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Mind bomb 2 stabilizes E-cadherin and the actin cytoskeleton to promote epithelial organization and cell migration in Drosophila

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Abstract:	Cell migration is essential in animal development and co-opted during metastasis and inflammatory diseases. Some cells migrate collectively which requires them to balance epithelial characteristics such as stable cell-cell adhesions with features of motility like rapid turnover of adhesions and dynamic cytoskeletal structures. How this is regulated is unclear but important to study. While investigating Drosophila oogenesis, we found that the putative E3 ubiquitin ligase, Mind bomb 2 (Mib2), is required to promote epithelial stability and the collective cell migration of border cells. mib2 mutant follicle cells have drastically reduced E-cadherin-based adhesion complexes and diminished actin filaments. Through biochemical analysis, we identified components of Mib2 complexes, which include E-cadherin and α- and β-catenins, as well as actin regulators. We also found that three Mib2 interacting proteins, RhoGAP19D, Supervillin, and Modulo, affect border cell migration. We conclude that Mib2 acts to stabilize E-cadherin-based adhesion complexes and promote a robust actin cytoskeletal network, which is important both for epithelial integrity and collective cell migration. Since Mib2 is well conserved, it may have similar functional significance in other organisms.
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Cover Letter

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Dear *Developmental Biology* Editors,

I write regarding the online submission of an original research manuscript that I believe is appropriate to be considered for publication in *Developmental Biology*. The work is a research article entitled **"Mind bomb 2 stabilizes E-cadherin-based adhesions and the actin cytoskeleton to promote epithelial organization and cell migration in Drosophila,"** and the authors are Sunny Trivedi, Mallika Bhattacharya and Michelle Starz-Gaiano (Corresponding author: starz@umbc.edu; 410-455-2217). We believe this original research study is well suited for the *Developmental Biology* for the following reasons:

It makes advances in understanding the dynamic regulation of collective cell migration: Tight regulation of stable tissue structure and a switch to cell motility is critical for animal development and immune function and is implicated in cancer metastasis. To unravel the regulation of this process, we investigated a well-characterized set of follicle cells in the Drosophila ovary that switch from an epithelial to motile cell type, called the border cells. We identified Mind bomb 2 (Mib 2) as a novel regulator that promotes epithelial character but is also required for collective cell migration. Mib 2 is known to function to prevent deterioration of muscle in Drosophila larva, and its orthologs have important roles regulation of Notch signaling in fish and mouse. RNAi-mediated depletion of *mib2* in follicle cells results in a cell migration failure, and null mutant clones of *mib2* in follicle cell leads to poor adhesion and cytoskeletal organization in the epithelium. These mutant phenotypes are distinct from those caused by disruptions in the Notch pathway. Notably, we found that Mib2 genetically and physically interacts with E-cadherin. Mass spectroscopy and other biochemical analysis indicates that Mib2 stabilizes E-cadherin-based adhesion complexes and the actin cytoskeleton. We additionally found novel roles for other interacting proteins - RhoGAP19D, Supervillin, and Modulo - in border cell migration. Our study suggests that Mib2 has a specific, required role in promoting adhesion complex stability and a robust actin cytoskeletal network.

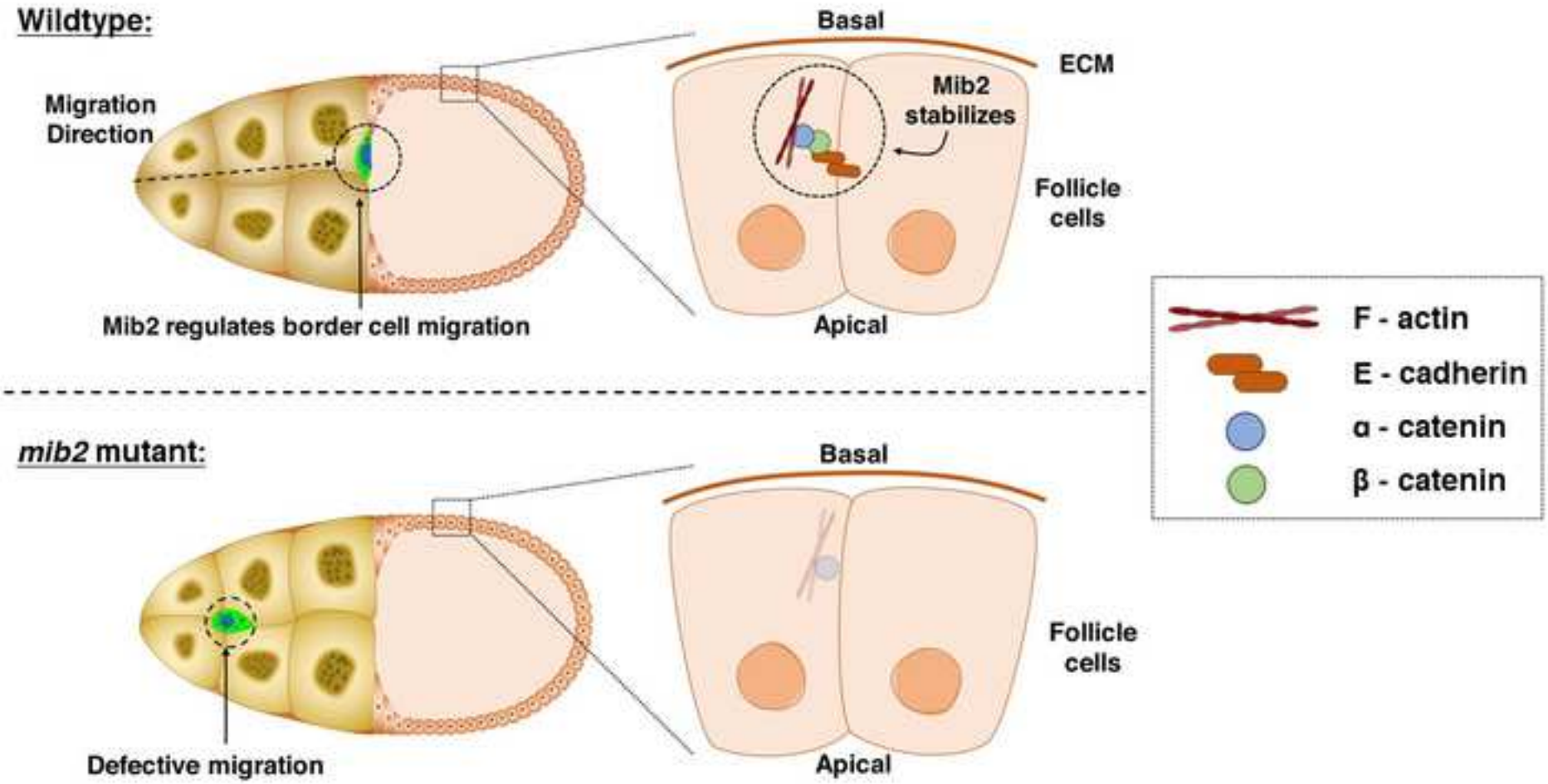
It is of broad interest to the scientific community: Mib 2 is highly conserved and its human homolog, Skeletropin, has been implicated in metastatic melanoma. In this paper, we utilized a well-characterized model system to elucidate how Drosophila Mib 2 functions as a positive regulator of adhesion and cytoskeletal stability. Since these types of cell junctions and cytoskeletal regulators are highly conserved, this work may shed light on E-cadherin regulation and epithelial morphogenesis more broadly. This idea is pertinent in many situations where epithelium must form stable tissues, as well as when cells undergo epithelial to mesenchymal transitions. Thus, we believe this study provides new insights that would be of interest to *Developmental Biology* readers, particularly those with interests in epithelial morphogenesis, collective cell migration, developmental biology, and immunology.

Lastly, as long term members of the Society of Developmental Biology, we have long supported the Society's Journal. We hope you agree that our work is suitable to be reviewed for publication in *Developmental Biology,* and we look forward to hearing from you.
Thank you,

Michelle Starz-Gaiano, PhD

- *Drosophila* Mind bomb 2, an E3 ligase, is highly expressed in ovarian follicle cells
- *mind bomb 2* is required for border cell migration and epithelial character
- Mind bomb 2 stabilizes adhesion complexes and the actin cytoskeleton
- Other Mind bomb 2 interactors, RhoGAP19D, Modulo, and Supervillin, act in migration

Graphical Abstract



Mind bomb 2 stabilizes E-cadherin and the actin cytoskeleton to promote epithelial organization and cell migration in Drosophila

Running title: Mib2 regulates cytoskeleton

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Keywords: Drosophila oogenesis, Mind bomb 2, collective cell migration, cytoskeletal dynamics, epithelial organization

Abstract

Cell migration is essential in animal development and co-opted during metastasis and inflammatory diseases. Some cells migrate collectively which requires them to balance epithelial characteristics such as stable cell-cell adhesions with features of motility like rapid turnover of adhesions and dynamic cytoskeletal structures. How this is regulated is unclear but important to study. While investigating *Drosophila* oogenesis, we found that the putative E3 ubiquitin ligase, Mind bomb 2 (Mib2), is required to promote epithelial stability and the collective cell migration of border cells. *mib2* mutant follicle cells have drastically reduced E-cadherin-based adhesion complexes and diminished actin filaments. Through biochemical analysis, we identified components of Mib2 complexes, which include E-cadherin and α- and β-catenins, as well as actin regulators. We also found that three Mib2 interacting proteins, RhoGAP19D, Supervillin, and Modulo, affect border cell migration. We conclude that Mib2 acts to stabilize E-cadherin-based adhesion complexes and promote a robust actin cytoskeletal network, which is important both for epithelial integrity and collective cell migration. Since Mib2 is well conserved, it may have similar functional significance in other organisms.

Introduction

Collective cell migration is a dynamic phenomenon required in animals during embryonic development and adulthood. The unchecked regulation of this normally well-orchestrated cell movement can result in pathological disorders such as metastasis, poor wound healing, and birth defects (Friedl et al., 2012; Friedl & Gilmour, 2009; Mehlen & Puisieux, 2006; Theveneau & Mayor, 2011). Fundamental aspects of cell migration are governed by cytokine and growth factor pathways that promote directed cytoskeletal and adhesive changes. Less is known, however, about how these changes are coordinated in groups of migrating cells since such groups often retain epithelial character (Seetharaman & Etienne-Manneville, 2020). Thus, further investigation is essential to gain insight into the key molecular signals controlling collective cell migration and to identify therapeutic targets when aberrant migration occurs.

Drosophila egg development serves as an excellent system to study epithelial organization and collective cell migration owing to tractable genetic tools, well-understood signaling mechanisms, and conserved, but relatively low genomic complexity (Chen et al., 2014; Hudson & Cooley, 2014; L. A. Manning et al., 2015; Prasad et al., 2007). The fly ovary contains ovariole chains made up of a series of developing egg chambers from stages 1 to 14 (King, 1970). Egg chambers consist of 15 nurse cells and an oocyte, surrounded by somatic, follicular epithelial cells (Fig. 1A). At mid-oogenesis, high levels of Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signaling in a small set of anterior follicle cells induce them to become the motile border cells (Beccari et al., 2002; Ghiglione et al., 2002; Silver & Montell, 2001). Along with two polar cells, the border cells detach from the epithelium and the group collectively migrates between nurse cells towards the oocyte at the posterior side. This process requires continuous STAT signaling and dynamic regulation of cytoskeletal and adhesion proteins including Actin, Myosin, E-cadherin, and β-catenin (Montell et al., 2012; Silver et al., 2005). Interestingly, this regulation must be controlled inter-and sub-cellularly to enable cluster cohesion while promoting movement, and how this occurs is not entirely clear.

Previous RNAi-based genetic screens showed that Mind bomb 2 (Mib2) can negatively regulate STAT activity (Müller et al., 2008) and may impact border cell migration (Saadin & Starz-Gaiano, 2016), making it a candidate regulator of collective cell movement. Mib2 is a putative E3 ubiquitin ligase evolutionarily conserved between *Drosophila,* vertebrates, and mammals, and it can bind select target proteins to drive their ubiquitination *in vitro* (Koo et al., 2005). Although polyubiquitination is best known for promoting protein turnover, the covalent addition of mono-ubiquitin can also stabilize proteins or alter their localization or activity (Ahearn et al., 2011; D. Chen et al., 2010; Hicke, 2001; Sewduth et al., 2020). Mib2 has five distinct conserved domains that are likely involved in protein-binding, including MIB-specific domains and RING domains (Domsch et al., 2017; Koo et al., 2005). *In vitro* ubiquitination assays with frog and mouse Mib2 suggest that the RING domains possess E3 ligase activity and, like Mind bomb, promote Notch ligand activation (Koo et al., 2005; Takeuchi et al., 2003). Mouse Mib2 physically interacts with α-Actin in skeletal muscles (Takeuchi et al., 2003). In the late

stages of fly embryogenesis, *mib2* mutants undergo major muscle deterioration independent of Notch signaling, suggesting a yet unidentified mechanism to regulate muscle integrity (Carrasco-Rando & Ruiz-Gómez, 2008; Nguyen et al., 2007). While fly Mib2 has been shown to bind directly to the non-muscle myosin component Zipper (Zip) (Carrasco-Rando & Ruiz-Gómez, 2008), its direct involvement in ubiquitination has not been shown, and a role in cell migration has not been characterized. Thus, the exact mode of action for fly Mib2 in ovarian follicle cells remains unclear.

Our current study indicates that Mib2 regulates cell migration and epithelial cell structure in *Drosophila* egg chambers by directly impacting adhesion complexes and actin organization. We found that Mib2 is highly expressed in follicle cells and is required for efficient border cell migration. Follicle cells mutant for *mib2* showed a dramatic reduction in E-cadherin-based adhesion components and cortical actin, and exhibit shortened epithelial cell heights. These *mib2* mutant phenotypes are distinct from Notch-related defects. We identified multiple cytoskeletal and adhesion proteins that physically and genetically interact with Mib2, including E-cadherin and regulators of actin organization. Our data suggest that Mib2 has a pleiotropic effect in the dynamic regulation of certain cytoskeletal components during Drosophila oogenesis. Thus, we hypothesize that the key role of Mib2 in this context is to bind to and promote the stability of E-cadherin-based adhesion complexes and promote certain types of actin filament organization.

Materials and Methods

Fly stocks and husbandry: All fly lines and crosses were kept at 25^{0}C. For fattening, the flies were fed dry yeast overnight at 29^{0}C for optimal UAS-GAL4 expression and high yields of stage 9-10 egg chambers. The next day the female flies were dissected and ovarioles were stained to observe stage 10 egg chambers for the extent of border cell migration and protein expression. In our study, we defined defective migration if the cluster is lagging by one cluster length in stage 9 or 10. For protein expression studies we performed intensity analysis using DAPI as our reference. See extended materials for genotypes.

Mosaic Clones: We used the flp-FRT system (Ito et al., 1997) to generate negatively marked mutant clones in follicle cells. To induce negatively marked mutant clones we crossed hsFLP flies (BDSC 6) with the *ubi-GFP* flies (BDSC 5629) followed by crossing their F1 males with *mib2^{1},FRT40A/CyO* virgins. The F2 flies were given heat shock at 37^{0}C twice a day for three days. Flies were kept at 25^{0}C for two days to get the maximum number of stage 10 mutant egg chambers. The flies were incubated at 29^{0}C overnight with yeast. The female flies with straight wings were dissected to extract and immunostain the ovaries.

Immunohistochemistry and microscopy: Ovarioles were extracted from fattened flies into dissection media (1x Schneider's Drosophila medium by Thermofisher Scientific (21720-001), 10% FBS, 0.6% Pen/Strep) and fixed with 4% paraformaldehyde (0.1M KPO4 buffer) for 10 mins at RT. Fixed ovarioles were rinsed and washed 3 times with NP40 buffer (0.05 M Tris HCl, pH 7.4, 0.15 M NaCl, 0.5% Nonidet P-40 (Igepal CA-630, Sigma-Aldrich), 1 mg/ml BSA) (McDonald et al. 2006). For staining, ovarioles were incubated with the respective primary antibody in NP40 buffer overnight at 4^{0}C. See extended materials for antibodies and concentrations. Ovarioles were rinsed and washed 3 times with NP40 buffer then incubated with Alexa Fluor secondary antibodies in NP40 buffer overnight at 4^{0}C followed by DAPI staining for 10 mins. Ovarioles were rinsed and washed 3 times with NP40 buffer and incubated then mounted in 70% Glycerol. Samples were observed using either the Zeiss LSM 900 confocal with Airyscan 2 or Carl Zeiss AxioImager Z1 and captured using the AxioVision or Zen acquisition system. For comparing staining intensities, antibody stainings were done at the same time, and images were captured at identical acquisition settings. Post image processing was performed using ImageJ and Adobe Illustrator.

Live imaging and analysis:

Live imaging was performed according to (Prasad et al., 2007) using a Zeiss AxioImager microscope with Zen Blue 3.0 by Zeiss. In short, flies of the appropriate genotype (*w^{1118}* or *mib2^{1}/CyO*) were fattened for about 16 hours at 29°C prior to dissection in insulin-

supplemented media. Egg chambers were then transferred to media containing a lipophilic dye, FM4-464 at a working concentration of 5μg/ml (ThermoFisher Scientific T13320) in 10mg/ml insulin (Applications.inc 128-100 to be diluted in acidified water). Single z-plane images (fluoresent and DIC) were acquired at 20X every three minutes. For velocity measurements, images were analyzed in FIJI/ImageJ. Migration distances were measured from the anterior of the egg chamber to the center of the border cell cluster in the first and last frames of the video. These distances were divided by the duration of the video to calculate velocity. Videos were exported at 5fps using FIJI/ImageJ

Cell height and intensity analysis:

We quantified cell height by measuring the distance between the apical and basal surfaces of the follicle cells. We analyzed *mib2*^{1} mutant clonal egg chambers and focused on the clone patches in the follicular epithelium present over the oocyte. For our study in each egg chamber, we selected 8 cells (4 mutant cells flanked by 2 wildtype cells on each side) and determined the average cell height and standard error of the mean (S.E.M). For both cell height and the F-actin intensity study, we used the Zen Blue 3.0 software by Zeiss to measure the fluorescent intensity of phalloidin 568 (TFS A12380, 1:500). We measure the intensity along a line that spans the cytoplasm of 3 cells including 2 lateral cell surfaces. In each egg chamber, the intensity was measured in the clone patch and also in wild-type cells on both sides of the clone patch. Intensities were measured in a.u. and quantified by averaging them.

Quantitative real-time PCR analysis: The HSP70-GAL4 virgin female flies were crossed with respective UAS RNAi lines at 25^{0}C. The F1 flies were heat-shocked at 37^{0}C 3 times a day for 3 days. Additionally, for other experiments, c306-GAL4 virgin female flies were crossed with respective UAS RNAi or overexpression lines at 25^{0}C. The mCherry RNAi lines were used as the control for all qRT-PCR experiments. All F1 progeny were fattened at 29^{0}C overnight and ~20 pairs of ovaries were dissected. The total RNA was extracted using the Qiagen RNeasy mini kit with DNase I digestion. Using BioRad iScript, ~1µg/µl cDNA was synthesized from total RNA. qRT-PCR reactions (3 biological and 3 technical

replicates for each sample) were set up with 1µg/µl cDNA, 2ul primer mix (10µM), and 10 µl iTaq Universal SYBR Green Supermix (BioRad) in a 20 *µ*l reaction in BioRad CFX96 qPCR instrument. Primers were designed according to the fly primer bank (Hu et al., 2013) (http://www.flyrnai.org/flyprimerbank). The statistical significance analysis and graphs were performed in Graphpad Prism.

Immunoprecipitation, Western blot, and tims TOM mass spectrometry:

Fattened flies were dissected on ice in IP buffer (PBS pH7.6, Halt protease and phosphatase inhibitor (78442, Thermofisher), 1mM PMSF, 1mM DTT). For each sample ~200 pairs of ovaries were dissected and snap-frozen in liquid nitrogen and stored at -80^{o}C. Frozen ovaries were homogenized using 400 µl lysis buffer (50mM tris-HCL pH 7.5, 100mM NaCl, 0.2% Np40, 5% glycerol, 1.5mM MgCl2, 1mM EDTA, 1mM EGTA, added fresh: 1mM DTT, Halt protease and phosphatase inhibitor (78442, Thermofisher), 1mM PMSF, 0.05mM Mg132) and incubated on ice for 20 mins. Samples were centrifuged at 16,000 RPM at 4^{o}C for 15 mins. The supernatant was incubated with pre-washed magnetic A/G beads (88803, Thermofisher) and respective primary antibodies at 4^{o}C overnight. The beads were washed three times with lysis buffer on ice. For mass spectrometry analysis, we immunoprecipitated the CantonS flies, FLAG-tagged ImpL2-expressing flies, and FLAG-GFP tagged Mib2 expressing flies using FLAG M2 antibody (Millipore Sigma, F1804). The immunoprecipitated protein complexes bound onto the beads were digested using a trypsin digestion kit (89895, Thermofisher). The samples were treated with a detergent kit (88305, Thermofisher) to remove any salt from the samples. The purified samples were then submitted to the MCAC core facility at UMBC for separation on the Bruker NanoElute HPLC, followed by dual Trapped Ion Mobility Spectrometry (TIMS) on the Bruker timsTOF pro-mass spectrometer to identify peptide abundance. Raw data was analyzed with PEAKs Studio to identify peptide abundance. Among the extensive list of proteins that bound to Mib2, we excluded the proteins that had peptide count lower than 5, ribosomal, chromatin related, uncharacterized proteins, and additional isoforms. We included proteins that were exclusively in the Mib2 IP or that had higher peptide counts in Mib2 IP compared to the controls. The relative abundance

is achieved by the following formula, standardize [Peptide count, average (peptide count of sample and both controls), standard deviation (peptide count of sample and both controls)]. The experiment was repeated twice, and the trends reported are representative of both experiments. Additionally, for Western blot analysis, the immunoprecipitated beads were boiled at 100^{0}C for 15 mins with 4X SDS loading dye. Samples were loaded onto 4-20% polyacrylamide pre-cast gels by BioRad for SDS gel electrophoresis. Next, proteins were transferred from the gel onto the PVDF membrane by Western blot overnight at 4^{0}C. The membranes were blocked with 3% BSA in TBST and incubated with primary antibody solutions made in TBST overnight at 4^{0}C. Membranes were washed three times in TBST for 5 mins and incubated with HRP conjugated secondary antibodies for 2 hrs at RT. Membranes were once again washed in TBST three times for 5 mins and developed using ECL developing solution (Biorad). Blots were imaged using a Biorad Chemidoc XRS+ imaging station, and further processed, cropped, and oriented in Adobe Illustrator.

Results

Mib2 is expressed throughout oogenesis and cytoplasmically enriched in follicle cells

To understand the potential roles of Mib2, we first examined its protein expression in egg chambers. Immunofluorescence staining using an antibody directed against Mib2 (M Carrasco-Rando et al., 2008) revealed expression throughout oogenesis (Fig. S1) and at stage 8, it is especially prominent within the cytoplasm of follicular epithelial cells (Fig. 1B-B’’’) and the oocyte. At stage 9 when the border cell cluster has been specified and detaches from the anterior epithelium, Mib2 expression is maintained at high levels in both polar cells and border cells (Fig. 1C-E’’’) and is enriched at the oocyte periphery. During the completion of the migration phase, stage 10, the border cell cluster retains Mib2 expression albeit at slightly reduced levels. The expression profile was confirmed using flies expressing a genetically tagged Mib2 (Sarov et al., 2016)(Fig. S2). Based on this expression pattern, we hypothesized that Mib2 functions in border cell migration and the maintenance of the follicular epithelium in ovaries.

mib2 is required for border cell migration

To test if Mib2 regulates border cell migration, we examined *mib2* mutants and RNAi knockdowns specifically in the anterior follicle cells. As *mib2*^{1} null mutants are homozygous lethal (Nguyen et al., 2007), we first characterized the effect of null mutations in heterozygosis. We stained egg chambers with antibodies directed against β-catenin (encoded by *armadillo* (*arm*) in flies) and E-cadherin (E-cad) (encoded by *shotgun* (*shg*)), which detects the border cell clusters. In controls, the border cells migrated in alignment with the flattened domain of outer follicle cells at stage 9 and arrived at the anterior of the oocyte by stage 10, having completed migration (Fig. 2A, F). In contrast, 37% of *mib2*^{1}/+ heterozygous mutant egg chambers showed delayed migration relative to outer follicle cells at stage 9, and 27% of *mib2*^{1}/+ heterozygous mutant egg chambers showed incomplete border cell migration in stage 10 egg chambers (Fig. 2C, F). Consistent with this static analysis, live imaging of this genotype revealed a fraction of *mib2*^{1}/CyO egg chambers with slower border cell migration velocities at stage 9 than a *w*^{1118}/*w*^{1118} control (Fig. S3). Separately, we used a border cell cluster-specific Gal4 (Brand & Perrimon, 1993) to express *mib2* RNAi under UAS control (Dietzl et al., 2007; Manseau et al., 1997). In these cases, we observed delayed border cell migration in 35-40% of egg chambers at stage 9, and incomplete migration in 25–30% of stage 10 egg chambers (Fig. 2B, F). qPCR analysis confirmed that Gal4-mediated *mib2* RNAi resulted in significantly less detectable *mib2* mRNA than in controls (Fig. S2). Overexpression of *mib2* did not have any effect on migration alone (Fig. 2D), but fully rescued the migration defect in *mib2*^{1}/+ heterozygotes (Fig. 2E, F). These results demonstrate that *mib2* is required for normal border cell migration.

In addition to overexpressing the full-length protein in anterior follicle cells, we overexpressed versions of Mib2 that had deletions in different domains (Domsch et al., 2017) within a wild-type background. Mib2 has eight conserved domains: two HERC2, a ZZ zinc finger domain, two Mib domains, eight ankyrin repeats, and two RING domains. Like full-length overexpression, expression of Mib2 with a ZZ deletion or HERC deletion did not disrupt border cell migration. However, Mib2 overexpression with deletions in the

MIB, ANK, or RING domains resulted in significant migration defects (Fig. 2G and Fig. S4). This supports the idea that Mib2 interacts with different partners to exert its function in cell migration and suggests that disruption of certain domains results in dominant-negative effects.

Mib2 is required for adhesion and cytoskeletal complex maintenance and epithelial organization

Next, we used mosaic clonal analysis to investigate the loss of *mib2* function in egg chambers. We initially aimed to observe mutant border cells; however, despite examining over one thousand clones in follicle cells, we identified no mutant border cell clusters. This suggests that *mib2* is required in initial border cell specification and/or survival. Nevertheless, mutant clones in the follicular epithelium turned out to be very informative about the molecular function of Mib2. In particular, we found that loss of *mib2* drastically affects the expression of proteins known to be important in border cell migration.

Cadherin-based adhesion complexes, including E- and N-cadherin and the adaptors α- and β-catenin that link them to the actomyosin network, have required roles in follicle cell organization and structural integrity (Cai et al., 2014; Niewiadomska et al., 1999a; Pacquelet & Rørth, 2005; Peifer M, 1993; Sarpal et al., 2012; Tanentzapf et al., 2000). Additionally, these complexes must be dynamically regulated during border cell migration since E-cadherin-based adhesions between border cells and nurse cells provide the traction for movement. Compared to neighboring wild-type cells, *mib2* mutant follicle cells showed a dramatic reduction in adhesion complexes and actin. Specifically, we observed severely reduced levels of the homophilic adhesion molecule E-cad in mutant follicle cells (Fig. 3 A-A''). Consistent with the nature of homophilic adhesions, non-cell-autonomous effects were also observed, where a wild-type cell in contact with a mutant cell showed a partial reduction of E-cad at the juxtaposed surface (Fig. 3 C-C''). In several instances when the mutant clone was large (more than 20 cells), we observed that the *mib2* mutant follicle cells were disorganized or led to bending in the epithelium (Fig. 3B-B''). Notably, such phenotypes are similar to what has been observed in *shg* mutant clones in follicle

cells (Niewiadomska et al., 1999a; Pacquelet & Rørth, 2005), however, the *mib2* mutant clonal phenotypes appear to be less severe. For instance, we never observed a mispositioned oocyte, which is characteristic of E-cadherin loss (Niewiadomska et al., 1999b), and the follicle cell layer was less disorganized in *mib2* mutants, suggesting some adhesion may be preserved.

We next examined the effects of loss of *mib2* on other adhesion complex components and the cytoskeleton. The adaptor protein β-catenin (Arm) associates with cadherins and α-catenin as part of a complex that links all of them to the actin cytoskeleton. In *mib2* mutant cells, we saw a near-complete loss of apical β-catenin while lateral expression was generally lower and restricted to a much smaller region (Fig. 3D-D''). Generally, mutant cells had a reduced lateral surface as they were often shorter in the apical-basal axis. The average cell height of wild-type follicle cells near the oocyte was 15μm (+/- 1.1μm SEM) at stage 10, while the average height of *mib2* mutant cells was 9.5μm (+/- 0.8μm SEM) (Fig. S5A). Additionally, cortical F-actin was markedly reduced in mutant follicle cells (Fig. 3E-E'') and the cytoplasmic level also appeared to be lower when assayed using fluorescent phalloidin. The average F-actin intensity of *mib2* mutant cells was 37% lower than in wild-type cells (Fig. S5B). This phenotype is distinct from *E-cad* (*shg*), *α-cat*, or *arm* mutant follicle cells where F-actin is unchanged or upregulated, although these mutant cells are also often shorter and less organized (Pacquelet & Rørth, 2005; Sarpal et al., 2012; Tanentzapf et al., 2000). Among egg chambers with distinct mutant clones, the reductions in Arm, F-actin, and E-cad expression/localization are 100% penetrant ($n > 200$ each). However, another cytoskeletal protein, the adducin-like protein, Hu li tai shao (Hts) (Yue & Spradling, 1992), appeared normal in *mib2* mutant clones (Fig. 3). Notably, we did not observe any phenotypes associated with the disruption in Notch signaling (for example, egg chamber fusions or changes in stalk/polar cell fates) (Duhart et al., 2017), indicating *mib2* is unlikely to act in this pathway in follicle cells. Since both *mib2* and *shg* (Niewiadomska et al., 1999b) are required for border cell migration, we attempted a simultaneous knockdown of both by respective RNAi targeting in anterior follicle cells (using c306-Gal4). None of the F1 generation bearing both *mib2*

and *shg* downregulation survived past pupal stages, suggesting a genetic interaction between these genes, and consistent with the idea that Mib2 could promote E-cad stability. These data indicate that Mib2 is crucial in the maintenance and/or correct subcellular localization of adhesion complexes and certain cytoskeletal components.

Mib2 associates with adhesion and cytoskeletal components

To understand Mib2's role in epithelial cell regulation better, we sought to identify the proteins to which it binds. We immunoprecipitated (IP) a FLAG-tagged Mib2 under control of its endogenous promoter (Sarov et al., 2016) from ovaries and used Trapped ion mobility spectrometry Time-of-Flight Mass Spectrometry (timsTOF MS) to identify proteins in this complex. As a comparison, we separately IPed another protein enriched in follicular epithelial cells, Imaginal morphogenesis protein-Late 2 (L. Manning et al., 2017), also fused to a FLAG-tag (FLAG-Tagged ImpL2) (Sarov et al., 2016). These were also compared to wild-type ovaries as a negative control (Canton-S strain). We found 48 proteins that were preferentially bound to FLAG-tagged Mib2 compared to controls and 11 that bound exclusively to the FLAG-tagged Mib2 but not to the controls. Proteins enriched in the FLAG-Mib2 complexes are represented in a heatmap in Fig. 4A. These include actin binding or cytoskeletal regulatory proteins like Rho GTPase activating protein at 19D (RhoGAP19D), Supervillin (Svil), Quail (Qua), β-Spectrin (β-Spec), and Hts, and cell adhesion complex components α-Cat, β-catenin (Arm), E-cad, and F-Actin capping protein (Gates et al., 2009). Although we used whole ovaries in this analysis, Mib2 is enriched in follicle cells, so we believe most of the interacting proteins we found are important in follicle cells. Several of these have been shown to be highly expressed in the follicular epithelium at these stages, including RhoGAP19D (Fic et al., 2021), Hts (Lin et al., 1994), β-Spec (Tanentzapf et al., 2000), Qua (Borghese et al., 2006; Mahajan-Miklos & Cooley, 1994)), α-Cat (Pacquelet & Rørth, 2005; Sarpal et al., 2012), Arm (Pacquelet & Rørth, 2005; Peifer M, 1993), E-cad (Niewiadomska et al., 1999b), while others, such as Svil, and Terribly reduced optic lobe (Trol), have not been previously characterized in the ovary. Among these potential interacting proteins, we confirmed direct binding of Arm and E-cad with Mib2 using IPs and Western blot analysis (Fig. 4C).

Altogether our biochemical analysis suggests that Mib2 physically interacts with adhesion proteins and actin network regulators, and we propose this increases their stability.

Mib2 associated proteins identify novel border cell migration regulators

Stabilization of E-cad, α- and β-catenin complexes could explain *mib2*'s requirement in the epithelial organization and could explain its role in border cell migration, in which E-cad based adhesion complexes are known to be required (Cai et al., 2014; Niewiadomska et al., 1999b; Pacquelet & Rørth, 2005; Sarpal et al., 2012)). E-cad adhesions are strong and stable between the cells within the border cell cluster but are required more transiently between border cells and nurse cells. Consistent with this, when we knocked down *α-Catenin,* border cell migration often failed (Fig. 5B). Since adhesions are more rapidly turned over in motile cells than stationary epithelial cells, migrating cells may be very sensitive to the destabilization of these complexes from the loss of *mib2.*

To determine if any of the other Mib2-interacting proteins could also help to explain Mib2's role in border cell migration, we used the UAS-GAL4 system to manipulate the expression of several candidates. We focused on candidates related to cytoskeleton or transcriptional regulation, given that these are known to be critical in border cells (Montell et al., 2012; Seetharaman & Etienne-Manneville, 2020). Strikingly, we uncovered roles for three novel genes in border cell migration. One encodes the DNA binding protein Modulo (Mod) (Krejci et al., 1989). RNAi-mediated reduction of *mod* led to migration defects in about 23% of stage 10 egg chambers (Fig. 5C). A second new candidate regulator is RhoGAP19D, which was recently shown to regulate Rho family protein Cdc42 and suppress follicle cell invasion (Fic et al., 2021). Interestingly, RhoGAP19D overexpression in anterior follicle cells resulted in severe border cell migration defects (Fig. 5D). However, *rhogap19D* knockdown in anterior follicle cells did not lead to significant border cell migration defects. We do not know if this is because of insufficient downregulation, or if this gene may function redundantly with other small GTPase regulators. Lastly, reduced expression of *svil*, which encodes a regulator of actin filament dynamics (Gaudet et al., 2011), resulted in poor border cell migration in stage 9 egg chambers. The migration

defects for these candidates are quantified in Fig. 5E. Besides these candidates, we also examined other proteins that we found associated with Mib2 in our immunoprecipitation, namely nuclear proteins JIL-1 anchoring and stabilizing protein (Jasper) and Elys, and cytoskeletal/membrane components Kramer, WupA, and β-Spectrin. However, reduction of these proteins using existing RNAi lines did not have any obvious effect on migration of the border cell cluster, suggesting they may not be required or may function redundantly for migration (data not shown). However, these may have other roles in the follicular epithelium. Since E-cad, *α*- and β-catenin, Mod, RhoGAP19D, and Svil (Fig. 5) can disrupt normal border cell migration when they are overexpressed or knocked down, and they can physically interact with Mib2, we propose that they are all stabilized and/or regulated by Mib2 function.

Discussion

Cell migration is an important aspect of both the development of an organism and disease pathophysiology. Collective cell migration is especially interesting as individual cells must balance stable and dynamic cell adhesions to stay associated with some cells while moving past others (Friedl et al., 2012; Friedl & Gilmour, 2009; Kraemer, 2000; Mehlen & Puisieux, 2006). Hence, to promote correct developmental events and to battle against migratory disorders, a deeper understanding of the mechanisms behind cell migration is required. Here, we identified critical roles for Mib2 in both collective cell migration and the maintenance of the epithelial organization.

In Drosophila egg chambers, we found that Mib2 acts in follicle cells to regulate adhesion complexes and the actin cytoskeleton by stabilizing them. Loss of *mib2* results in a dramatic decrease of E-cad expression, reduced Arm on the lateral surface of follicle cells, and downregulation of cortical actin. Notably, *mib2* mutant clones of a smaller size do not disrupt follicle cell structure. However, in the larger clones, we observed cell extrusion and multilayering of follicular epithelium, probably owing to loss of adhesion over time. While these phenotypes resemble those of null mutant follicle cell clones for E-cadherin or catenins, the effects are less severe. Thus, we suspect that these adhesion

complex proteins are being produced but are destabilized, leading to a much faster turnover. Additionally, the dramatic reduction of actin in *mib2* clones is distinct from what occurs due to loss of adhesion complex components and may suggest a more direct interaction between Mib2 and the cytoskeleton. Since we never observed border cell mutant clones, we speculate that the loss of *mib2* prevents cluster formation. However, as E-cad based adhesion is a key mediator of border cell migration via interactions within the cluster as well as with the nurse cell-substrate over which they move (Cai et al., 2014), changes in the stability of these adhesion complexes likely explain Mib2's role in the migration process. Rapid turnover of adhesion complexes is known to occur in motile cells, so the border cells may be more sensitive to additional destabilization of adhesion compared to other follicle cells.

Zebrafish, mouse, and human Mib2 orthologs act as E3 ubiquitin ligases (Koo et al., 2005; Nguyen et al., 2007), leading to ubiquitination of several different targets, but this activity has not been directly shown in Drosophila. However, catalytic residues in the ligase domain of fly Mib2 are required for muscle development (Nguyen et al., 2007). Our genetic and physical interaction data suggest that Mib2 directly binds and stabilizes certain adhesion complex components, in particular those containing E-cad. It is possible that Mib2 acts as a scaffold component, stabilizing the complex simply by association, or that it acts through its ligase activity. While polyubiquitination leads to protein degradation, mono-ubiquitination can increase protein stability or alter subcellular localization (Sadowski & Sarcevic, 2010), so Mib2 may promote this post-translational modification on its targets. Interestingly, the defects we found due to loss of *mib2* function are different than those due to changes in Notch signaling, indicating fly Mib2 in oogenesis acts differently than its fish or frog homologs (Koo et al., 2005). More work will be needed to elucidate the roles of the different Mib2 domains involved in protein-protein interaction, and if the RING domain, in fact, confers Ubiquitin ligase activity.

While the direct activity of Mib2 remains to be determined, our biochemical analysis suggests it can interact with several important follicle cell components. These include

cytoskeletal regulatory proteins such as RhoGAP19D, Troponin1, Svil, Hts, β-Spectrin, α-Catenin, and Quail. Among these proteins, α-Catenin has been shown to be involved in border cell migration, while Quail is expressed in border cells and is thought to act redundantly with another actin regulator, Singed (Borghese et al., 2006; Omelchenko, 2012; Pacquelet & Rørth, 2005). Using RNAi analysis, we newly demonstrate that *svil* and *mod* also have a required role in border cell migration regulation. Additional cytoskeletal regulation is likely. Prior studies suggest that fly Mib2 binds and stabilizes the non-muscle myosin subunit Zipper (Carrasco-Rando & Ruiz-Gómez, 2008), and tight regulation of the actinomyosin cytoskeleton is necessary for proper border cell migration (Edwards & Kiehart, 1996; Lucas et al., 2013; Majumder et al., 2012; Montell et al., 2012). In our study, both non-muscle Myosin subunits Spaghetti-squash (Sqh) and Zipper (Zip), as well as actin, appeared in our IP-MS analysis, but these were also detected in the negative controls, and hence not included in our dataset. Interestingly, Skeletrophin can physically interact with α-actin monomers and act as a novel suppressor for invasion in myeloma cells (Takeuchi et al., 2006). Thus, it is compelling to predict that Mib2's regulation of the actinomyosin network may be a conserved function of Mib2.

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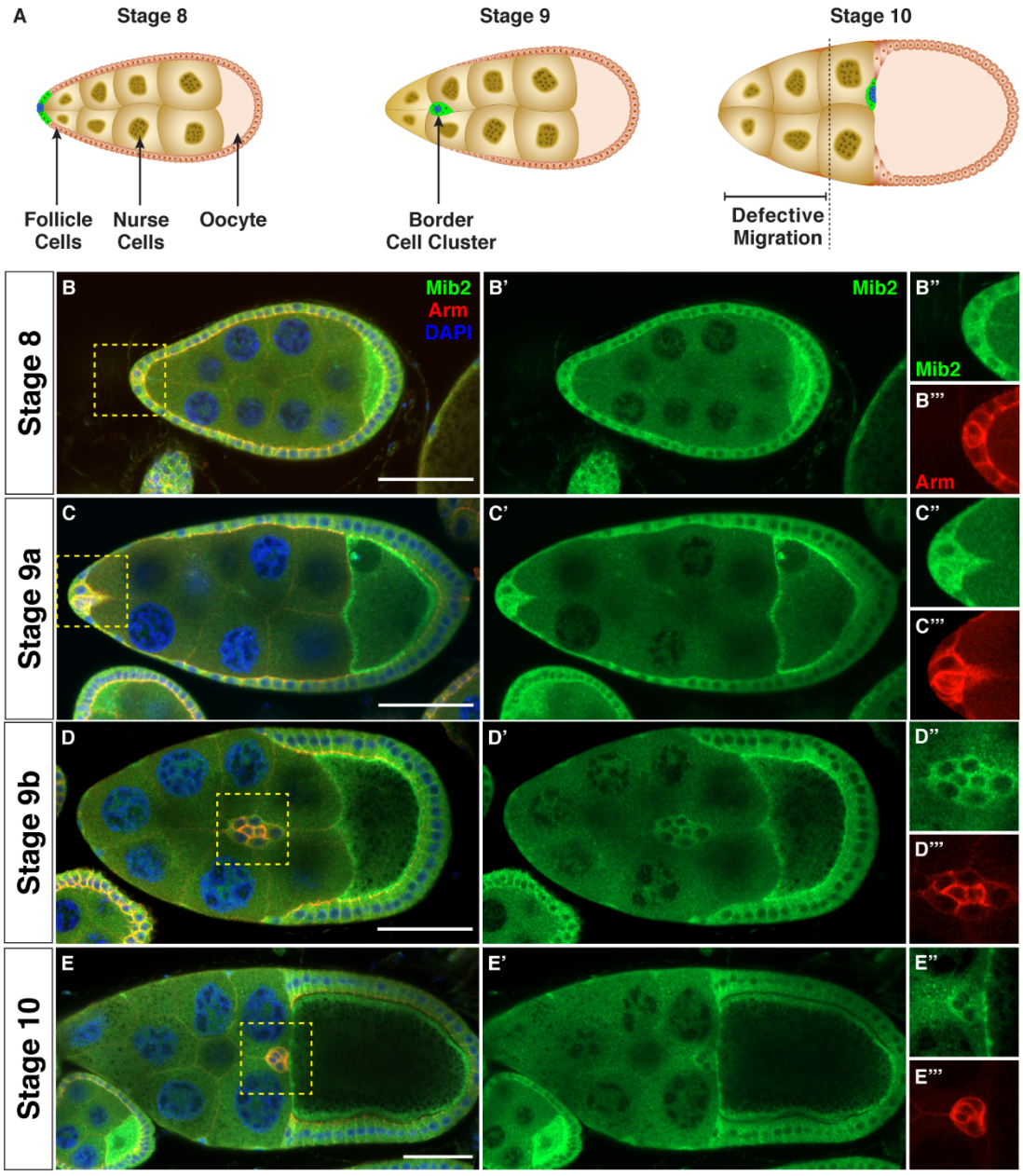

Fig. 1. Mib2 expression is enriched in the follicular epithelium of developing egg chambers. A) Schematic representation of *Drosophila* egg chambers of stages 8, 9, and 10, which is when border cells transition from epithelial cells to motile cells and migrate to the oocyte. Green cells represent border cells and blue cells represent polar cells. The follicular epithelium surrounds the germline cells. **B-E)** Immunofluorescence staining shows that Mib2 is expressed in a spatially restricted pattern at different stages of oogenesis as indicated. Mib2 protein (green) is highly localized at the cortex and in

the cytoplasm of follicle cells including border cells (boxed) and is also detected cytoplasmically in the oocyte early and later at the oocyte cortex. β catenin (Arm) expression detected by antibody staining is shown in red, and nuclei are stained with DAPI shown in blue. The insets focus on fated or migrating border cell clusters. The scale bar is 50μm.

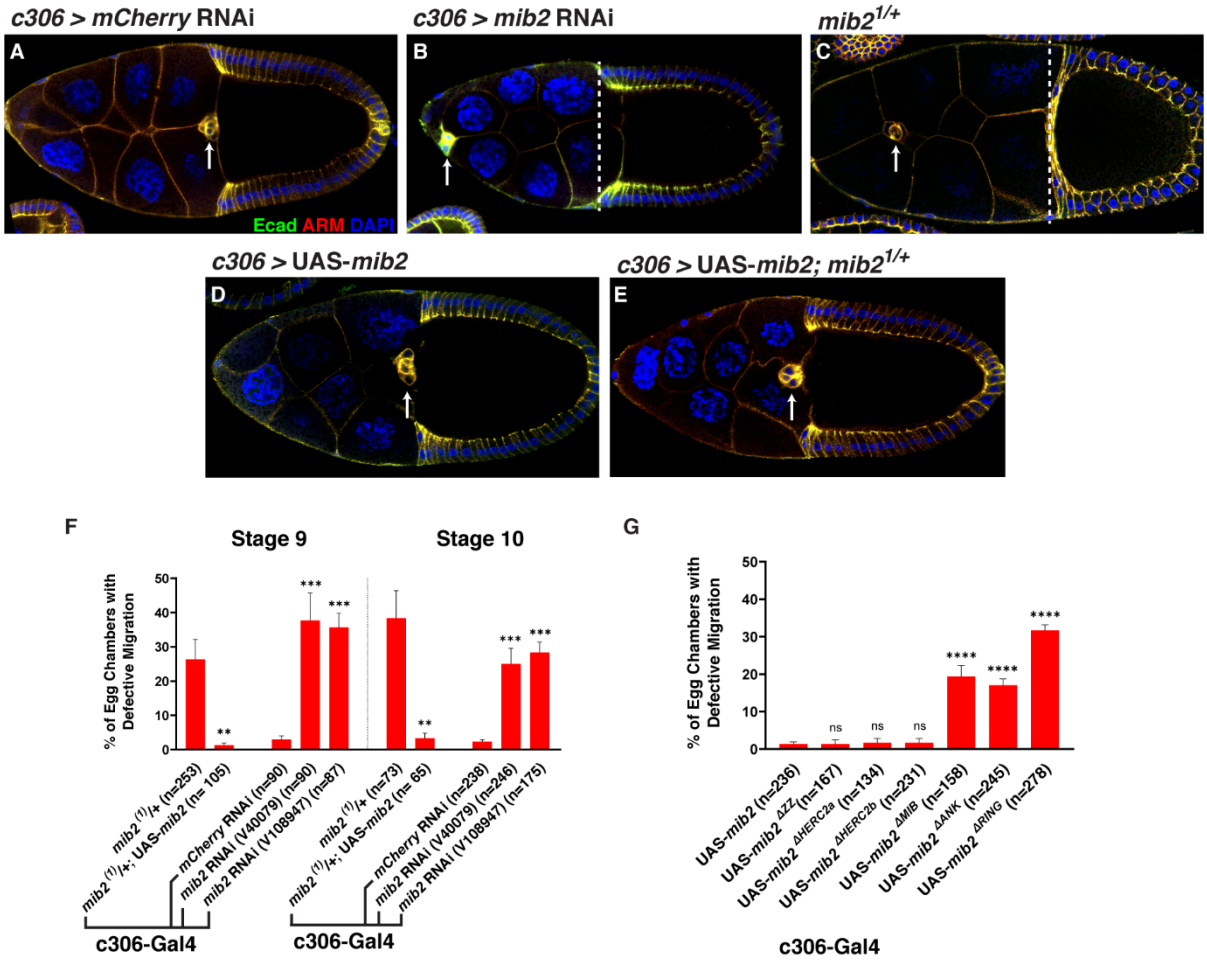

Fig. 2. Mib2 regulates border cell migration. A-E) Egg chambers immunofluorescently stained to detect Arm protein, shown in red, and E-cad, shown in green; nuclei are shown by DAPI staining in blue. **A)** The anterior follicle cell-specific Gal4 line, c306, promotes expression of *mCherry* RNAi (control), resulting in normal migration of the border cell cluster. **B)** Expression of *mib2* RNAi in anterior follicle cells results in defective border cell migration, with cells remaining close to their anterior starting point (arrow), instead of aligned with the columnar epithelium (dashed line). **C)** An egg chamber from a heterozygous null mutant, *mib2*^{1}/+, shows defective border cell migration, with cells having moved only about 25% of the normal migration distance

(arrow). **D)** Overexpression of *mib2* in anterior follicle cells with UAS-*mib2* does not disrupt border cell migration or egg chamber morphology. **E)** Overexpression of *mib2* in the *mib2*^{1}/+ mutant background shows normal cell migration, indicating rescue of the defect shown in (C). **F)** Quantification of the penetrance of defective border cell migration at stages 9 and 10 in the indicated genotypes. **G)** Quantification of the penetrance of defective border cell migration due to overexpression of different Mib2 domain deletion lines in anterior follicle cells. Statistical analysis was performed using a one-way ANOVA test and t-test, ** is p<= 0.0021, *** is p<=0.0002, and **** is p ≤ 0.0001. n= number of egg chambers analyzed.

hs-flp/+;Ub-gfp,frt40a/mib2^{1},frt40a

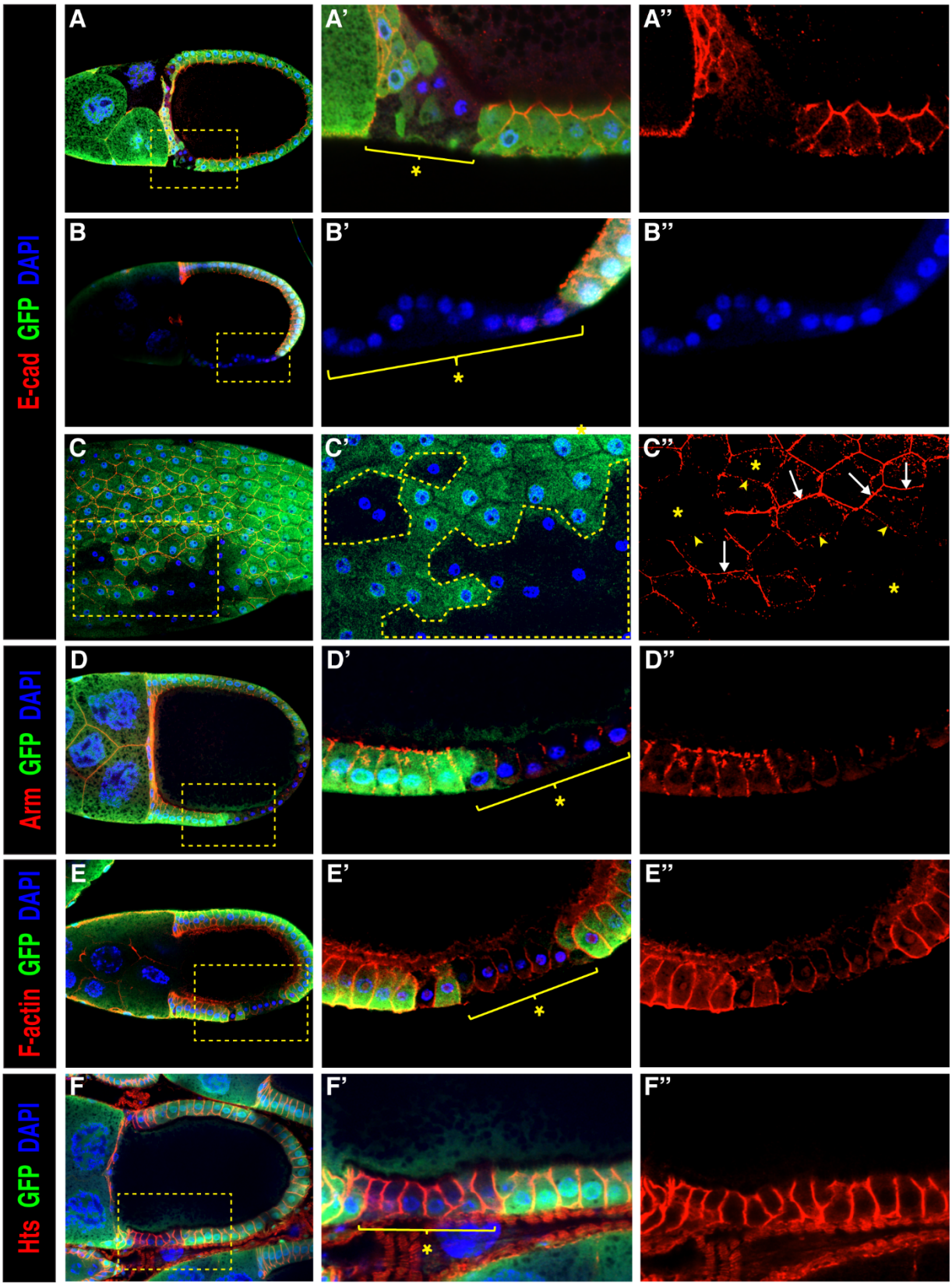

Fig. 3. *mib2* is required for stable and correctly localized adhesion complexes and actin filament expression. A-E) Negatively marked *mib2*^{1} homozygous mutant clones in the follicular epithelium of stage egg chambers lack GFP and are marked by a bracket and an asterisk in A-D and outlined in E. Wild-type cells are marked in green and nuclei are shown in blue. **A-A'')** *mib2* mutant follicle cells have undetectable E-cad expression (shown in red) at the cell surface. **B-B'')** An egg chamber at stage 10 with a large *mib2*^{1} mutant clone (more than 20 cells) shows a disrupted, uneven epithelial layer. **C-C'')** Patches of *mib2*^{1} mutant cell clones in a stage 13 egg chamber show greatly reduced E-cad expression, and neighboring wild-type cells are also affected. The asterisks mark mutant cells, arrows point to wild-type/wild-type cell boundaries, and arrowheads point to wild-type-mutant cell boundaries. **D-D'')** Mutant follicle cells show a dramatic reduction of Arm expression (shown in red) at the apicolateral and lateral surface of follicle cells in a stage 10 egg chamber. **E-E'')** F-actin, detected by phalloidin staining (shown in red), is reduced at the cell surface and the expression also appears to be lower in the cytoplasm in mutant cells at stage 10. **F-F'')** Hts (shown in red) remains unchanged in mutant follicle cells and shows the columnar epithelial organization of these cells.

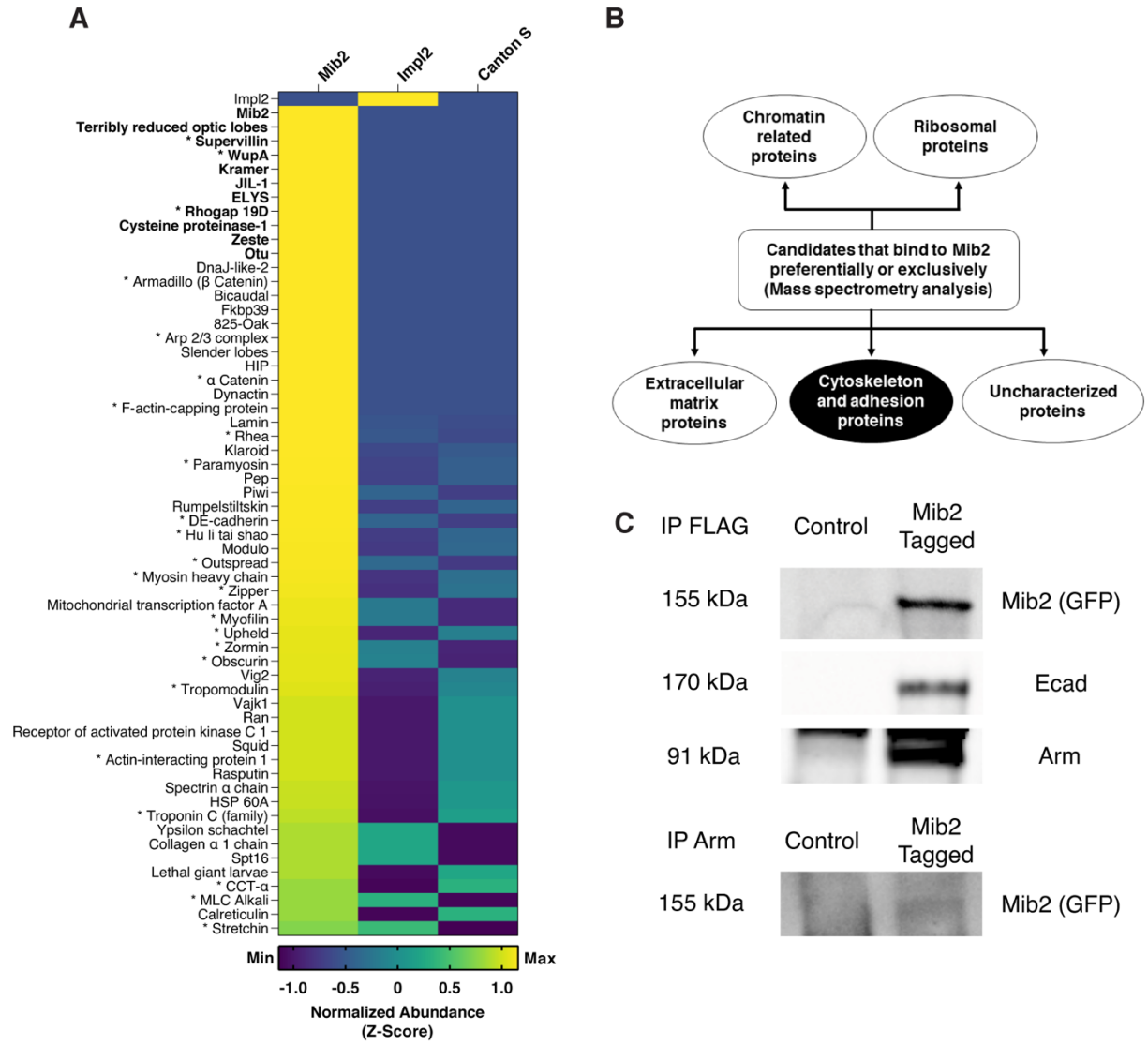

Fig. 4. Mib2 physically interacts with E-cadherin and cytoskeletal proteins. A) Mass spectrometry data identifies proteins associated with Mib2 in immunoprecipitation from ovary extracts. The heatmap shows a selection of interacting proteins according to the relative abundance of each. Immunoprecipitation with FLAG-tagged Mib2 is compared to the negative controls Canton S and FLAG-tagged ImpL2. The proteins in bold exclusively bound to Mib2, not controls, and rest were found in higher abundance than in controls. The proteins with a * are cytoskeletal and adhesion proteins. **B)** Classes of protein families that bound preferentially or exclusively to Mib2 in the IP. Among them, we further characterize the cytoskeleton and adhesion proteins for epithelial maintenance. **C)** Western blots show that immunoprecipitation of Mib2 results

in a complex that includes Arm (β-catenin) and E-cad (top 3 panels). Conversely, immunoprecipitation of Arm results in a complex that includes Mib2 (bottom panel). The control lane contained protein extract from Canton S ovaries, and the Mib2 lane shows the results from extracts from flies with GFP and FLAG-tagged Mib2 controlled under an endogenous regulatory region.

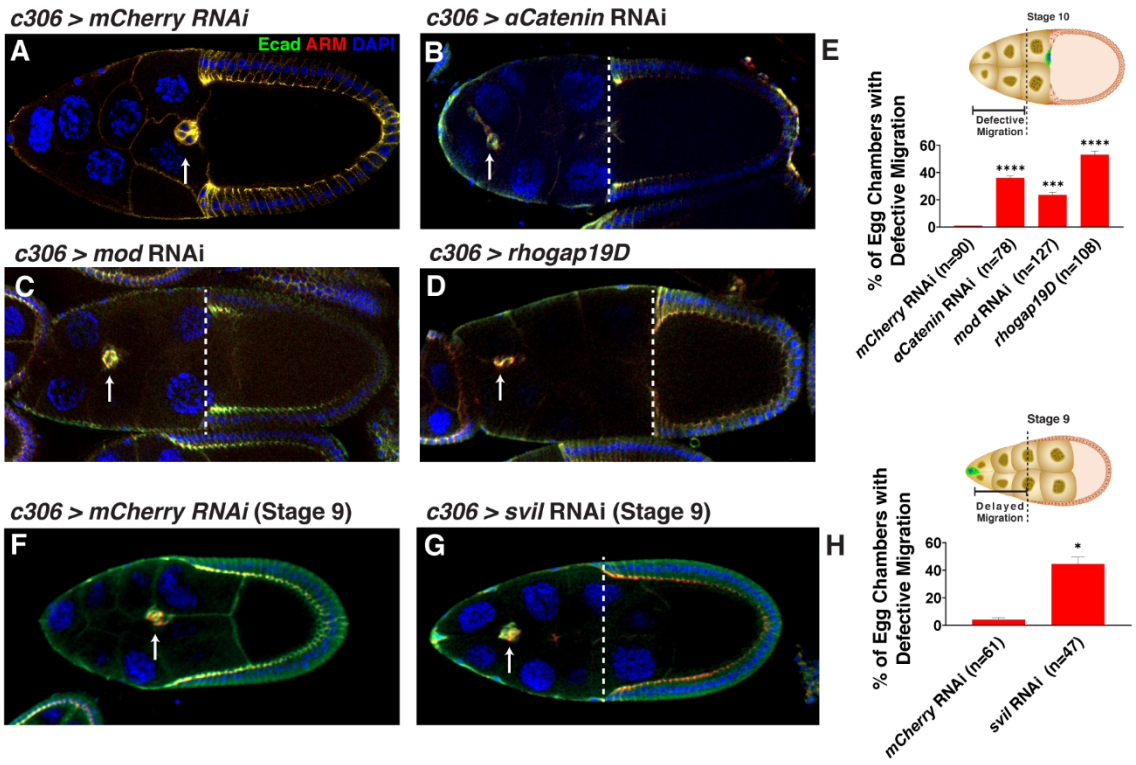

Fig. 5. Novel proteins are involved in border cell migration. A-D) Egg chambers of the indicated genotypes immunofluorescently stained with antibodies directed against Arm protein (red), E-cad (green); and DAPI (blue). **A)** The knockdown of *mCherry* in anterior follicle cells serves as wild-type control for border cell migration at stage 10. **B, C)** The knockdown of *mod* and *α-catenin,* respectively, in anterior follicle cells caused severe migration defects shown by the lagging border cell clusters in stage 10 egg chambers. The arrow points to cluster location; the dotted line indicates the expected location at the oocyte border by this stage. **D)** The overexpression of *Rhogap19D* in the anterior follicle cells results in defective border cell migration. **E)** Quantification of the penetrance of migration defects from (**A, B, C,** and **D**) in egg chambers with respective genotypes. **F**) The knockdown of *mCherry* in anterior follicle cells of stage 9 egg chambers shows no migration delay. **G**) The knockdown of *svil* in anterior follicle cells caused a severe migration delay in stage 9 egg chambers. **H**) The quantification of stage 9 migration delay in *svil* knockdown (**F** and **G**). Statistical analysis was performed using a one-way ANOVA t-test and t-test, * is p< or equal to 0.0332, *** is p<=0.0002, and **** is p< 0.0001; n = number of egg chambers analyzed.

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