Abl suppresses cell extrusion and intercalation during epithelium folding

Jeanne N. Jodoin and Adam C. Martin*
Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

ABSTRACT Tissue morphogenesis requires control over cell shape changes and rearrangements. In the Drosophila mesoderm, linked epithelial cells apically constrict, without cell extrusion or intercalation, to fold the epithelium into a tube that will then undergo epithelial-to-mesenchymal transition (EMT). Apical constriction drives tissue folding or cell extrusion in different contexts, but the mechanisms that dictate the specific outcomes are poorly understood. Using live imaging, we found that Abelson (Abl) tyrosine kinase deletion causes apically constricting cells to undergo aberrant basal cell extrusion and cell intercalation. abl deletion disrupted apical–basal polarity and adherens junction organization in mesoderm cells, suggesting that extruding cells undergo premature EMT. The polarity loss was associated with abnormal basolateral contractile actomyosin and Enabled (Ena) accumulation. Depletion of the Abl effector Enabled (Ena) in abl-depleted embryos suppressed the abl phenotype, consistent with cell extrusion resulting from misregulated ena. Our work provides new insight into how Abl loss and Ena misregulation promote cell extrusion and EMT.

INTRODUCTION
Embryonic development requires morphogenetic events that alter the shape of cells and tissues (Lecuit et al., 2011). Defects in tissue morphogenesis result in abnormal organ formation, and genes and processes that reactivate morphogenetic processes later in life have been linked to metastasis (Yamada and Cukierman, 2007; Wang et al., 2012). Thus it is important to understand how morphogenesis is precisely regulated. Epithelial tissue folding is driven by apical constriction, a cell-shape change that alters a columnar cell into a wedged-shape cell (Leptin and Grunewald, 1990; Leptin, 1995). During constriction, nonmuscle myosin II (MyoII) contracts a network of actin filaments (F-actin) spanning the apical surface, generating a force that is propagated across the tissue via catenin-cadherin–containing adherens junctions (AJs) (Franke et al., 2005; Martin et al., 2009, 2010). In addition, apical constriction drives other morphogenetic events, such as cell extrusion and delamination (Marinari et al., 2012; Slattum and Rosenblatt, 2014), although the mechanism determining the morphogenetic outcome of apical constriction is poorly understood.

In Drosophila, transcription factors Twist and Snail promote both apical constriction of ventral cells to induce tissue folding into an epithelial tube within the embryo (called the ventral furrow; Figure 1A) and then epithelial-to-mesenchymal transition (EMT) of the epithelial tube (Leptin, 1991; Martin et al., 2009; Clark et al., 2011). Twist and Snail promote EMT, in part, via repression of E-cadherin (E-cad) transcriptional activation (Vesuna et al., 2008; Shamir et al., 2014). E-cad repression promotes the loss of apical–basal polarity and cell–cell junctions and induces cell migration of the mesoderm, features recapitulated in metastatic cancer (Onder et al., 2008). Reactivation of the EMT program later in life results in this disease state. Apically constricting cells are mechanically coupled to each other during tissue folding (Martin et al., 2010), and no cell extrusion occurs, suggesting that a mechanism functions to prevent cell extrusion before EMT.

Abl tyrosine kinase has conserved roles in tissue morphogenesis and disease states (Koleske et al., 1998; Fox and Peifer, 2007). Drosophila Abl regulates apical F-actin organization during apical constriction and tissue folding via negative regulation of Enabled (Ena; Fox and Peifer, 2007). Ena binds to F-actin barbed...
RESULTS

Abl prevents cell extrusion and intercalation of ventral cells during apical constriction

During Drosophila gastrulation, ventral cells apically constrict in a coordinated manner; cells constrict their apical surface at similar rates, such that apical surface areas are homogeneous (Figure 1, A and B). Abl is required for this coordinated apical constriction; abl4 null mutants exhibited uncoordinated constriction, resulting in populations of larger, unconstricted cells (Supplemental Figure S1B, green arrow) and smaller, constricted cells (Supplemental Figure S1B, red arrow; Fox and Peifer, 2007). Flies expressing small hairpin RNA (shRNA) targeting abl, which effectively depletes ∼83% of abl transcript (Jodoin et al., 2015), recapitulated the uncoordinated-constriction ends to promote actin elongation and restrict actin capping (Bear and Gertler, 2009; Hansen and Mullins, 2010). Abl also promotes AJ dynamics during tissue elongation via β-catenin (β-cat; Drosophila armadillo) phosphorylation (Tamada et al., 2012). In mammalian systems, misregulation of Abl is associated with metastasis; Abl overexpression leads to hyperproliferation and tumor formation, and Abl depletion results in metastasis and invasion (Hayes et al., 2013). Moreover, increased levels of mammalian Ena increase the occurrence of metastasis in animal models and breast cancer (Philippar et al., 2008; Oudin et al., 2016). Here we identify a mechanism by which loss of Abl and Ena misregulation, combined with Snail and Twist expression, promotes EMT-like behavior during tissue folding.

FIGURE 1: Abl suppresses cell extrusion and intercalation during apical constriction. (A) Schematic of ventral furrow formation. Green denotes Twist and Snail expressing–ventral cells. (B) Schematic of cell behavior during apical constriction. Colored dots track cells. Red cell denotes cell extrusion. (C, D) Time-lapse images of constricting cells from embryos expressing indicated UAS-shRNA and Utr::GFP (F-actin, cell outlines) at indicated depths below the apical surface. White arrow denotes a constricting cell. Red arrow denotes basal cell extrusion after loss of abl. (C’, D’) Schematicized cells denoted by the white and red arrows in C and D, respectively. (E, F) Time-lapse images of embryos expressing indicated UAS-shRNA and Gap43::CH (membrane). White arrowheads and colored dots track an interface and cells, respectively. (E’, F’) Schematicized interface denoted by white arrowheads in E and F, respectively. (G) Number of cells extruding during tissue folding. (H) Number of T1 transitions during tissue folding. Box plots (in this and all subsequent figures): red line, median; bottom and top, 25th and 75th percentiles, respectively; black dashed lines, lowest and highest values; red crosses, outliers beyond 1.5 times the interquartile range of the box edges. *p < 0.00001. n.s., not significant. Scale bars, 5 μm. See Materials and Methods for data point numbers for all experiments in this and all subsequent figures.
phenotype (Supplemental Figure S1A), confirming that this is an effective tool with which to study Abl during tissue folding.

We previously performed a live-imaging RNA interference screen targeting known actomyosin regulators (Jodoin et al., 2015) and identified a surprising and unreported phenotype in abl-shRNA embryos. Live imaging of abl-depleted embryos revealed that ventral cells frequently undergo basal cell extrusion during tissue folding, a phenotype not observed in wild-type or control embryos (Figure 1, B–D, and G, Supplemental Figure S1, E, F, J, and K, and Supplemental Movie S1). Extrusion was not observed in cells adjacent to the ventral region that do not express Twist and Snail (nonventral cells; Figure 1G). This suggests that Abl promotes the maintenance of cells within the epithelium during tissue folding. Loss of abl results in a disorganized, apical actomyosin meshwork, with some cells lacking apical actomyosin (Fox and Peifer, 2007). However, apical actomyosin pulses were observed in extruding cells (Supplemental Figure S1H; 17 of 17 embryos). Nuclei of extruding cells were not fragmented, suggesting that extrusion is not due to an apoptotic signal (Supplemental Figure S1K). Moreover, before the onset of tissue folding, embryos depleted for abl exhibit reduced cell packing (ctl-shRNA, 24 ± 1.1, and abl-shRNA, 19.9 ± 0.74, cells per 35-μm² area; p < 0.00001), suggesting that cell extrusion is not due to cell crowding. In addition to extrusion, intercalation events known as T1 transitions, in which junctions aligned along the dorsal–ventral axis collapse and extend new junctions along the anterior–posterior axis (Bertet et al., 2004), were observed specifically in ventral cells during tissue folding in abl-depleted embryos (Figure 1, F and H, and Supplemental Figure S1G). T1 transitions were not observed in control embryos during tissue folding in either ventral or nonventral cells because this is before tissue elongation (Figure 1, E and H, and Supplemental Figure S1G). Aberrant ventral region T1 transitions occur with preferred direction, with junctions oriented along the dorsal–ventral axis shrinking and junctions along the anterior–posterior axis lengthening despite a lack of planar polarized actomyosin, which is important for intercalation during tissue elongation (Bertet et al., 2004; Zallen and Wieschaus, 2004). The elongation is in the direction in which tension is normally highest in the ventral furrow (Martin et al., 2010), suggesting that intercalation may be a response to global tissue tension. Together these data suggest that abl functions to prevent cell extrusion and intercalation specifically in Twist- and Snail-expressing cells during tissue folding.

Abl regulates apical–basal polarity of ventral cells

After tissue folding and tube formation, ventral cells lose apical–basal polarity and undergo EMT (Clark et al., 2011). To determine whether cell extrusion is due to early EMT-like behavior, we tested whether abl depletion altered apical–basal polarity. During apical constriction, the cell polarity protein Par-3 (Drosophila Bazooka) localizes apically and establishes the apical domain by setting the position of AJs (Figure 2D, ctl-shRNA, yellow arrows; Harris and Peifer, 2004, 2005). abl depletion resulted in the basal–lateral accumulation of Par-3 specific to the ventral region (Figure 1A, apically constricting; Figure 2, B–E, red arrows, and Supplemental Figure S1). This accumulation below that apical surface due to the loss of abl occurred after the onset of tissue folding (Figure 2C, red arrows). In contrast, Par-3 is restricted apically and not present in the basolateral domain of ctl-shRNA embryos (Figure 2, A, and C–E, yellow arrows). These data suggest that abl maintains apical–basal polarity in ventral cells during tissue folding.

Apical and basal E-cadherin organization of ventral cells requires abl

Apical AJs are essential for segregating apical and basal E-cadherin (Harris and Peifer, 2004). A reduction of AJ components in apically constricting cells results in a loss of apical identity (Harris and Peifer, 2004) and aberrant T1 transitions (Martin et al., 2010). Thus we next assessed whether loss of abl disrupts apical AJs. In fixed images, E-cad is enriched at apical junctions in a punctate pattern, with relatively low enrichment in the center of the apical surface or the medioapical domain (Figure 2, F, I, and J, ctl-shRNA; and Supplemental Figure S1I). In contrast, abl depletion resulted in a ventral cell–specific defect in which E-cad was localized across the entire apical surface (Figure 2, F, I, and J, abl-shRNA). Live imaging revealed that junctional E-cad observed in ctl-shRNA embryos was greatly decreased in abl-shRNA embryos, and E-cad became more diffusely localized (Figure 2, K and L). We also observed a striking defect in basolateral E-cad organization after loss of abl. In ctl-shRNA embryos E-cad is distributed along basolateral junctions, with little variability in its intensity (Figure 2, G and H). Loss of abl significantly increased variability in E-cad intensity (Figure 2H), with junctions displaying either a reduction in E-cad levels (Figure 2G, white arrow) or an overaccumulation of E-cad levels (Figure 2G, white arrowhead). Basolateral E-cad levels corresponded to varied basolateral F-actin levels (Figure 2G). Taken together, these data suggest that abl promotes proper intercellular junction organization both apically and basolaterally.

Failure to restrict E-cad to cell–cell interfaces and maintain a uniform basolateral organization are likely due to the loss of apical–basal polarity in abl-depleted embryos. Basal cell extrusion from an epithelium is often associated with a change in E-cad levels and distribution (Friedl and Wolf, 2003; Slattum and Rosenblatt, 2014). Thus we propose that E-cad defects could play a role in promoting cell extrusion and rearrangements. In other mutants, such as diaphanous mutants, E-cad localization is similarly disrupted, but such mutants do not result in cell extrusion (Mason et al., 2013), suggesting that defects in E-cad localization alone are not sufficient to drive extrusion.

Abl restricts contractile actomyosin pulsing to the apical domain in constricting cells

Pulses of contractile actomyosin that drive apical constriction are restricted to the apical domain during tissue folding (Harris et al., 2013). Increased basolateral contractility can drive cell extrusion from the epithelium (Slattum and Rosenblatt, 2014; Wu et al., 2014, 2015). Because we found that Abl promotes apical–basal polarity, we sought to determine whether the apical contractile machinery is localized improperly in abl-shRNA embryos. In control embryos, pulsed actomyosin is not present in the basolateral domain (Figure 3, A, C, E, G, and H, ctl-shRNA, yellow arrow; and Supplemental Figure S1C). abl depletion disrupted the position of contractile actomyosin. Dynamic F-actin, MyoII, and the MyoII activator Rho kinase (ROCK) accumulated basolaterally in abl-depleted embryos (Figure 3, B, D, and F–J, abl-shRNA, red arrows; and Supplemental Figure S1D, red arrows), suggesting that actomyosin contractility occurs below the apical surface. Basally extruding cells exhibited basolateral pulsed actomyosin, although the presence of basolateral actomyosin in cells did not always result in extrusion (17 of 17 embryos). Moreover, basolateral MyoII and ROCK accumulation occurred at the onset of tissue folding (Figure 3, G and H, red arrows, and Supplemental Movie S2), indicating that the phenotype is not due to delayed folding after loss of abl (Figure 3K). On the basis of these findings, we propose that the loss of abl
Defects in AJ integrity result in basolateral MyoII accumulation

To test whether disrupted apical–basal polarity and AJs could cause actomyosin mislocalization, we mildly reduced β-catenin (β-cat) levels via Abl depletion in Twist- and Snail-expressing cells, such that force generation occurs basolaterally in addition to apically (Supplemental Figure S1H), which, coupled with AJ defects, leads to cell extrusion and rearrangements.

Defects in AJ integrity result in basolateral MyoII accumulation

To test whether disrupted apical–basal polarity and AJs could cause actomyosin mislocalization, we mildly reduced β-catenin levels via Abl depletion in Twist- and Snail-expressing cells, such that force generation occurs basolaterally in addition to apically (Supplemental Figure S1H), which, coupled with AJ defects, leads to cell extrusion and rearrangements.

FIGURE 2: Abl depletion disrupts apical–basal polarity in ventral cells. (A–D) Embryos expressing indicated UAS-shRNA and GFP::Bazooka (Par-3). (A, B) Time-lapse images of basolateral domain of ventral cells (~21 μm below the apical surface). Red arrows denote basolateral Par-3. (A', B') Zoomed-in region indicated by the white-dashed boxes in A and B, respectively. Red arrows denote dynamic basolateral Par-3. (C) Kymographs of embryos expressing indicated UAS-shRNA and Par-3. Kymographs of basolateral line along the anteroposterior axis. Red arrows denote basolateral Par-3, and blue arrowhead indicates the beginning of tissue folding. (D) Cross-sections. Yellow arrows denote apical Par-3, and red arrows denote basolateral Par-3. (E) Ratio of ventral vs. nonventral basolateral Par-3 intensity. (F) Fixed images of the apical domain in embryos expressing the indicated UAS-shRNA stained for E-cad. Apical E-cad, AJs (green). Subapical E-cad, cell outline (gray). White arrowheads denote pools of medioapical E-cad. (G) Fixed images of the basolateral domain (~21 μm below the apical surface) in embryos expressing the indicated UAS-shRNA and stained for E-cad and phalloidin (F-actin). White arrows denote junctions lacking E-cad, and white arrowheads denote junctions overaccumulated with E-cad. (H) Basolateral E-cad intensity, coefficient of variance. (I) Cross-sections of fixed embryos expressing the indicated UAS-shRNA stained for E-cad. White arrows denote junctional pools of E-cad, and white arrowhead denotes medioapical pool of E-cad. (J) Ratio of junctional vs. medioapical E-cad intensity in fixed embryos. (K) Kymographs of embryos expressing the indicated UAS-shRNA and GFP::E-cad (E-cad). Kymographs are of basolateral line along the anteroposterior axis. Images are maximum intensity projections of the top eight slices of each embryo (~8 μm below apical surface). White arrows denote junctional E-cad. (L) Ratio of junctional vs. diffuse E-cad intensity in live embryos. **p < 0.00001, *p < 0.04, n.s., not significant. Scale bars, 5 μm.
Molecular Biology of the Cell

J. N. Jodoin and A. C. Martin

of the cell, suggesting that the accumulation of basolateral actomyosin in abl-shRNA embryos was a downstream consequence of defective AJs. Of importance, basal cell extrusion was not observed in mildly β-cat-depleted embryos (Supplemental Figure S2E), suggesting that defects in AJ integrity and basolateral MyoII were not sufficient to induce extrusion. Thus an additional mechanism might be required.

Abl restricts Ena from the basolateral cortex in ventral cells

Ena was identified as an essential effector of abl during tissue folding (Fox and Peifer, 2007). In wild-type and control embryos, Ena localized diffusely in the cytoplasm (Figure 4, A and E) but became injection of β-cat double-stranded RNA (dsRNA), previously shown to effectively deplete β-cat (Tamada et al., 2012), into the embryo. Abl depletion causes partial disruption in AJ organization (Figure 2, F and G). Thus we mildly disrupted AJs to mimic abl-shRNA embryos. Complete down-regulation of β-cat results in a dramatic separation of ventral cells at the onset of apical constriction (Martin et al., 2010), whereas mild β-cat depletion resulted in only slight separations at the poles during the initiation of tissue folding (Supplemental Figure S2A, white arrow). Mild β-cat reduction also resulted in aberrant T1 transitions (Supplemental Figure S2D), recapitulating previous reports (Martin et al., 2010) and indicating that AJ integrity is disrupted. In ventral cells, we observed increased basolateral MyoII after mild β-cat depletion compared with buffer-only (Ctl) injection (Supplemental Figure S2, B and C, wild-type + β-cat dsRNA, red arrow), suggesting that the accumulation of basolateral actomyosin in abl-shRNA embryos was a downstream consequence of defective AJs. Of importance, basal cell extrusion was not observed in mildly β-cat-depleted embryos (Supplemental Figure S2E), suggesting that defects in AJ integrity and basolateral MyoII were not sufficient to induce extrusion. Thus an additional mechanism might be required.

Abl restricts Ena from the basolateral cortex in ventral cells

Ena was identified as an essential effector of abl during tissue folding (Fox and Peifer, 2007). In wild-type and control embryos, Ena localized diffusely in the cytoplasm (Figure 4, A and E) but became
ena depletion suppresses cell extrusion and rearrangements in an abl mutant

To test whether misregulated Ena contributed to cell extrusions and intercalation that occurred in abl-depleted embryos, we down-regulated ena via injection of ena-dsRNA, previously shown to effectively deplete ena (Kiger et al., 2003), in abl-shRNA embryos. We found that the timing of ventral furrow formation was reduced significantly in abl/ena–depleted embryos compared with abl-depleted embryos (Figure 5C), showing that dsRNA-mediated depletion of ena in abl-shRNA embryos suppressed gross tissue-folding defects (Fox and Peifer, 2007). Of importance, reducing ena levels in abl-shRNA embryos suppressed basal cell extrusion and T1 transitions in ventral cells, as well as ventral cell–specific Par-3 and AJ defects (Figure 5, E–H). The ratio of junctional to medioapical E-cad levels increased significantly after ena-dsRNA injection compared with Ctl injection, indicating restoration of normal junctional enrichment (Figure 5F). Apical actomyosin meshwork defects and localization

enriched at intercellular junctions in abl mutants (Figure 4, A and E, yellow arrowhead; Fox and Peifer, 2007). We observed that apical Ena was not present uniformly at junctions in abl-shRNA embryos, similar to apical E-cad localization (Figures 2F and 4A, yellow arrowhead). In addition, Ena accumulated basolaterally in ventral cells with a localization similar to Par-3 and E-cad after abl depletion (Figure 2, B and G, and Figure 4, B and F, yellow arrowhead). Furthermore, basolateral Ena accumulation at junctions was not observed in nonventral cells, although Ena localized to apical junctions in these cells (Figure 4, C–F). This is consistent with the basolateral mislocalization of Ena being specific to Twist- and Snail-expressing cells in the ventral region but not apical Ena accumulation. Ectopic Ena expression locally increases contractility (Furman et al., 2007). On the basis of these data, we hypothesized that the accumulation of basolateral Ena contributes to the cell extrusion present in abl-depleted embryos by increasing basolateral contractility.

FIGURE 4: abl inhibits basolateral, cortical Ena localization in ventral cells. (A–D) Fixed embryos expressing the indicated UAS-shRNA stained for Ena (green) and phalloidin (F-actin, gray) showing either ventral cells (A, B) or nonventral, lateral cells (C, D). Yellow arrowheads denote Ena accumulation at junction. (E) Apical junctional vs. medioapical Ena intensity. (F) Basolateral Ena intensity, coefficient of variance. **p < 0.00001, *p < 0.003, n.s., not significant. Scale bars, 5 μm.
Reduction of the Abl effector ena in abl mutants suppressed gross morphological defects in tissue folding (Fox and Peifer, 2007). Here our work identifies different roles for Abl in maintaining cells within the epithelium during tissue folding, as well as in promoting apical–basal polarity and apical AJ positioning. We found that loss of abl results in aberrant basal cell extrusion and cell intercalation events. Correlation between basal cell extrusion and T1 transitions was not observed after loss of abl. We found that T1 transitions do not preferentially occur near or after extrusion of a cell or vice versa. Loss of adhesion results in T1 transitions (Supplemental Figure S2D), which occur independently of basal cell extrusion (Supplemental Figure S2E). Together these results suggest that cell extrusion and intercalation are two different outcomes that occur due to the loss of abl.

Moreover, abl-depleted embryos exhibited loss of polarity and AJ organization (Figure 6A), hallmarks of EMT. Because cell extrusion happens specifically in Twist- and Snail-expressing cells, we suggest

**DISCUSSION**

Previous work described the role of Abl in promoting proper coordinated constriction and apical actomyosin organization during tissue folding (Fox and Peifer, 2007). abl-null mutants exhibit populations of both unconstricted and constricted cells and defects in tissue invagination and display discontinuous apical MyoII and F-actin meshworks across the ventral region of the embryo (Fox and Peifer, 2007). Reduction of the Abl effector ena in abl mutants suppressed gross morphological defects in tissue folding (Fox and Peifer, 2007). Here our work identifies different roles for Abl in maintaining cells within the epithelium during tissue folding, as well as in promoting apical–basal polarity and apical AJ positioning. We found that loss of abl results in aberrant basal cell extrusion and cell intercalation events. Correlation between basal cell extrusion and T1 transitions was not observed after loss of abl. We found that T1 transitions do not preferentially occur near or after extrusion of a cell or vice versa. Loss of adhesion results in T1 transitions (Supplemental Figure S2D), which occur independently of basal cell extrusion (Supplemental Figure S2E). Together these results suggest that cell extrusion and intercalation are two different outcomes that occur due to the loss of abl. Moreover, abl-depleted embryos exhibit loss of polarity and AJ organization (Figure 6A), hallmarks of EMT. Because cell extrusion happens specifically in Twist- and Snail-expressing cells, we suggest
that loss of abl promotes premature initiation of EMT during the tissue folding process.

Loss of abl and cell extrusion are accompanied by relocalization of the apical contractile machinery to basolateral junctions, suggesting that contractility occurs abnormally below the apical surface (Figure 6A), which can drive basal cell extrusion (Wu et al., 2014, 2015). Ena, which can locally increase contractility (Furman et al., 2007), also accumulates along basolateral junctions in abl mutants (Figure 6A), further suggesting that the loss of abl promotes basolateral contractility. We hypothesize that basolateral contractility, which, in combination with apical contractility, drives basal cell extrusion after loss of abl (Figure 6B; Wu et al., 2014, 2015). The heterogeneous nature of Ena and actomyosin accumulation along basolateral junctions could result in random cell extrusion within the ventral region rather than premature EMT of all ventral cells. Understanding why loss of polarity and E-cad organization defects, as well as basolateral actomyosin and Ena accumulation, is not ubiquitous across the ventral region will be important for understanding which cells undergo extrusion and premature EMT. Cells exhibit heterogeneity in their behavior at the onset of apical constriction (Xie and Martin, 2015). This could also play a role in determining which cells undergo extrusion in abl mutants.

Because we did not observe clear correlation between local basolateral actomyosin accumulations and cell extrusion, we suggest that extrusion could result from a nonautonomous effect of basolateral contractility, which could possibly increase lateral pressure on cells in the epithelial sheet. Alternatively, E-cad localized along lateral junctions can organize actin to generate tension; changes in lateral E-cad levels and altered tension are associated with cell extrusion (Wu et al., 2014). Thus changes in E-cad organization along basolateral junctions in abl mutants could potentially alter basolateral tension, promoting extrusion. More work is needed to understand the mechanical differences that result in cells and tissues lacking abl function. In addition, changes in E-cad organization after loss of abl could also play a role in driving basal cell extrusion. Loss of E-cad protein levels is sometimes associated with EMT (Onder et al., 2008). Defects in E-cad organization in abl-shRNA embryos could be due to a loss of E-cad protein, thus triggering premature EMT during tissue folding. Altered E-cad localization, namely higher levels of apical E-cad, could also support extrusion by allowing the apical surface to make contact with neighboring cells. Finally, a reduction in basolateral stiffness can lead to cell extrusion (Slattum and Rosenblatt, 2014). Pulsatile actomyosin along basolateral junctions could potentially result in more-rapid F-actin turnover along junctions, as shown in cultured mammalian cells (Wu et al., 2014), reducing the junctional stiffness and promoting cell extrusion. Further work is needed to test these potential models and determine the mechanisms driving basal cell extrusion before EMT after the loss of abl.

We show here that depletion of ena in abl-shRNA embryos suppressed all of the observed phenotypes in abl-depleted embryos, suggesting that Abl negatively regulates Ena in order to promote epithelial maintenance, polarity, and AJ positioning during tissue folding. During tissue elongation, β-cat was found to be an essential Abl substrate (Tamada et al., 2012); however, because ena downregulation suppresses all observed phenotypes (Figure 5), we hypothesize that Ena is the critical Abl effector during tissue folding. Abl is required to inhibit cell intercalation during tissue folding (Figure 1) but functions to promote intercalation during elongation (Tamada et al., 2012). This difference could be due to the expression of Twist and Snail or a difference in the organization of the contractile machinery between tissue folding and elongation. Moreover, loss of abl disrupts Par-3 and actomyosin apical–basal polarity during tissue folding (Figures 2 and 3) but not during elongation (Tamada et al., 2012).

Our findings are consistent with those of Fox and Peifer (2007). However, our data show how Abl and Ena regulate an EMT-like process, which provides insight into how Abl and Ena may influence metastasis (Philippar et al., 2008; Hayes et al., 2013; Oudin et al., 2016). Our work highlights the importance of proper Ena regulation via Abl in maintaining cells within the epithelium during tissue folding, before EMT. Kinase-dependent and -independent roles have been identified for Abl in regulating F-actin–dependent processes (Henkemeyer et al., 1990; Dudek et al., 2010; Courtemanche et al., 2015). Whether Abl inhibits cell extrusion during tissue folding via direct regulation of Ena function and/or localization or indirectly via regulation of polarity or junctional components will be of interest for understanding how Abl regulates epithelial integrity and polarity.

MATERIALS AND METHODS

Fly stocks

UAS-β-gal-shRNA (control-shRNA) and UAS-abl-shRNA expression were driven by the following maternal driver lines: [Mat67, Utr::green fluorescent protein (GFP)] (Rauzi et al., 2013), [Mat67, sqh::GFP; Mat15, Gap43::CFHP], [Mat67, utor::GFP; Mat15, sqh::CHFP], [Mat67, ubi-GFP::ROCK)] (Bardet et al., 2013), [Mat67, Histone::GFP], [Mat67, Ecad(knock in)::GFP] (Huang et al., 2009), and [Mat67, UAS-GFP::Bazooka] (Benton and St Johnston, 2003). To image abl mutants and control (ctl) embryos, germine clones were generated using the FLP-DHS technique by heat shocking mutant/OvoD larvae for 2 hr at 37°C for 3–4 d (Chou and Perrimon, 1992). For abl mutants, [yw,hsflp; FRT2A, OvoD/TM3] embryos were imaged (Supplemental Figure S1B). For ctl, [yw,hsflp; abl, FRT2A/TM3] embryos were imaged (Supplemental Figure S1B). For ctl, [yw,hsflp; abl, FRT2A/TM3] embryos were imaged (Supplemental Figure S1B). For ctl, [yw,hsflp; abl, FRT2A/TM3] embryos were imaged (Supplemental Figure S1B). For ctl, [yw,hsflp; abl, FRT2A/TM3] embryos were imaged (Supplemental Figure S1B). For ctl, [yw,hsflp; abl, FRT2A/TM3] embryos were imaged (Supplemental Figure S1B).

Making dsRNA

The following primers were used to generate ~500–base pair fragments of each gene of interest: β-cat (as described and shown to deplete β-cat in Tamada et al., 2012), β-cat forward, 5′-TAA TAC
GAC TCA CTA TAG GGA GAC CAC CAA GAA GCG GCT ATC CAT AFA GC-3, and β-cathe reverse, 5′-TAA TAC GAC TCA CTA TAG GGA GAC CAC CAA TCG GTA TAC CAG CCA GC-3; and Ena (as described and shown to deplete Ena in Kiger et al., 2003), Ena forward, 5′-TAA TAC GAC TCA CTA TAG GGG CCC CCT TTC CTT TGA TT-3, and Ena reverse, 5′-TAA TAC GAC TCA CTA TAG GGC GAG CAG ATG TAT CCT TAT T-3. Each primer contains a T7 sequence (TAA TAC GAC TCA CTA TAG GG) at its 5′-end. dsRNA was made using the MEGAscript T7 transcription kit (Ambion, Austin, TX) and resuspended in injection buffer (0.1x phosphate-buffered saline [PBS] in RNase-free water).

dsRNA injections
Embryos were dechorionated, mounted ventral side up, and then desiccated for 5 min (Drierite, Drierite Company, Salt Lake City, UT). A 3:1 halocarbon 700/halocarbon 27 oil was added on the embryo for injection. The 0–30-min-old embryos (before pole cell formation) were injected laterally with a drop size of ~40 μm, with estimated a 1:200–1:300 dilution for the dsRNA. ena-dsRNA and β-cate–dsRNA were injected into embryos at 2 μg/μl and 1.5 ng/μl, respectively. For buffer-only control, 0.1x PBS was injected.

Microscope image acquisition
Live and fixed images were acquired on a Zeiss LSM 710 confocal microscope with a 40×numerical aperture 1.2 Apochromat water objective (Zeiss, Jena, Germany) and argon-ion, 561-nm diode, 594-nm HeNe, and 633-nm HeNe lasers. Pinhole settings were 1–2 Airy units. For two-color live imaging, simultaneous excitation was used with band-pass filters set at 499–561 nm for GFP and 599–696 nm for CHFP. All images are representative of at least six embryos unless otherwise stated.

Live imaging. Embryos were dechorionated with 1:1 bleach/water for 2 min, washed with water, and mounted ventral side up onto a slide coated with embryo glue (double-sided tape soaked in heptane). Spacer coverslips (No. 1.5) were attached using glue, and a coverslip was attached to create a chamber so that embryos were not compressed. Halocarbon 27 oil was added to the chamber. Imaging was performed at room temperature (~23°C) on a Zeiss LSM 710 confocal microscope.

Fixed imaging. Embryos were dechorionated with 1:1 bleach and water solution for 2 min, washed with water, and then fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 with 50% heptane for 30 min and manually devitellinized. To visualize F-actin, embryos were then incubated with Alexa Fluor 568–conjugated phalloidin (Invitrogen, Carlsbad, CA) diluted in PBS plus 0.1% Triton X-100 (PBST) overnight (~12 h) at 4°C. Embryos were blocked with 10% bovine serum albumin (BSA) in PBST. Primary and secondary antibodies were diluted in 5% BSA in PBST. Embryos were mounted ventral side up using AquaPolymount (Polysciences). The following antibodies were used for immunofluorescence: primary, anti–E-cadherin (Dcad2, 1:25; Developmental Studies Hybridoma Bank, Iowa City, IA) and anti-Enabled (SG2, 1:100; Developmental Studies Hybridoma Bank); and secondary, Alexa Fluor 488 (1:500; Invitrogen).

Microscopy image processing
Images were processed with Photoshop CS6 (Adobe, San Jose, CA) and Fiji (http://fiji.sc/wiki/index.php/Fiji). Figures were prepared using Illustrator C6 (Adobe). A Gaussian filter (kernel = 1 pixel) was applied to images. Apical images are maximum intensity projections of multiple z-sections (~2–3 μm), unless otherwise noted. Subapical images are one z-slice, ~1–2 μm below the apical sections. Cross-sections of embryos were generated using the reslice command in Fiji.

Statistical analysis
Significance between data sets was determined using the Student’s t test. Data sets are represented with box-and-whisker-plots. A red line indicates the median. Bottom and top boxes mark the 25th and 75th percentiles of the data set, respectively. Black dashed lines mark the lowest and highest values of the data set, respectively. Red crosses indicate outliers that are beyond 1.5 times the interquartile range of the box edges. Box plots are presented in Figures 1, G and H, 2, E, H, J, and L, 3, J and K, 4, E and F, and 5, C–H, and Supplemental Figures S1, F and G, and S2, C–E.

Microscopy image analysis
Cell extrusion was quantified by manually tracking cells leaving the epithelium, using Gap34::CHFP (membrane) or Utr::GFP (F-actin) to visualize cell outlines. To quantify cell extrusion, the ventral region and the ectoderm adjacent the ventral region (nonventral cells) were examined. For the ventral region, the average field of view was 29.4 × 9 and 25.1 × 7.5 cells for control and abl-depleted embryos, respectively. All visible cells within the field of view were quantified. For ctrl-shRNA and abl-shRNA, 13 and 17 embryos were analyzed, respectively. For ctrl and abl mutants, three and five embryos were analyzed, respectively. For abl-shRNA plus buffer-only injection and abl-shRNA plus ena-dsRNA injection, six embryos per condition were analyzed. For wild-type plus buffer-only injection and wild-type plus β-cat dsRNA injection, four embryos per condition were analyzed. The number of extruding cells during tissue folding per individual embryo was counted, and the number of cell extrusion events for each data set is presented in Figures 1G and 5G and Supplemental Figures S1, F and G, and S2, C–E.

T1 transitions were quantified by manually tracking the reorientation of cell–cell contacts along the dorsoventral axis to the anteroposterior axis using Gap34::CHFP (membrane) or Utr::GFP (F-actin) to visualize the cell outlines. For ctrl-shRNA and abl-shRNA, 13 and 17 embryos were analyzed, respectively. For ctrl and abl mutants, three and five embryos were analyzed, respectively. For abl-shRNA plus buffer-only injection and abl-shRNA plus ena-dsRNA injection, six embryos per condition were analyzed. For wild-type plus buffer-only injection and wild-type plus β-cat dsRNA injection, four embryos were analyzed. Number of T1 transitions during tissue folding is presented in Figures 1H and 5H and Supplemental Figures S1G and Figure S2D.

Cell crowding was assessed by manually counting the number of cells within a 35 μm x 35 μm area just before onset of apical MyoII. Only embryos with normal cellularization after loss of abl were counted. Three regions within one embryo were counted and averaged together; for ctrl-shRNA and abl-shRNA, six embryos were assessed.

Dynamic Par-3, F-actin, MyoII, and Rho kinase in the basolateral domain were identified by imaging embryos expressing indicated UAS-shRNA and GFP::Par-3 (Par-3), Utr::GFP (F-actin), Sqh::GFP (MyoII), or ubi-ROCK::GFP (ROCK) ~21 μm below the apical surface. For ctrl-shRNA, at least five embryos were analyzed per fluorescent marker. For abl-shRNA, six Par-3–, eight F-actin–, nine MyoII–, and six ROCK-expressing embryos were analyzed. For ctrl and abl mutants expressing ur::GFP, three and five embryos were analyzed, respectively. Representative images are presented in Figures 2, A–D (Par-3), and 3, A, B, and I, Supplemental Figure S1, C and D (F-actin),
were measured in ventral cells. The coefficient of variance (SD/mean) of basolateral Ena intensity in fixed cells is presented in Figure 4F. For abl-shRNA, at least five embryos were analyzed per fluorescent marker. For abl-shRNA, six and nine embryos were analyzed for Par-3 and MyoII, respectively. For abl-shRNA plus buffer-only injection and abl-shRNA plus ena-dsRNA injection, five embryos per condition were analyzed. For wild-type plus buffer-only injection and wild-type plus β-cat dsRNA injection, four embryos per condition were analyzed. The ratio of MyoII intensity in the ventral versus the nonventral region is presented in Figures 3J and SD and Supplemental Figure S2C. The ratio of Par-3 expression in the ventral versus the nonventral region is presented in Figure 2E. To compare embryos at the same developmental timing, embryos with invagination occurring at the poles were compared.

Basolateral Ecad variability. Embryos fixed and stained for E-cad were quantified using Fiji by measuring E-cad or Par-3 intensity along basolateral junctions. For ctl-shRNA and abl-shRNA, at least five embryos were analyzed, and the junctions of at least 30 cells were measured in ventral cells. The coefficient of variance (SD/mean) of basolateral E-cad intensity is presented in Figure 2, E and H.

Junctional-to-miodiapical E-cad intensity. Fixed embryos stained for E-cad were quantified using Fiji by measuring E-cad expression intensity at the junctions and in the medioapical domain. For ctl-shRNA and abl-shRNA, at least six embryos were analyzed, and 50 junctional versus medioapical comparisons were made. The ratio of E-cad intensity in fixed cells is presented in Figure 3J.

Live embryos expressing E-cad(KI)::GFP (E-Cad) were analyzed with Fiji by generating kymographs of maximum intensity projections of the top eight slices (~8 μm below the apical surface) and measuring the intensity of E-cad at and between junctions (diffusely localized) at a 85-μm line scan at three locations within each kymograph. For ctl-shRNA and abl-shRNA, three embryos were analyzed, and 90 junctional versus medioapical comparisons were made. For abl-shRNA plus buffer-only injection and abl-shRNA plus ena-dsRNA injection, at least four embryos were analyzed, and 90 junctional versus medioapical comparisons were made per condition. The ratio between junctional and diffuse E-cad intensity in live cells is shown in Figures 3L and 5F.

Ena localization quantification

Junctional-to-miodiapical Ena. Fixed embryos stained for Ena were quantified using Fiji by measuring Ena intensity at the junctions and in the medioapical domain. For ctl-shRNA and abl-shRNA, at least seven embryos were analyzed, and 50 junctional versus medioapical comparisons were made in both ventral and lateral cells. The ratio of Ena intensity in fixed cells is presented in Figure 4E.

Basolateral Ena intensity. Fixed embryos stained for Ena were quantified using Fiji by measuring Ena intensity along basolateral junctions. For ctl-shRNA and abl-shRNA, at least five embryos were analyzed, and the junctions of at least 30 cells were measured in both ventral and lateral cells. The coefficient of variance (SD/mean) of basolateral Ena intensity in fixed cells is presented in Figure 4F.

ACKNOWLEDGMENTS

We thank the Bloomington Drosophila Stock Center (Bloomington, IN) for fly stocks and antibodies, as well as Thomas Lecuit, Johanns Bellaiche, Mark Peifer, and Eric Wieschaus for generously providing stocks used in this study. We also thank Matthew R. Broadus, Frank Gertler, Terry Orr-Weaver, and the Martin lab for helpful comments and discussion of the manuscript. This work was supported by National Institute of General Medical Sciences Grants F32GM113425 to J.N.J. and R01GM105984 to A.C.M.

REFERENCES

Bardet P-L, Guirao B, Paolleti C, Serman F, Léopold V, Bosveld F, Goya Y, Minouse V, Graner F, Bellaiche Y (2013). PTEN controls junction lengthening and stability during cell rearrangement in epithelial tissue. Dev Cell 25, 534–546.

Bear JE, Gertler FB (2009). Ena/VASP: towards resolving a pointed controversy at the barbed end. J Cell Sci 122, 1947–1953.

Benton R, St Johnston D (2003). A conserved oligomerization domain in Benten R, St Johnston D (2003). A conserved oligomerization domain in Drosophila Bazooka/Par-3 is important for apical localization and epithelial polarity. Curr Biol 13, 1330–1334.

Bentet C, Sulak L, Lecuit T (2004). Myosin-dependent junction remodeling controls planar cell intercalation and axis elongation. Nature 429, 667–671.

Chou TB, Perrimon N (1992). Use of a yeast site-specific recombinase to produce female germline chimeras in Drosophila. Genetics 131, 633–644.

Clark IBN, Muha V, Klingensmith A, Leptin M, Müller H-AJ (2011). Fibroblast growth factor signaling controls successive cell behaviours during mesoderm layer formation in Drosophila. Development 138, 2705–2715.

Courtemanche N, Gifford SM, Simpson MA, Pollard TD, Koleske AJ (2015). Abi2/Abi-related gene stabilizes actin filaments, stimulates actin branching by actin-related protein 2/3 complex, and promotes actin filament severing by cofilin. J Biol Chem 290, 4038–4046.

Dudek SM, Chiang ET, Camp SM, Guo Y, Zhao J, Brown ME, Singleton PA, Wang L, Desai A, Arce FT, et al. (2010). Abl tyrosine kinase phosphorylates nonmuscle Myosin light chain kinase to regulate endothelial barrier function. Mol. Biol Cell 21, 4042–4056.

Fox DT, Peifer M (2007). Abl2/Abi-related gene stabilizes actin filaments, stimulates actin branching by actin-related protein 2/3 complex, and promotes actin filament severing by cofilin. J Biol Chem 290, 4038–4046.

Franke JD, Montague RA, Kiehart DP (2005). Nonmuscle myosin II generates forces that transmit tension and drive contraction in multiple tissues during dorsal closure. Curr Biol 15, 2208–2221.

Friedl P, Wolf K (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer 3, 362–374.

Furman C, Seminski AL, Kwiatkowski AV, Rubinson DA, Vasile E, Bronson RT, Fassler R, Gertler FB (2007). Ena/VASP is required for endothelial barrier function in vivo. J Cell Biol 179, 761–775.

Hansen SD, Mullins RD (2010). VASP is a processive actin polymerase that regulates monomeric actin for barbed end organization. J Cell Biol 191, 571–584.

Harris TJ, Peifer M (2004). Abl regulates junctional and independent steps in the establishment of epithelial cell polarity in Drosophila. J Cell Biol 167, 135–147.

Harris TJ, Peifer M (2005). The positioning and segregation of apical cues during epithelial polarity establishment in Drosophila. J Cell Biol 170, 813–823.

Hayes KE, Walk EL, Ammer AG, Kelley LC, Martin KH, Weed SA (2013). Abl2/Abi-related gene stabilizes actin filaments, stimulates actin branching by actin-related protein 2/3 complex, and promotes actin filament severing by cofilin. J Biol Chem 290, 4038–4046.

Henkemeyer M, West SR, Gertler FB, Hoffmann FM (1990). A novel tyrosine kinase-independent function of Drosophila abl correlates with proper subcellular localization. Cell 63, 949–960.

Huang J, Zhou W, Dong W, Watson AM, Hong Y (2009). Directed, efficient, and versatile modifications of the Drosophila genome by genomic engineering. Proc Natl Acad Sci USA 106, 8284–8289.

Jodoin JN, Coravos JS, Chanet S, Vasquez CG, Tworoger M, Kingston ER, Perkins LA, Perrimon N, Martin AC (2015). Stable force balance between epithelial cells arises from F-actin turnover. Dev Cell 35, 685–697.
Philippar U, Roussos ET, Oser M, Yamaguchi H, Kim HD, Giampieri S, Wang Y, Goswami S, Wyckoff JB, Lauffenburger DA, et al. (2008). A Mena invasion isoform potentiates EGF-induced carcinoma cell invasion and metastasis. Dev Cell 15, 813–828.

Rauzi M, Hočevar Brezaviček A, Zihel P, Leptin M (2013). Physical models of mesoderm invagination in Drosophila embryo. Biophys J 105, 3–10.

Shamir ER, Pappalardo E, Jorgens DM, Coutinho K, Tsai W-T, Aziz K, Auer M, Tran PT, Bader JS, Ewald AJ (2014). Twist1-induced dissemination preserves epithelial identity and requires E-cadherin. J Cell Biol 204, 839–856.

Slattum GM, Rosenblatt J (2014). Tumour cell invasion: an emerging role for basal epithelial cell extrusion. Nat Rev Cancer 14, 495–501.

Tamada M, Farrell DL, Zallen JA (2012). Abl regulates planar polarized junctional dynamics through β-catenin tyrosine phosphorylation. Dev Cell 22, 309–319.

Vesuna F, van Diest P, Chen JH, Raman V (2008). Twist is a transcriptional repressor of E-cadherin gene expression in breast cancer. Biochem Biophys Res Commun 367, 235–241.

Wang C-C, Jamal L, Janes KA (2012). Normal morphogenesis of epithelial tissues and progression of epithelial tumors. Wiley Interdiscip Rev Syst Biol Med 4, 235–241.

Wu SK, Gomez GA, Michael M, Verma S, Cox HL, Lefevre JG, Parton RG, Hamilton NA, Neufeld Z, Yap AS (2014). Cortical F-actin stabilization generates apical-lateral patterns of junctional contractility that integrate cells into epithelia. Nat Cell Biol 16, 167–178.

Wu SK, Lagendijk AK, Hogan BM, Gomez GA, Yap AS (2015). Active contractility at E-cadherin junctions and its implications for cell extrusion in cancer. Cell Cycle 14, 315–322.

Xie S, Martin AC (2015). Intracellular signalling and intercellular coupling coordinate heterogeneous contractile events to facilitate tissue folding. Nat Commun 6, 7161.

Yamada KM, Cukierman E (2007). Modeling tissue morphogenesis and cancer in 3D. Cell 130, 601–610.

Zallen JA, Wieschaus E (2004). Patterned gene expression directs bipolar planar polarity in Drosophila. Dev Cell 6, 343–355.