for two data sets) or one-way analysis of variance (ANOVA; for more than two data sets) using the statistical functions available in Prism 6. All images were prepared for publication using Photoshop CS6 v13.0 (Adobe Systems).

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Formins regulate cell–cell adhesion

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