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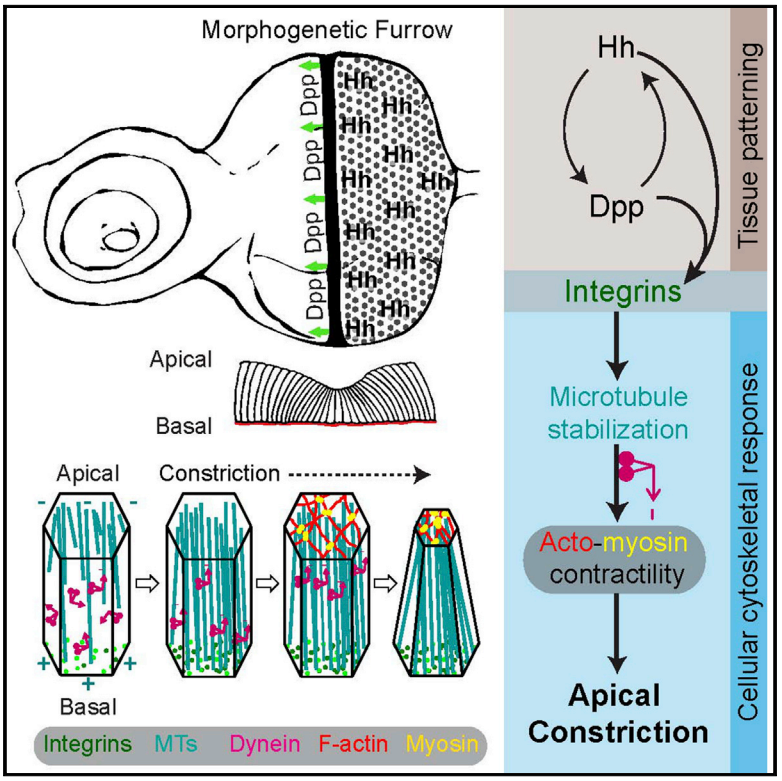
Integrins Regulate Apical Constriction via Microtubule Stabilization in the *Drosophila* Eye Disc Epithelium

Graphical Abstract



Highlights

- Hh and Dpp signaling regulates integrin expression
- Integrins promote apical constriction in the morphogenetic furrow
- Integrins regulate apical constriction by promoting microtubule stability
- Microtubule minus-ends are apical, and dynein is required for apical constriction

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In Brief

During *Drosophila* eye development, Hh and Dpp signaling drives a wave of apical constriction across the epithelium. Fernandes et al. find that integrins are downstream of these pathways and that integrins promote apical constriction by stabilizing microtubules. Thus, integrins genetically link tissue patterning and morphological change at the cellular level.

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Integrins Regulate Apical Constriction via Microtubule Stabilization in the *Drosophila* Eye Disc Epithelium

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SUMMARY

During morphogenesis, extracellular signals trigger actomyosin contractility in subpopulations of cells to coordinate changes in cell shape. To illuminate the link between signaling-mediated tissue patterning and cytoskeletal remodeling, we study the progression of the morphogenetic furrow (MF), the wave of apical constriction that traverses the *Drosophila* eye imaginal disc preceding photoreceptor neurogenesis. Apical constriction depends on actomyosin contractility downstream of the Hedgehog (Hh) and bone morphogenetic protein (BMP) pathways. We identify a role for integrin adhesion receptors in MF progression. We show that Hh and BMP regulate integrin expression, the loss of which disrupts apical constriction and slows furrow progression; conversely, elevated integrins accelerate furrow progression. We present evidence that integrins regulate MF progression by promoting microtubule stabilization, since reducing microtubule stability rescues integrin-mediated furrow acceleration. Thus, integrins act as a genetic link between tissue-level signaling events and morphological change at the cellular level, leading to morphogenesis and neurogenesis in the eye.

INTRODUCTION

Coordinated cellular shape changes drive morphogenesis, which is the development of 3D structures from tissues that often start as 2D sheets (reviewed by Munjal and Lecuit, 2014). Signal transduction pathways initiate morphogenetic events such as the bending, folding, and narrowing of tissue; these events are dependent on polarized subcellular actomyosin-based contractile networks and cellular adhesion, both between cells and with the extracellular matrix (ECM) (Munjal and Lecuit, 2014). During development, polarized actomyosin contractile networks drive cellular deformations in processes as diverse as cytokinesis, tissue invagination, and cell motility.

Localized activation of the small GTPase, Rho, controls actomyosin-based contractility (reviewed by Jordan and Canman, 2012; Munjal and Lecuit, 2014). In its GTP-bound form, Rho activates a number of downstream effectors, including the formins and formin-related proteins, which nucleate the formation of actin filaments and promote their elongation, and kinases such as Rho-associated kinase or Rho-associated protein kinase (ROCK), which promotes the motor activity of Myosin II (Myo II) (Jordan and Canman, 2012; Munjal and Lecuit, 2014). It is the localization of Rho activators, guanine nucleotide exchange factors (GEFs), within the cell that activates Rho (Jordan and Canman, 2012; Munjal and Lecuit, 2014). Connections between the microtubule cytoskeleton and Rho activation have been studied extensively during cytokinesis. Microtubules of the anaphase spindle promote the localization of the RhoGEF, Ect2/Pebble, to the equatorial region of the cell (Jordan and Canman, 2012). Once activated locally, Rho causes local polymerization of F-actin and activation of Myo II to form and constrict the contractile ring (Jordan and Canman, 2012).

When cellular deformations are coordinated across cells, they drive tissue morphogenesis. Tissue bending or invagination is seen in numerous developing embryos, including during ascidian (Sherrard et al., 2010), sea urchin (Davidson et al., 1995), *Drosophila* (Kiehart et al., 1990) and vertebrate (Lee and Harland, 2007) gastrulation, *Drosophila* tubulogenesis (Booth et al., 2014), and vertebrate neural tube closure (Schroeder, 1970). A morphogenetic mechanism commonly used to achieve tissue bending is apical constriction (Martin and Goldstein, 2014; Sawyer et al., 2010). Here, signaling pathways localize Rho activity to the apical surfaces of subsets of cells within the tissue (Martin and Goldstein, 2014; Munjal and Lecuit, 2014; Sawyer et al., 2010). In turn, this promotes actomyosin-driven apical constriction in these cells, which coupled to cadherin-based cell-cell adhesion exerts local mechanical stress and ultimately tissue invagination (Brasch et al., 2012; Martin and Goldstein, 2014; Sawyer et al., 2010). While the contribution of cell-cell adhesion has been well characterized in the context of apical constriction (Guillot and Lecuit, 2013; Martin and Goldstein, 2014; Sawyer et al., 2010), the contribution of cell-ECM adhesion to this process is poorly understood. Here we use the pseudostratified columnar epithelium of the *Drosophila* eye imaginal disc to explore the connections between upstream signaling pathways that control tissue patterning during organogenesis, the

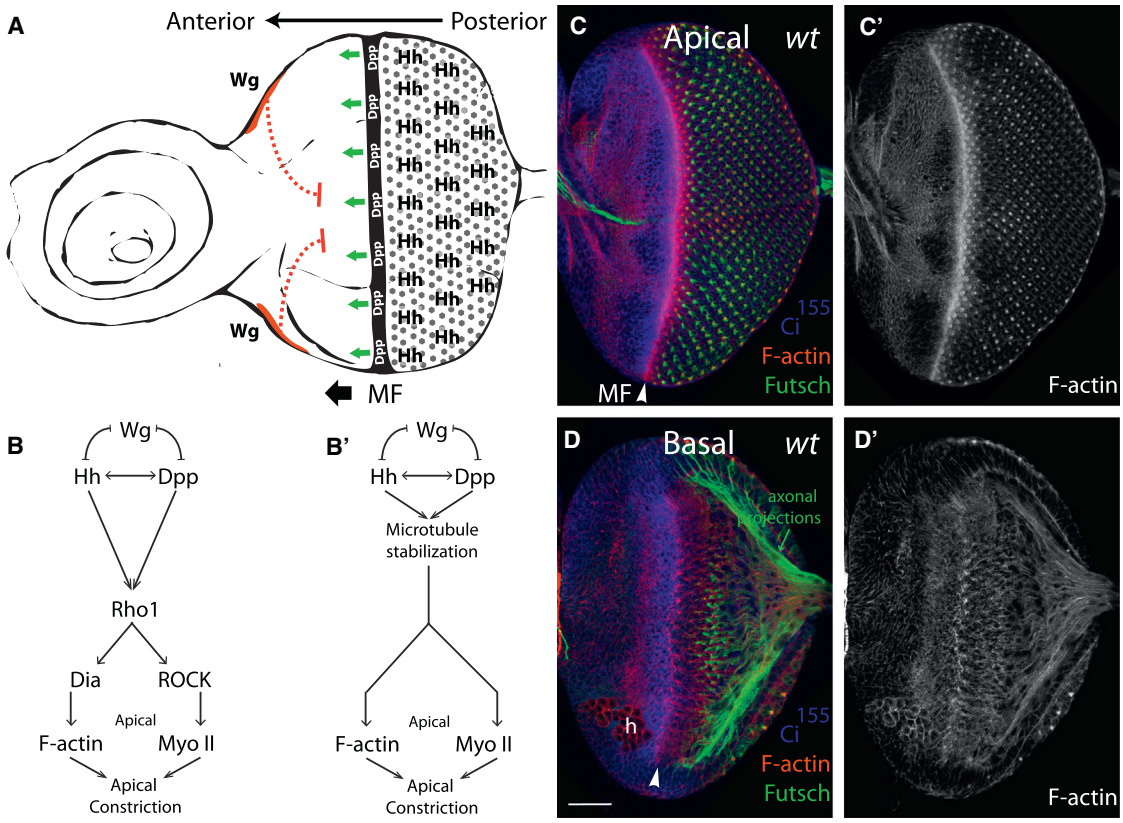

Figure 1. An Overview of the Known Interactions between Tissue Patterning and Actomyosin-Based Contractility during MF Progression in the *Drosophila* Eye Disc

(A) In the *Drosophila* eye disc (posterior is right here and in all images), the Hh, Dpp, and Wg signaling pathways regulate tissue patterning and MF progression. Developing PRs secrete Hh; Hh promotes *dpp* expression within the MF. Together the two pathways promote MF progression, which Wg signaling from the anterior disc margins inhibits.

(B) Summary schematic of the current understanding of how Hh and Dpp signaling redundantly promote apical actomyosin-based contractility by the localized activation of Rho in MF cells (Corrigall et al., 2007; Escudero et al., 2007). (B') Corrigall et al. (2007) showed that these pathways induce microtubule stabilization in MF cells and that MF microtubules are necessary for the apical accumulation of F-actin and Myo II activation, which promotes apical constriction.

(C) A focal plane at the apical side of a WT *(wt; w^{1118})* eye disc stained for F-actin, which is enriched in apically constricting MF cells, the Hh transcriptional effector Ci^{155} (a commonly used MF marker), and the neuronal marker Futsch (22C10), which shows PRs posterior to the MF.

(D) A focal plane at the basal side of the same disc shown in (C). F-actin is mildly enriched in a broad band in the region of the MF. Futsch shows PR axonal projections exiting the basal surface of the disc proper. Hemocyte immune cells (h) are F-actin rich cells often found on the basal surface of eye imaginal discs. Scale bar is 20 μm.

cytoskeletal response, and the contribution of cell-ECM adhesion to this process.

During the *Drosophila* third larval instar (L3), photoreceptor (PR) neuron development initiates at the posterior margin of the eye disc and moves anteriorly in a wave of morphogenesis and differentiation called the morphogenetic furrow (MF; Figure 1) (Ready et al., 1976). The MF consists of a propagating band of apically constricting cells undergoing simultaneous apicobasal contraction, leading to the appearance of a physical groove moving across the epithelium (Ready et al., 1976). Cells anterior to the furrow exist in an undifferentiated proliferative state. These cells apically constrict with the passage of the furrow before differentiating as PRs toward the posterior (Ready et al., 1976). The MF, therefore, represents the leading edge of retinal differentiation. Proper eye development depends on the MF moving at the correct speed (Kumar, 2011).

The Hedgehog (Hh) and Decapentaplegic (Dpp, a bone morphogenetic protein homolog) pathways promote furrow progression (reviewed by Roignant and Treisman, 2009). Developing PR neurons posterior to the MF secrete Hh (Figure 1A), which diffuses toward the anterior to promote target gene expression via its transcriptional effector Cubitus interruptus (Ci) (Heberlein and Moses, 1995). Hh signaling initiates *dpp* transcription within the furrow (Figure 1A); Dpp relies on its own transcriptional effector, Mothers against Dpp (Mad), to promote target gene expression and redundantly regulate MF progression (Pichaud, 2014; Roignant and Treisman, 2009). Wingless (Wg) signaling from the anterior disc margins opposes the activity of the Hh and Dpp pathways (Figure 1A), and the balance in activity of these three pathways regulates the pace of furrow progression across the eye field (Roignant and Treisman, 2009).

Actomyosin-based contractility drives apical constriction within the eye disc downstream of Hh and Dpp signals (Figure 1B) (Corrigall et al., 2007; Escudero et al., 2007). It requires the small GTPase Rho1, the formin Diaphanous (Dia), and ROCK (Corrigall et al., 2007) (Figure 1B). Additionally, stabilized microtubules are enriched in cells within the MF (Corrigall et al., 2007). Depolymerization of these microtubules leads to decreased recruitment of F-actin and to decreased Myo II activation and therefore to defects in apical constriction (Corrigall et al., 2007). There are obvious parallels between apical constriction of cells within the MF and that of other systems such as the ventral furrow. Further, the involvement of stabilized microtubules that are coupled to apical F-actin enrichment and Myo II activation is reminiscent of the contractile ring formed during cytokinesis, the subcellular position of which is also dependent on a population of stabilized microtubules (Jordan and Canman, 2012).

Cell-ECM adhesion occurs at the basal surfaces of epithelia and is mediated primarily by integrin receptors (Narasimha and Brown, 2006). Integrins are α/β-heterodimeric trans-membrane ECM receptors found in all metazoans (Narasimha and Brown, 2006). They bind every major component of the ECM. Cytoplasmic linker proteins connect transmembrane integrins to the cytoskeleton and act as adapters for the bidirectional transmission of force across the plasma membrane (Narasimha and Brown, 2006).

In this study, we establish links between stabilized microtubules in the MF, the integrin adhesion receptors, and the Hh and Dpp signaling pathways. We find that the Hh and Dpp pathways are upstream of integrin expression in the eye disc and that integrins enriched within the MF promote furrow progression. We show that microtubule stability correlates with integrin levels at the basal surface of cells within the MF. Indeed, we find that integrins promote apical F-actin accumulation, Myo II activation, and apical constriction within the furrow via the regulation of microtubule stability. We demonstrate that within cells of the MF, microtubules orient their minus ends at the apical cell surface, the region where actomyosin contractility is activated. Additionally, we show that the apical enrichment of F-actin needed for apical constriction requires cytoplasmic dynein, the motor protein responsible for nearly all minus-end directed microtubule-based transport in eukaryotes (Vale, 2003). Thus, integrins link tissue patterning events with the subcellular actomyosin-based contractility that governs apical constriction and furrow progression.

RESULTS

Hh and Dpp Signaling Are Upstream of the Spatially Distinct α-PS Integrin Expression Domains in the Eye Disc

We were interested in exploring the connection between upstream signaling pathways that drive MF progression and the cytoskeletal response underlying apical constriction. We reasoned that cytoskeletal-interacting proteins with distinct expression domains in the region of the MF represent good candidates to link upstream signaling to the cytoskeletal response. Integrins are conserved transmembrane cell-ECM adhesion receptors, which function as obligate α/β heterodimers. α-PS1 (encoded by *multiple edematous wings* or *mew* in *Drosophila*), α-PS2 (*inflated* or *if* in *Drosophila*), and β-PS (*myospheroid* or *mys* in *Drosophila*) are the most conserved across species (Narasimha and Brown, 2006). Early reports of integrin expression in *Drosophila* described distinct spatially restricted domains for α-PS1 and α-PS2 in the eye disc (Brower et al., 1985), though no further in-depth or high-resolution subcellular localization has been reported. We used the accumulation of apical and basal F-actin (Figures 1C and 1D) as landmarks for the MF to precisely define the basal (Figures 2A–2C) and apical (Figures S1A–S1C) expression domains of α-PS1, α-PS2, and β-PS integrin in the L3 eye disc. Basally, α-PS1 and α-PS2 occupy distinct domains (Figures 2A and 2B). α-PS1 is enriched anterior to and within the MF, but is present at low levels posterior to the furrow (Figures 2A and 2B). In contrast, basal α-PS2 expression initiates within the MF, where it achieves its highest expression before decreasing slightly in the posterior of the eye field (Figures 2A and 2B). β-PS integrin is expressed throughout the disc with enrichment in the MF region at the basal surface (Figure 2C). In summary, at the basal surface of the disc proper, the highest levels of integrins are observed in cells within the MF. This is the region with the highest levels of β-PS and overlapping domains of α-PS1 and α-PS2 (Figure 2D).

It is not known which signaling factors regulate α-PS integrin expression in the eye disc. Since α-PS integrin expression domains are dynamic and evolve with MF progression and subsequent PR differentiation, which are downstream of Hh and Dpp signaling, we hypothesized that these pathways may regulate their expression. We observed reduced α-PS2 expression in clones lacking the Hh signal transducer, Smoothened (Smo), which spanned the MF (Figure 2E). Consistent with other reports, *smo* mutant clones showed disrupted F-actin and apical constriction (Corrigall et al., 2007; unpublished data). Conversely, enhancing Hh signaling anterior to the MF by overexpressing an active form of Ci (*UAS-ci^{5M}*; Price and Kalderon, 1999) in clones resulted in ectopic expression of α-PS2 (Figure 2F) in regions of the clone where ectopic apical constriction and ectopic PRs were induced (Figure S1D). As the principal inducer of actomyosin-based apical constriction, Hh signaling is thought to transcriptionally promote MF progression, in part through promoting Dpp signaling, which functions redundantly with Hh (Corrigall et al., 2007; Escudero et al., 2007). Consistent with this, we observe ectopic α-PS2 expression in clones anterior to the MF in which Dpp signaling is increased by expressing an activated form of the Dpp coreceptor, Thickveins *(UAS-tkv^{QD})*. As with Ci^{5M}-expressing clones, Tkv^{QD}-expressing clones showed ectopic α-PS2 (Figure 2G) in regions of the clone where ectopic apical constriction and ectopic PRs were induced (Figure S1E). Unfortunately, hemocyte aggregates were found on the basal surface of every disc we examined for the above conditions (*smo* mutant clones, and Ci^{5M}- and Tkv^{QD}-expressing clones; n > 20 for each genotype). These obscured examination of α-PS1 and β-PS expression in these genotypes because hemocytes express high levels of α-PS1/β-PS integrins (Irving et al., 2005) and deform the basal surface of the epithelium. Collectively, these data show that Hh and Dpp signaling or the tissue-level patterning events triggered by these pathways are upstream of α-PS2 expression.

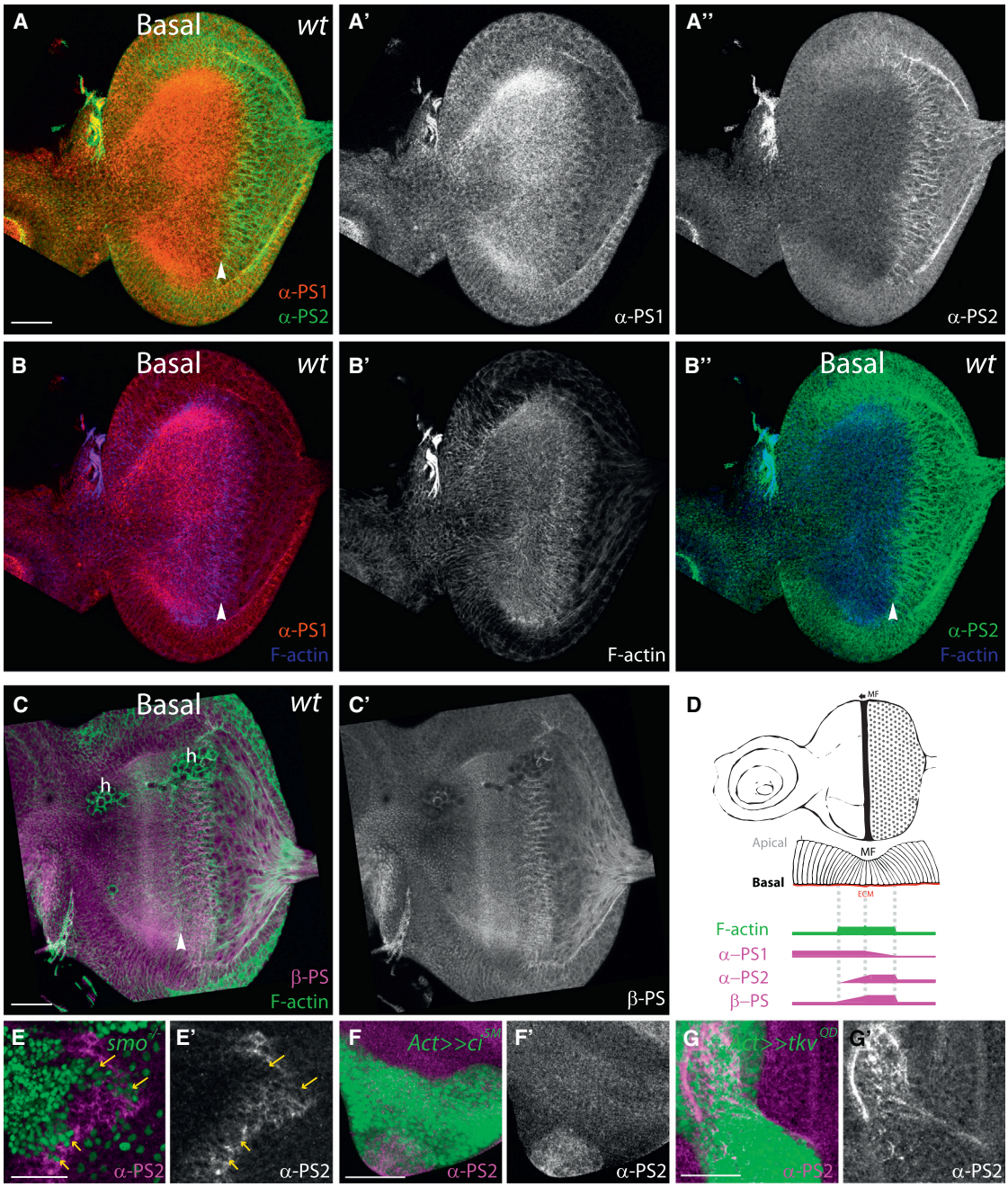

Figure 2. α-PS Integrin Subunits Are Expressed in Distinct Basal Domains Downstream of Hh and Dpp Signaling

(A and B) A basal focal plane of a WT eye disc showing the domains of α-PS1 integrin (red in A and B), α-PS2 integrin (green in A and B''), and F-actin (blue in B and B'') relative to the MF (arrowhead marks the middle of the MF). α-PS1 and α-PS2 expression overlap within the MF.

(C) The basal surface of a WT eye disc showing β-PS integrin (magenta) and F-actin (green). Hemocytes (h) at the basal surface are indicated in (C).

(D) Schematic indicating the enrichment of F-actin, α-PS1, α-PS2, and β-PS within the eye disc relative to the position of the MF.

(E) Basal α-PS2 expression (magenta) in mosaic eye discs containing cells homozygous for a mutation in the Hh receptor, *smo*, (GFP marks $smo^{Q/Q}$ cells).

(F and G) Basal α-PS2 expression (magenta) in eye discs containing GFP positive flipout clones overexpressing (F) an active form of Ci, $UAS\text{-}ci^{5M}$ or (G) an activated form of the Dpp coreceptor, Tkv, $UAS\text{-}tkv^{QD}$.

Scale bars are 20 μm. See Figure S1.

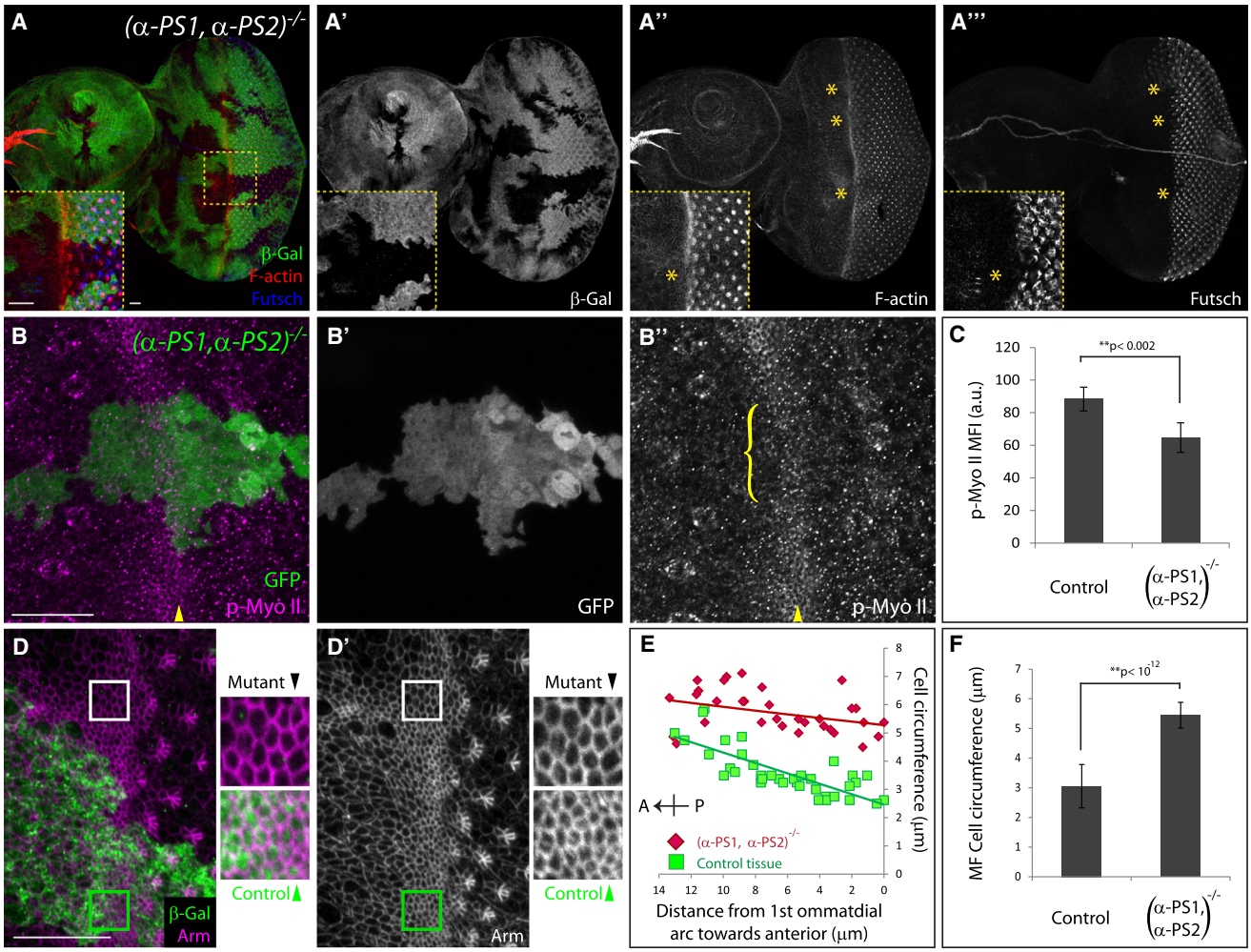

Figure 3. Integrins Are Required for Apical Constriction and the Progression of the MF at the Proper Speed

(A–D) Apical focal planes of $(\alpha\text{-}PS1,\alpha\text{-}PS2)^{-/-}$ mosaic eye discs showing (A) F-actin (red) and Futsch (blue), which labels differentiated PRs.

(B) Phospho-Myo II (p-Myo II; magenta), which is normally at cell membranes in constricting MF cells and (D) Arm (magenta), which marks cell outlines. Homozygous mutant (*α-PS1*, *α-PS2*) cells lack β-Gal (A and D) or are GFP positive (B). (A) Asterisks indicate lagging regions of the furrow (reduced F-actin along with delayed PR development). (B) Bracket marks the clone region overlapping the MF with reduced p-Myo II.

(C) Quantification of p-Myo II MFI (arbitrary units [a.u.]) between $(\alpha\text{-}PS1,\alpha\text{-}PS2)^{-/-}$ and control tissue ($p < 0.002$; $n = 5$).

(D) MF cells appear to constrict less in $(\alpha\text{-}PS1,\alpha\text{-}PS2)^{-/-}$ cells.

(E) Quantification of cell circumference as a function of distance from the first emerging ommatidial arc toward the anterior (test for homogeneity of regression slopes: $F[1,65] = 8.45$; $p < 0.006$).

(F) Average circumference for cells in the MF region ($p < 10^{-12}$; $n^{\alpha\text{-}PS\ mutant} = 19$; $n^{control} = 24$).

Scale bars are 20 μm. See Figure S2.

Integrins Promote Apical F-Actin Enrichment and Apical Constriction within the MF

Early studies of integrins in *Drosophila* showed that integrins are required for proper organization of PRs in the eye in clusters called ommatidia (Brower et al., 1995; Longley and Ready, 1995; Zusman et al., 1990). In adult eye tissue mutant for integrins, all ommatidial cell types were present, but rhabdomere structure was severely perturbed (Longley and Ready, 1995; Zusman et al., 1990). These data rule out a role for integrins in cell type specification, and rhabdomere defects have since been traced to adhesion deficiencies in the cone cell plate and the retinal floor during rhabdomere morphogenesis (Longley and Ready, 1995). While these studies demonstrated a clear role for integrins during rhabdomere morphogenesis, they did not examine the effects of disrupting integrins during MF progression. Based on the enrichment of integrins in cells within the MF, we asked whether they might play a role in MF progression within the larval eye disc. We induced homozygous mutant clones of the different integrin subunits (*α-PS1*, *α-PS2*, *α-PS1* and *α-PS2*, *or β-PS* integrin) in the eye disc and examined MF progression and PR development by probing for F-actin and activated Myo II, which accumulate apically in constricting cells of the MF, and the neuronal marker Futsch or 22C10 (Figures 3 and S2). Interestingly, while loss of either *α-PS* subunit alone

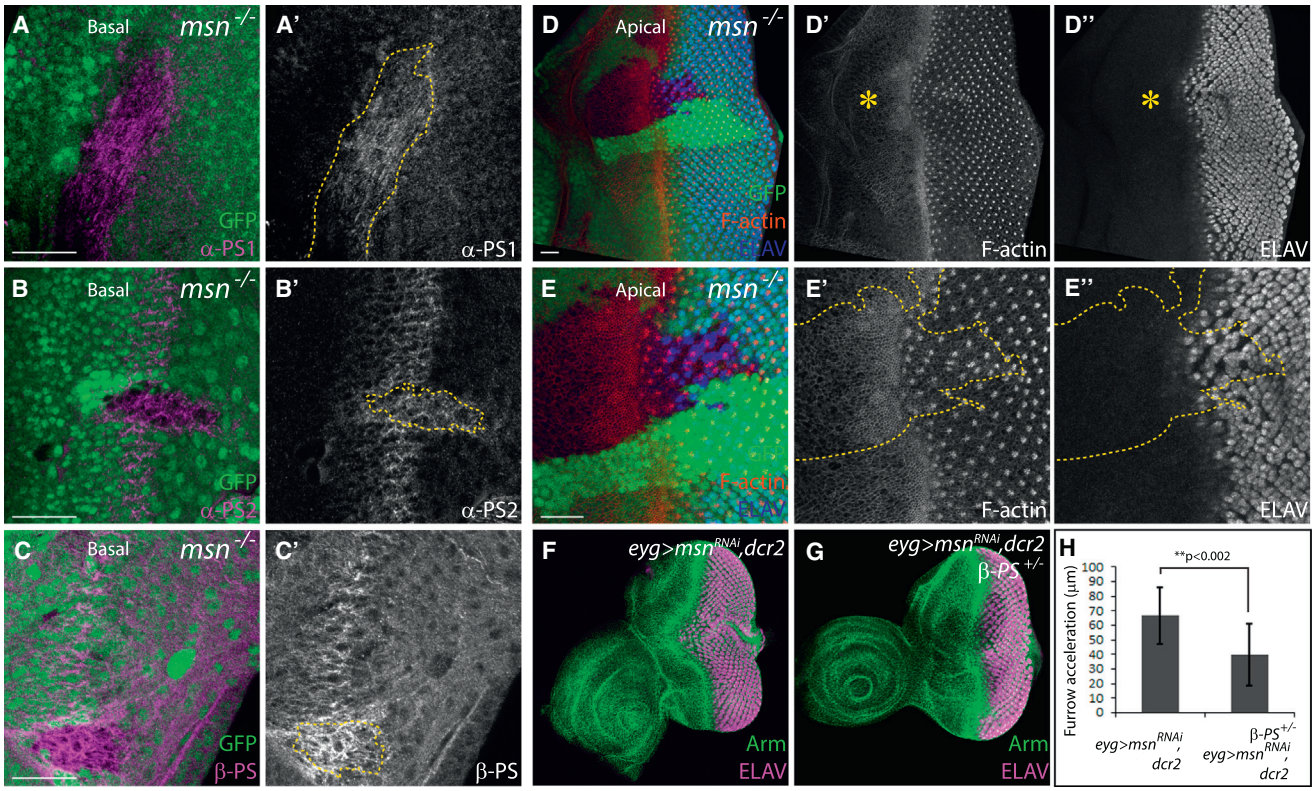

Figure 4. The Ste20 Kinase, Misshapen (Msn), Negatively Regulates Integrin Levels and Furrow Progression within the Eye Disc

(A–C) Basal focal planes of mosaic *msn*$^{-/-}$ (*msn*^{172}) eye discs show elevated (A) α-PS1, (B) α-PS2, and (C) β-PS integrins in *msn*$^{-/-}$ cells (marked by the absence of GFP).

(D) An apical focal plane of a mosaic *msn*$^{-/-}$ eye disc shows accelerated MF progression and PR development through *msn*$^{-/-}$ cells (lacking GFP). F-actin is enriched in constricting cells within the MF and ELAV marks developing PRs.

(E) Close-up view of the region surrounding the *msn*$^{-/-}$ cells shown in (D). More cells constrict apically and contribute to the MF in *msn*$^{-/-}$ clones.

(F and G) Apical focal planes of eye discs expressing *UAS-msn*^{RNAi} under the control of the *eyg-GAL4* driver either alone (F) or in a heterozygous *β-PS* mutant background (G). Discs are stained with antibodies against Armadillo (green) and ELAV (magenta). Scale bars are 20 μm.

(H) Quantification of the degree of furrow acceleration observed in the conditions shown in (F) and (G). Error bars represent SD. *eyg* > *UAS-msn*^{RNAi}, *UAS-dcr2* (n = 15) and *βPS*$^{+/-}$, *eyg* > *UAS-msn*^{RNAi}, *UAS-dcr2* (n = 15), t test p value < 0.02).

See Figure S3.

had no effect on MF progression or PR development (Figures S2A and S2B), loss of both *α-PS1* and *α-PS2* resulted in reduced accumulation of F-actin, decreased Myo II activation, along with reduced apical constriction and delayed PR differentiation (Figure 3). Similar delays in MF progression and PR differentiation were observed with loss of the obligate heterodimer partner, *β-PS* integrin (Figure S2C). Thus, integrins appear to be required for proper apical enrichment of F-actin, apical constriction, and subsequent PR differentiation.

Next, we asked whether elevating integrins was sufficient to induce ectopic apical F-actin enrichment, increased apical constriction, and precocious PR development. To do this, we took advantage of the negative regulation of integrins by the Ste20 kinase Misshapen (Msn). Msn inhibits basal integrin localization in the *Drosophila* follicular epithelium (Lewellyn et al., 2013). We find that Msn plays a similar role in the eye disc, since clones mutant for *msn* (*msn*^{172}) in the eye disc have elevated α-PS1, α-PS2, and β-PS integrins, specifically at the basal surface (Figures 4A–4C and S3D–S3F with mean fluorescence intensity [MFI] quantified in Figures S3A–S3C and S3G–S3I).

Because loss of Msn increases the levels of integrins specifically at the basal surface, we examined the effects of this perturbation on apical constriction, MF progression, and PR development. Strikingly, MF progression and PR differentiation accelerate through *msn* mutant (elevated integrin) clones, as demonstrated by a more advanced position in static snapshots of the MF and of PRs marked by the pan-neuronal marker, ELAV (Figures 4D and 4E). Additionally, increased levels of apical F-actin (Figures 4D and 4E) and myosin contractility (revealed by a protein trap for the heavy chain of Myo II, Zipper-YFP; Figure S3J) result in ectopic apical constriction. Thus, while traversing *msn* mutant tissue, the width of the MF is wider relative to control tissue as more cells apically constrict (Figures 4D and 4E). Similarly, knocking down Msn by RNAi in a wedge along the equator of the disc using the *eyg-Gal4* driver (the expression domain of which is shown in Figure S3K) also results in accelerated MF progression and PR differentiation (Figure 4F).

In addition to regulating integrins, Msn has been implicated in numerous signaling processes, including as a regulator of c-Jun N-terminal kinase (JNK) signaling (Su et al., 2000), bone morphogenetic protein (BMP) signaling (Kaneko et al., 2011), and Wnt signaling (Mahmoudi et al., 2009). Perturbing the JNK pathway had no effect on MF progression (Figure S3L). Similarly, readouts of the Hh, Dpp, and Wg pathways were not affected in *msn* mutant clones beyond changes caused by MF acceleration (see Supplemental Results and Figures S3M–S3P). Collectively, these results strongly suggest that Msn acts independently of JNK, Hh, Dpp, and Wg signaling to regulate integrin expression and MF progression.

We reasoned that if the acceleration of MF progression downstream of *msn* loss was due to increased basal integrin expression, then reducing integrins in this genetic context should restore the normal pace of MF progression and PR development. Consistent with this hypothesis, using the *eyg-Gal4* driver to deplete Msn by RNAi in a *β-PS* heterozygous mutant background results in a statistically significant suppression of MF acceleration and PR development ($p < 0.02$, $n = 15$; Figures 4F–4H). Taken together, our data identify a role for integrins in promoting apical constriction and MF progression.

Integrins Promote the Accumulation of Stable Microtubules within the Furrow

The implication that integrins are involved in promoting apical constriction within the furrow is surprising. How are basally localized adhesion receptors able to regulate actomyosin contractility at the apical surface? Integrin function has been studied extensively in the context of cell migration, where connections have been made between integrins and the microtubule cytoskeleton (Ezratty et al., 2005, 2009; Kaverina et al., 1999; Palazzo et al., 2004; Stehbens et al., 2014). Previous work demonstrated that depolymerization of microtubules in the MF via the misexpression of the microtubule severing protein, Spastin, completely blocked accumulation of F-actin, Myo II, and apical constriction (Corrigall et al., 2007). We therefore tested whether integrins could be regulating microtubules within the MF. We used an antibody raised against the acetylated form of α-tubulin to examine stable or long-lived microtubule regions with slow dynamics (Westermann and Weber, 2003).

A lateral view through WT cells in eye discs showed acetylated microtubules present parallel to the apicobasal axis in cells within the MF, many of which span the full apicobasal length of the epithelium (Figure 5A). In contrast, clones lacking *β-PS* integrin in the same disc have fewer and shorter acetylated microtubules (Figures 5B and 5C), consistent with decreased microtubule stability in integrin mutant cells. Next, we induced *msn* mutant clones in order to elevate integrin levels basally. Strikingly, in *msn* mutant tissue, apicobasally polarized acetylated microtubules are observed in a wider region (i.e., more cells) than control tissue (Figures 5D–5F); furthermore, these cells show an increase in the density of acetylated microtubules relative to WT cells in the same disc, indicating that microtubules are ectopically stabilized when *msn* is lost (i.e., elevated basal integrins). This elevation in stable microtubules is also visible at the basal surface of the disc proper, which shows an increase in acetylated tubulin in the *msn* mutant clone relative to WT tissue (Figure 5G, MFI quantified in Figure 5H). Together, these data suggest that integrins promote microtubule stability within the MF.

Integrins Regulate Apical Constriction and Furrow Progression by Increasing Microtubule Stability

Loss of *msn* generates cells with increased levels of basal integrins, an enrichment of stable acetylated microtubules, precocious accumulation of F-actin, and accelerated MF progression. Since microtubules are required within the MF, we asked whether integrins could be promoting apical constriction and MF progression via their ability to stabilize microtubules; if this is the case, destabilizing microtubules should rescue the premature apical constriction and accelerated MF progression, independent of basal integrin levels (Figure S4A).

Microtubules are dynamic filaments that extend in phases of growth or polymerization and shorten in phases of catastrophe or depolymerization, predominantly at their plus-ends while their minus-ends typically remain fixed. Previous work demonstrated that eliminating microtubules leads to a dramatic loss of F-actin accumulation and apical constriction within the MF (Corrigall et al., 2007); however, the strength of this perturbation precludes epistatic analysis of integrin function, microtubule stabilization, and apical constriction. We therefore sought to shift the existing balance between microtubule growth and catastrophe through modulation of the microtubule plus-end binding protein, EB1 (Figure S4A). Knocking down EB1 increases microtubule catastrophe in multiple contexts in vivo (Busch and Brunner, 2004; Ligon et al., 2003; Tirnauer et al., 2002; Wen et al., 2004).

As with *msn* mutant clones, depleting Msn by RNAi in flipout clones elevated basal levels of β-PS integrin, increased acetylated microtubules, and resulted in a corresponding acceleration in apical constriction and MF progression in affected cells (Figures 6A and 6B). Clones in which EB1 was knocked down by RNAi showed a decrease in acetylated tubulin at the basal surface (Figure 6D; MFI: EB1 RNAi = 549.3 ± 45.1 compared with control 1048.8 ± 79; $p < 0.001$; $n = 6$ each); this is consistent with a shift in the balance between microtubule growth and shrinkage. However, apical constriction and MF progression appeared unaffected, suggesting that the effect of EB1 depletion on microtubule dynamics was not sufficient to affect apical constriction (Figures 6C and 6D). Additionally, depleting EB1 did not affect basal levels of β-PS integrin (Figure 6C). In clones in which both Msn and EB1 were knocked down, basal levels of acetylated tubulin were restored to WT levels, and the MF progressed at its normal pace (Figure 6F). Notably, however, basal β-PS expression remained elevated in double knockdown clones (Figure 6E), suggesting that EB1 acts downstream of integrin expression. A similar rescue was obtained using the *eyg-Gal4* driver (Figures S4B–S4E), allowing the rescue of MF pace to be quantified ($p < 10^{-8}$; compared to Msn RNAi alone). We previously showed that reducing the genetic dose of *β-PS* integrin rescued MF acceleration caused by knocking down Msn (Figures 4F–4H). To confirm the link between integrin upregulation and microtubule stabilization in promoting MF acceleration, we examined acetylated tubulin in clones expressing RNAi against Msn in a *β-PS* heterozygous background and

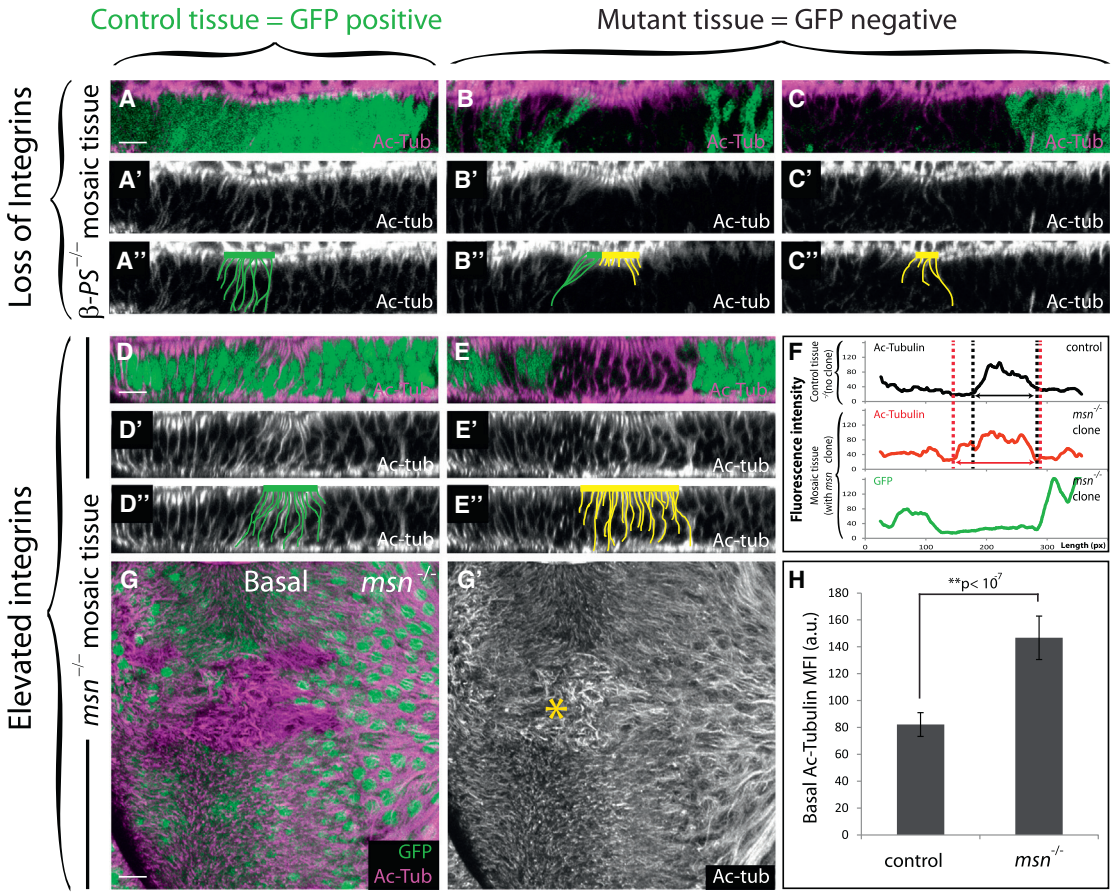

Figure 5. Integrins Regulate Stable Microtubule Enrichment within the MF

(A–C) Lateral views of different regions of the same β-*PS*$^{-/-}$ mosaic eye disc stained with an antibody against acetylated tubulin (magenta). β-*PS*$^{-/-}$ homozygous mutant cells are indicated by a lack of GFP (green). Lines (A'', B'', and C'') highlight the number and length of microtubules within cells found in the MF. In B'', the green lines indicate microtubules that are found within control cells, and the yellow lines indicate microtubules in the mutant tissue.

(D–F) Lateral views of different regions of the same *msn*$^{-/-}$ mosaic eye disc stained with an antibody against acetylated tubulin (magenta). *msn*$^{-/-}$ cells are indicated by a lack of GFP (green). Lines (D'' and E'') highlight the number and length of microtubules within cells found in the MF, which are quantified in (F) by fluorescence intensity profiles (smoothened with a moving average of 25 pixels) for acetylated tubulin across lateral cross-sections of control and *msn* mutant tissue.

(G) Basal focal plane of the *msn*$^{-/-}$ mosaic eye disc shown in (D) and (E).

(H) MFI (a.u.) of acetylated tubulin at the basal surface in control and *msn*$^{-/-}$ tissue ($p < 10^{-7}$; n = 8). Scale bars are 20 μm.

found that acetylated microtubules of the MF detected both in lateral cross-sections and at the basal surface are restored along with normal MF width and progression, suggesting integrins act upstream of microtubule stabilization (Figures 6G–6I; quantified in Figure S4F). Collectively, these data suggest that integrins are able to exert their effects on apical constriction via the regulation of microtubule stability.

Apical F-Actin Enrichment and Apical Constriction in the MF Require Cytoplasmic Dynein

We show that microtubule stability downstream of integrins positively contributes to apical constriction, complementing previous data showing that microtubules within the MF are required for the apical enrichment of F-actin and activation of Myo II (Corrigall et al., 2007). Another cellular process that requires microtubules to position actomyosin contractility is cytokinesis (Jordan and Canman, 2012). Efficient cytokinesis is proposed to be regulated by the motor-dependent delivery of contractility activators along microtubules to the cell cortex at the division plane (Jordan and Canman, 2012). We hypothesized that microtubules in the MF may also serve as tracks for the motor-dependent delivery of contractility activators to the apical surface to promote apical constriction.

To explore this hypothesis, we first examined microtubule polarity in the furrow using an antibody against γ-tubulin, which nucleates microtubules and so serves as a minus-end marker. We find that γ-tubulin is localized to the apical cortex of MF cells (Figures 7B, 7C, and S5A), indicating that within the MF, microtubules emanate from the apical surface and grow basally. Interestingly, consistent with previous reports of microtubule orientation in PRs (Mosley-Bishop et al., 1999), we observed that γ-tubulin reoriented in differentiating PRs posterior to the MF

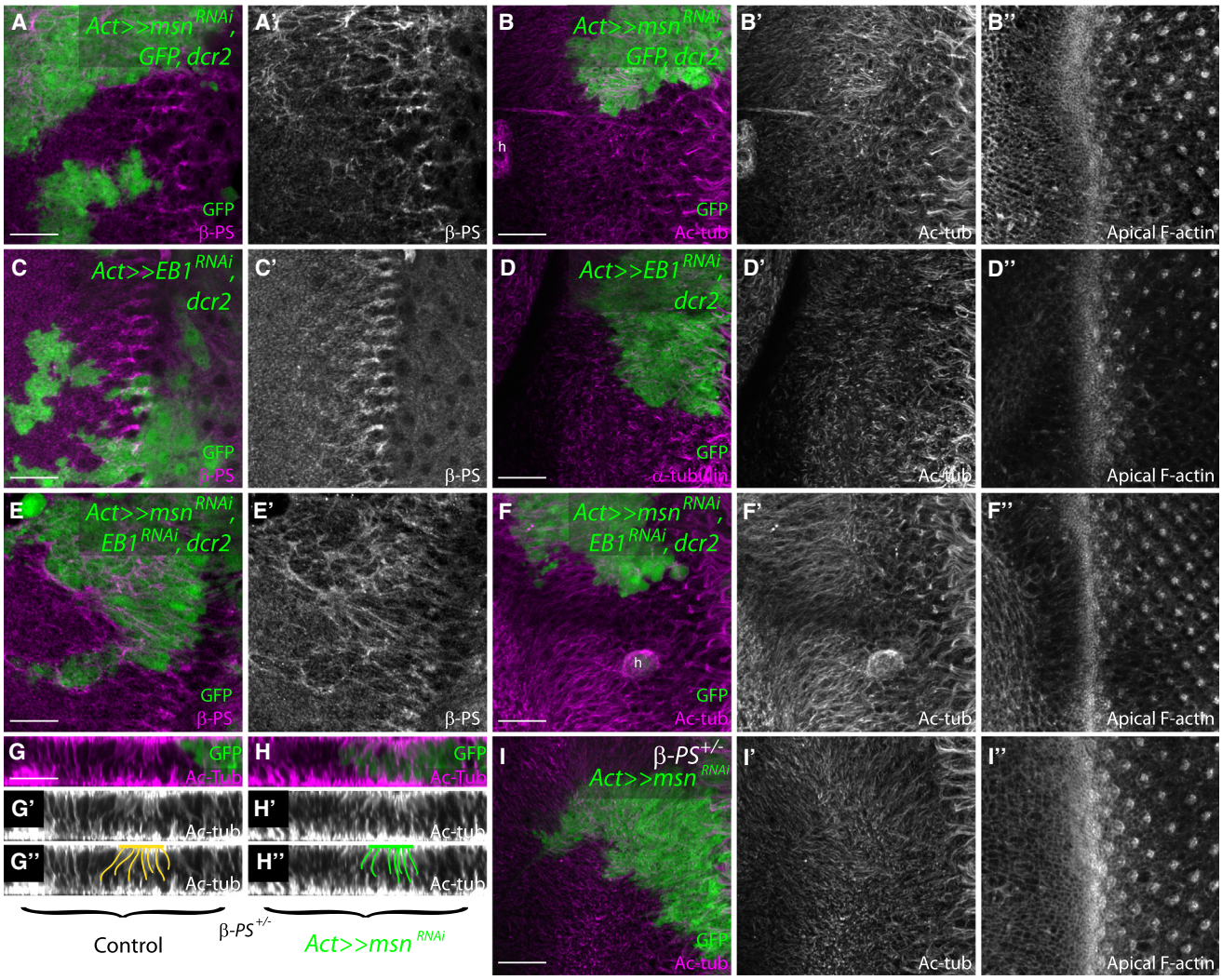

Figure 6. Codepletion of EB1 Rescues Elevated Integrin-Induced *(msn^{RNAi})* Ectopic Microtubule Stabilization and Apical Constriction

(A–F) Larval eye discs containing flipout clones of cells depleted of (A and B) Msn alone (*UAS-GFP* as a titration control), (C and D) EB1 alone, or (E and F) Msn and EB1.

(G–I) *msn^{RNAi}* flipout clones were also generated in a heterozygous *β-PS* mutant background (*β-PS$^{+/-}$*). Depleted cells are marked by GFP (green). Discs were stained with antibodies against β-PS integrin (A, C, and E) or acetylated tubulin (B, D, F, and G–I). Phalloidin labels F-actin (B'', D'', F'', and I''). (G and H) Lateral views of different regions of the same mosaic eye shown in (I). Lines (G'' and H'') highlight the number and length of microtubule bundles within cells found in the MF (quantified in Figure S4F).

Scale bars are 20 μm. See Figure S4F.

and became enriched at the basal surfaces of these cells (Figure S5B; also refer to the Supplemental Results).

If, as we speculate, microtubules serve as tracks for the motor-dependent delivery of contractility activators to the apical surface, then based on their orientation within the furrow, minus-end directed motor proteins must be implicated in apical constriction. In eukaryotes, cytoplasmic dynein is responsible for nearly all microtubule minus end-directed motor protein-based transport (Vale, 2003). In *Drosophila,* the heavy chain of cytoplasmic dynein is encoded by *Dhc64C* (Rasmusson et al., 1994). We tested whether loss of Dhc64C would alter apical constriction within the MF. Unfortunately, we were unable to induce *Dhc64C* mutant mitotic clones due to its requirement in cytokinesis (unpublished data). To circumvent this, we used the mosaic analysis with a repressible cell marker technique to express the baculovirus antiapoptotic protein p35 in clones that were homozygous for the *Dhc64C* mutation, thereby preventing Caspase-dependent cell death in those cells. *Dhc64C* mutant cells (overexpressing p35) within the furrow showed a clear loss of apical F-actin enrichment and reduced apical constriction relative to WT tissue (Figures 7D, 7E, and S5C). Thus, cytoplasmic dynein function is required for the apical enrichment of F-actin and for apical constriction.

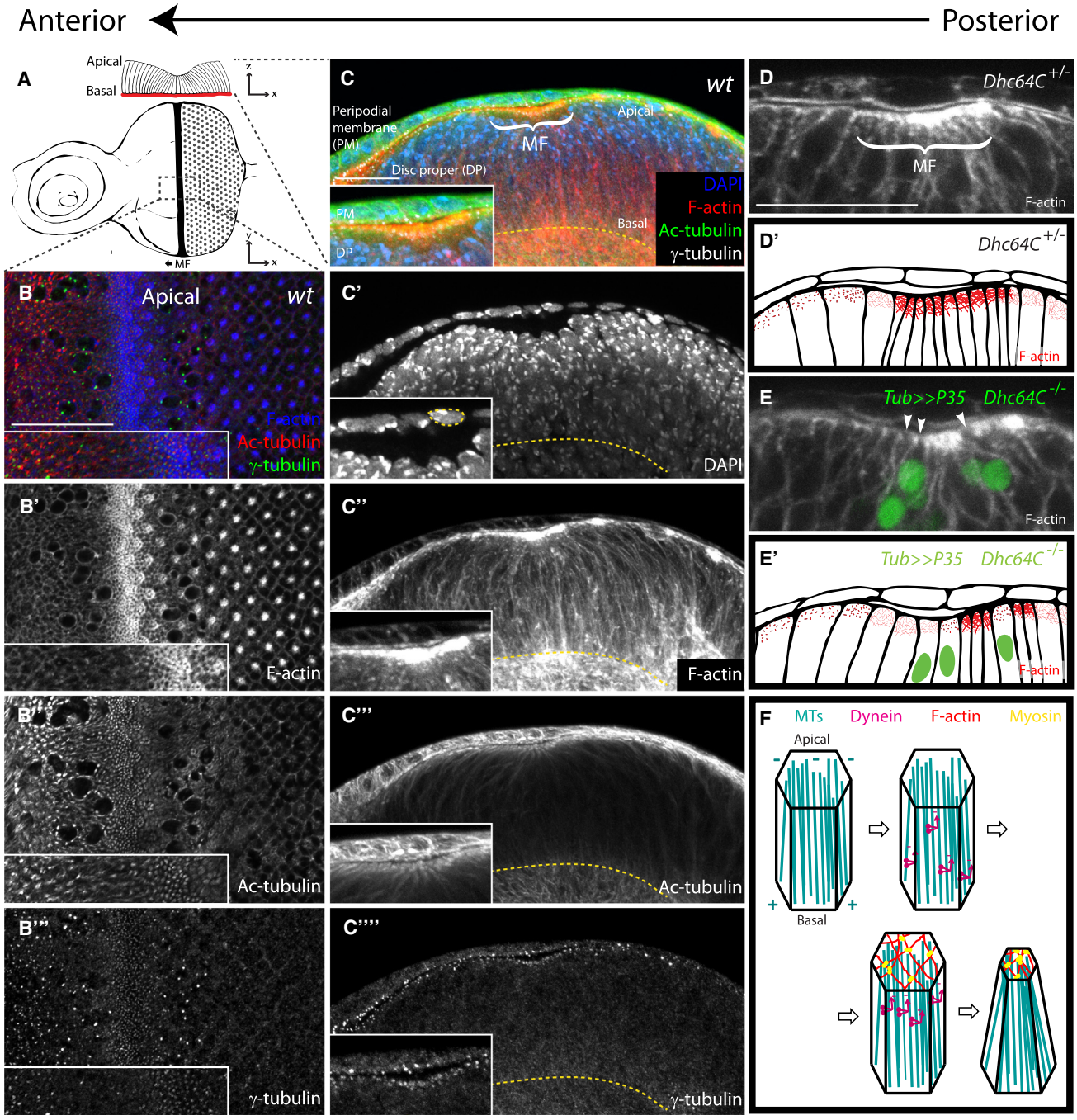

Figure 7. Apical Constriction of Cells within the MF Depends on the Minus-End Directed Motor, Cytoplasmic Dynein

(A) An illustration of an apical (xy) focal plane and a lateral (xz) plane view of the disc proper shown for (B) and (C), respectively.

(B) An apical focal plane of a region spanning the MF in a WT disc stained for F-actin, acetylated tubulin (Ac-tub), and γ-tubulin. γ-tubulin is apically localized anterior to and within the MF where it appears punctate; it is not observed apically posterior to this region. Bright punctae just anterior to and posterior to the MF mark the centrosomes of dividing cells.

(C) A lateral (xz) plane view of the same WT disc shows the squamous epithelium called the peripodial membrane overlying the disc proper. The MF is a physical groove in the tissue with basally positioned nuclei relative to neighboring tissue. A DAPI-stained nucleus is outlined by dashed lines in the peripodial membrane; the bright punctae in the nuclei mark the nucleolus. F-actin is enriched apically. Stable microtubules (identified by acetylated tubulin staining) are seen growing basally from the apical surface where γ-tubulin punctae are localized within the MF.

(D) A lateral (xz) plane view through control tissue (heterozygous for the cytoplasmic dynein heavy chain, *Dhc64C*) focused on the apical F-actin rich region of the MF and an illustration outlining the same features.

(legend continued on next page)

DISCUSSION

This study demonstrates links between basal integrin expression, microtubule stability, and apical constriction. In the MF the actomyosin contractility that drives apical constriction is coincident with microtubule minus-ends and correlates with microtubule stability. Corrigall et al. (2007) demonstrated that apical F-actin accumulation and constriction in the MF require microtubules; we show that, in addition, they require the minus-end microtubule motor, cytoplasmic dynein. Cytoplasmic dyneins are multifunctional motors implicated in transporting cargo, exerting tension on cellular structures such as centrosomes and in spindle assembly (reviewed by Roberts et al., 2013). While our data do not explicitly address whether dynein-dependent cargo delivery is required for apical constriction, they are consistent with a model in which dynein transports contractility activators, such as RhoGEFs, along microtubules to the apical surface to promote apical constriction. This model predicts that dynein-dependent transport, and therefore, apical constriction can be regulated by modulating microtubule stability. Indeed, our data on integrin-mediated microtubule stability demonstrate that apical constriction correlates with microtubule stability. The specific RhoGEF(s) involved in MF apical constriction is not known, but at least two RhoGEFs, Lfc and GEF-H1, are known to associate with Tctex-1, a dynein light chain (in mammals) (Meiri et al., 2012, 2014). Tctex-1 binding inhibits GEF activity, which is restored upon release (Meiri et al., 2012, 2014). Such dynein-associated RhoGEFs therefore represent promising candidates for Rho1 activators during MF apical constriction and warrant investigation.

Microtubule-Dependent Actomyosin-Based Contractility Is a Conserved Developmental Feature

Studies documenting examples of microtubule-dependent actomyosin-based contractility in the field of morphogenesis are numerous and the count is rising (Booth et al., 2014; Lee and Harland, 2007; Lee et al., 2007). In *Xenopus*, analogous actomyosin-driven cellular deformations are observed during both neurulation and gastrulation, where apical actomyosin contractility, driving apical constriction, depends on apicobasally polarized microtubule networks (Lee and Harland, 2007; Lee et al., 2007). Another topologically similar process occurs during *Drosophila* salivary gland tubulogenesis, where apical constriction is dependent on dynamic microtubule reorganization from dense apical arrays into apicobasally polarized microtubules, concomitant with the onset of apical constriction (Booth et al., 2014). In the salivary gland, microtubule arrays emanate from the apical surface in an acentrosomal manner (Booth et al., 2014). In the eye, we observe a strikingly similar switch in apical microtubule organization (Figure 7B'', inset), which corresponds to the approach of the MF and apical constriction. Similarly, microtubules emanating from the apical surface of MF cells appear normal in *SAS4* homozygous mutant flies, which lack centrosomes (Gopalakrishnan et al., 2011), suggesting that MF microtubules may also be acentrosomal (unpublished data).

The apicobasal microtubule polarity in the MF of the eye disc appears to be a conserved feature of pseudostratified epithelia, as it is observed in the zebrafish retina (Norden et al., 2009) and in chick (Spear and Erickson, 2012) and mammalian neuroepithlia (Chenn et al., 1998). Recently, Akhtar and Streuli (2013) demonstrated that the epithelial repolarization event that occurs during mammary gland formation involves stabilization of acentrosomal apicobasally oriented microtubules and furthermore showed that microtubule stabilization is mediated by integrins.

Restricted Expression of Cytoskeletal Interacting Proteins Coordinates Tissue-Level Patterning with Morphogenesis

During MF progression, the Hh and Dpp pathways induce actomyosin-based apical constriction (Corrigall et al., 2007; Escudero et al., 2007). How these pathways modulate cytoskeletal remodeling, when they chiefly regulate gene expression, is mysterious. Cadherin, Cad86C, is an example of a cytoskeletal interacting protein that acts downstream of Hh and Dpp signaling to promote MF progression (Schlichting and Dahmann, 2008). The exact mechanism by which Cad86C functions to induce epithelial folds is not clear, but Schlichting and Dahmann (2008) propose that it depends on heterophilic interactions with an unknown cadherin.

Here, we present evidence that implicates another set of cytoskeletal interacting proteins, the evolutionarily conserved integrins, as regulators of apical constriction and MF progression. We find that the basal expression domains of the two most conserved α-PS integrin subunits (α-PS1 and α-PS2) overlap within the MF, thus producing a unique domain of paired integrin expression within the eye disc. Our data suggest that the onset of α-PS2 integrin expression within the furrow is either directly or indirectly downstream of Hh and Dpp signaling (Figure S6). Elevated integrin expression at the basal cell surface within the MF promotes microtubule stability. Consistent with a model in which microtubules act as tracks for dynein-dependent delivery of contractility-promoting factors to the apical surface, microtubule stabilization promotes apical constriction (Figure S6). Examples of integrin-microtubule interactions are numerous and include integrin-mediated microtubule nucleation and stability in other cellular contexts (Akhtar and Streuli, 2013; Bökel et al., 2005; Colello et al., 2012; Palazzo et al., 2004; Wen et al., 2004). In mammary epithelia, integrin-mediated microtubule stabilization is dependent on integrin-linked kinase (ILK), which promotes the association of β1 integrin with microtubule plus ends via EB1 (Akhtar and Streuli, 2013). However, we find that ILK is dispensable for microtubule stabilization and apical constriction in the MF (unpublished data). In migrating fibroblasts, integrins in focal adhesions regulate Rho activation of

(E) A lateral (xz) plane view through the same disc as (D) containing *Dhc64C* homozygous cells (also expressing p35; marked by GFP, green) and an illustration outlining the decrease in apical F-actin enrichment and reduced apical constriction in mutant cells marked by arrowheads in (E). Scale bars are 20 μm.
(F) Combined with previous work (Corrigall et al., 2007), our data support a model in which microtubules stabilized within the MF provide tracks for the dynein-dependent delivery of contractility-inducing factors to the apical surface of MF cells.
See Figures S5 and S6.

mDia, which in complex with EB1 and APC, binds to and stabilizes microtubule plus-ends (Palazzo et al., 2004; Wen et al., 2004). While Corrigall et al. (2007) demonstrated a requirement for Rho and Dia in MF apical constriction, their potential roles in promoting apical constriction via microtubule stability, in addition to their function in the actomyosin contractility cascade, remain to be evaluated. Indeed, further studies are required to address the mechanism by which integrins regulate microtubule stability within the MF.

Our data highlight how the spatially restricted expression of a set of cytoskeletal interacting proteins, as a consequence of upstream signaling, aids in the coordination of tissue patterning with morphogenesis (model in Figure S6). It is unlikely that integrins and Cad86C represent the only links between genetic expression and cytoskeletal action in the eye disc. Future studies that identify factors in this category will improve our understanding of organogenesis. Given the extensive similarities between microtubule-dependent apical constriction across diverse systems, testing for integrin regulation of microtubule stability in other contexts will be particularly interesting.

EXPERIMENTAL PROCEDURES

Fly Husbandry and Immunohistochemistry

Flies and crosses were raised on standard media at 25°C. In all RNAi misexpression experiments, we coexpressed *UAS-dicer 2* (dcr-2) to enhance the knockdown efficiency. Antibody staining was performed as detailed in the supplement.

Quantification

We used Nikon instruments software Elements to measure MFI between clones and neighboring control tissue. MF acceleration and cell circumference were measured in ImageJ.

Refer to Supplemental Experimental Procedures for a complete list of fly strains, antibodies, and microscopy techniques used.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.11.041.

AUTHOR CONTRIBUTIONS

V.M.F. designed the experiments, performed them with K.M., and analyzed the data. L.L. provided key reagents. V.M.F. and L.L. wrote the manuscript with E.M.V., who supervised the project.

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