Essential roles of myosin phosphatase in the maintenance of epithelial cell integrity of *Drosophila* imaginal disc cells

Tomoaki Mitonaka[a], Yoshiyuki Muramatsu[a], Shin Sugiyama[a], Tomoaki Mizuno[a, *] and Yasuyoshi Nishida[a,*]

[a] Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan.

[*] Authors for correspondence (e-mail: nishida@bio.nagoya-u.ac.jp and 6tmizuno@bunshi3.bio.nagoya-u.ac.jp)

Summary

Reorganization of the actin cytoskeleton and contraction of actomyosin play pivotal roles in controlling cell shape changes and motility in epithelial morphogenesis. Dephosphorylation of the myosin regulatory light chain (MRLC) by myosin phosphatase is one of the key events involved. Allelic combinations producing intermediate strength mutants of the *Drosophila* myosin-binding subunit (*DMBS*) of myosin phosphatase showed imaginal discs with multilayered disrupted morphologies, and extremely mislocated cells, suggesting that DMBS is required to maintain proper epithelial organization. Clonal analyses revealed that DMBS null mutant cells appear to retract basally and localization of apical junction markers such as DE-cadherin are indetectable in most cells, whereas phosphorylated MRLC and F-actin become heavily concentrated apically, indicating misconfiguration of the apical cytoskeleton. In agreement with these findings, DMBS was found to concentrate at the apical domain

suggesting its function is localized. Phenotypes similar to *DMBS* mutants including increased migration of cells were obtained by overexpressing the constitutive active form of MRLC or Rho-associated kinase signifying that the phenotypes are indeed caused through activation of Myosin II. The requirement of DMBS for the integrity of static epithelial cells in imaginal discs suggests that the regulation of Myosin II by DMBS has a role more general than its previously demonstrated functions in morphogenetic events.

Key words: Myosin II, MBS, apical-basal polarity, motility, epithelial morphology.

Introduction

Dynamic changes in cell shapes and motility of epithelial tissues contribute to the formation of organ and body morphologies. Epithelial morphogenesis is driven by differential actomyosin contractility (reviewed by Schock and Perrimon, 2002), and non-muscle actomyosin is composed of two major components, actin and Myosin II. Myosin II is a hexamer composed of two each of three subunits; the heavy chain, the regulatory light chain (MRLC) and the essential light chain. The force-generating activity of actomyosin is mainly regulated by phosphorylation and dephosphorylation of MRLC. Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) and Rho-associated kinase/Rokα, one of the effectors of the Rho GTPase, are responsible for the phosphorylation of MRLC (Tan et al., 1992; Amano et al., 1996). Myosin light chain phosphatase (MLCP) dephosphorylates MRLC, leading to inactivation of Myosin II. MLCP itself is a heterotrimer composed of

the catalytic subunit belonging to protein phosphatase 1c (PP1c), the myosin-binding subunit (MBS), and M20 (Alessi et al., 1992; Hartshorne et al., 1998). MBS regulates MLCP as a target of upstream signals and as a determinant of substrate specificity. MLCP is negatively regulated through phosphorylation of MBS by Rho-associated kinase/Rokα (Kimura et al., 1996; Kawano et al., 1999). Thus, Rho-associated kinase/Rokα doubly activates Myosin II through direct phosphorylation of MRLC and the inactivation of MLCP by phosphorylating MBS (Kaibuchi et al., 1999; Supplentary fig. 1).

The *Drosophila* homolog of *MBS* (*DMBS*), which has been also described as *DMPT* (Tan et al., 2003), has been cloned and its mutant phenotypes have demonstrated its essential roles in dynamic processes such as dorsal closure during embryogenesis, ring canal growth during oogenesis, and the retention of photoreceptor cells within the eye imaginal disc during axon growth (Mizuno et al., 2002; Tan et al., 2003; Lee and Treisman, 2004).

To further the insights into the regulation of Myosin II activity and its effects on epithelial organization, roles of *DMBS* were analyzed in the undifferentiated

and static cells of the imaginal disc epithelia. *Drosophila* imaginal discs are monolayers of epithelial cells with an obvious apical-basal polarity and a well organized actin cytoskeleton (Muller and Bossinger, 2003; Gibson et al., 2005). We demonstrate that *DMBS* is essential for maintaining the integrity of epithelial cells and the organization of epithelial tissues through regulation of Myosin II activity.

Materials and methods

Fly stocks and culture

DMBS mutant alleles used in this study were: $l(3)72Dd^{3}$ ($DMBS^{E1}$), $l(3)72Dd^{03802}$ ($DMBS^{P1}$), and $DMBS^{P2r31}$ (Mizuno et al., 2002). Mbs^{T541}, Mbs^{T666} and Mbs^{T791} were provided by J. E. Treisman (Lee and Treisman, 2004). Other stocks used were as follows: *patched-GAL4* and *hsFLP* (Brand and Perrimon,

1993), *P{w[+mC]=AyGAL4}25* *P{w[+mC]=UAS-GFP.S65T}T2*, *MS1096-GAL4* (Capdevila and Guerrero, 1994), and *P{w[+mC]=AyGAL4}25 P{w[+mC]=UAS-GFP.S65T}T2* (Ito et al., 1997). Fly cultures and crosses were carried out at 25 ℃ unless otherwise stated. $DMBS^{P1}$ and $DMBS^{E1}$ yielded the same results in this study.

Transgenic constructs

The *hs-DMBS* and *UAS-Drok* transgenic lines have been described previously (Mizuno et al., 2002). To generate a kinase-negative version of the *Drok* transgene ($UAS\text{-}Drok^{KA}$), the conserved lysine residue (K116) required for ATP binding was changed to alanin by site-directed mutagenesis. The $UAS\text{-}sqh^{WT}$ construct was made by cloning a full-length cDNA into the pUAST vector. To generate an activated form of the *sqh* transgene ($UAS\text{-}sqh^{DD}$), the conserved phosphorylation sites of Sqh (Thr20Ser21) were both exchanged for aspartic acid. The constructs were injected into embryos following standard procedures.

Clonal analyses of the DMBS mutant clones

FLP/FRT-mediated recombination (Xu and Rubin, 1993) was used to generate clones homozygous for the *DMBS* mutation. To generate clones in imaginal discs, *w*; $DMBS^{P1}$, *P{FRT(w[hs])}2A/TM6B* virgin females were mated to *y, f, hsflp*/Y; *P{Ubi-GFP.nls}3L1P {Ubi-GFP.nls}3L2 P{FRT(w[hs])}2A* males. For *Mbs* alleles, *w*/Y*; FRT80, Mbs/TM6B* males were mated to *hsflp*/*w; P{w[+mC]=Ubi-GFP(S65T)nls}3L P{ry[+t7.2]=neoFRT}80B*/*TM3, Sb* virgin females. Mitotic clones were generated in the imaginal disc 36 and 72 h after egg laying (AEL) using two 60-min heat shocks at 37 ℃, separated by a 60-min interval at 25 ℃. The same was done with $DMBS^{E1}$.

Generation of flip-out clones

The flip-out GAL4-UAS system (Ito et al., 1997) was employed to overexpress

transgenes within imaginal discs. To generate clones in imaginal discs, *y, f, hsflp*; *UAS-sqh^{DD}, y, f, hsflp*; *UAS-Drok* or *y, f, hsflp* virgin females were mated to *P{w[+mC]=AyGAL4}25* *P{w[+mC]=UAS-GFP.S65T}T2* males, respectively. Flip-out clones were generated in imaginal discs 36 or 72 h AEL by two consecutive 60-min heat shocks at 37 ℃ with a 60-min interval at 25 ℃.

Immunostaining

Third instar-wandering larvae were dissected and fixed in 4% formaldehyde in PBS. F-actin was stained with 200nM phalloidin-Alexa^{568nm} (Molecular Probes) in PBT. Primary antibodies used were rat anti-DE-cadherin, mouse anti-Dlg, mouse anti-Crumbs (1/5, each. Developmental Studies Hybridoma Bank, IA), rabbit anti-phospho-MRLC (1/10. Cell Signaling Technology Inc., MA), guinea pig anti-coracle (1/2000, from Rick Fehon (Fehon et al., 1994)) and rabbit anti-phospho-ERM (1/100. Cell Signaling Technology Inc. MA).

Antibodies to DMBS were rabbit antisera raised against a polypeptide containing 161 amino acids corresponding to the residue 220-380 and tagged with six His residues. Secondary antibodies were anti-rat-Cy3 (Jackson Labs, 1:200), anti-mouse-Alexa488, anti-rabbit-Alexa568, and anti-guinea pig-Alexa568 (Molecular Probes, 1:200, each). Images were collected with a Zeiss LSM510 laser scanning microscope and processed with Adobe Photoshop CS.

Results

DMBS is essential for epithelial cell integrity

To investigate the roles of DMBS in the morphogenesis of epithelial tissues, we examined imaginal discs in *DMBS* mutant larvae. Since the homozygotes for strong alleles such as $DMBS^{E1}$ or $DMBS^{P1}$ are lethal during early larval stages, we made transheterozygotes between these strong alleles and a weaker allele,

DMBS^{P2r31}. These animals grew slowly and became wandering third-instar larvae six to seven days after egg laying (AEL) as compared to the normal five days AEL. After starting to wander, the larvae were semiparalyzed, and survived for several days without forming pupariums. In such larvae, imaginal discs continued to grow and developed to a slightly larger size than that of wild-type discs. These imaginal discs were disorganized and multilayered (Fig. 1A, D, G and J) compared to wild-type discs (Fig. 1B, E, H and K). Furthermore, adjacent imaginal discs such as the wing and halter discs, or neighboring leg discs were frequently fused to each other suggesting a tumorous growth of imaginal cells (Fig. 1M and N). These phenotypes were fully rescued by expressing the wild-type *DMBS* transgene (Fig. 1C, F, I and L).

It has been reported that phenotypes such as the formation of multilayered epithelial cell sheets or the fusion of epithelial tissues are due to aberrant apical-basal polarity (reviewed by Tepass et al., 2001; Muller and Wieschaus, 1996; Bilder et al., 2000; Klebes and Knust, 2000). Mitotic clones of the *DMBS*

mutants were produced in wing discs and stained with antibodies against apical junction markers. The wild-type cells are columnar, and DE-cadherin is enriched at the apical cortex (Fig. 2A, Suplementary fig. 2). Similarly, Discs Large (Dlg) is detected in the sub-apical region of the cells (Fig. 2D). Mutant clones were distinguished by the absence of green fluorescent protein (GFP), and cells within *DMBS* mutant clones lost their columnar morphology and seemed to retract basally judging from the position of the nuclei, or were at least showing greatly altered cell shape (Fig. 2 A-C and D-F, Supplementary fig. 2). Observation of the apical distribution of F-actin suggested that the surrounding wildtype cells extended over the edges of basally retracted mutant clones so that mutant cells were exposed apically in only a small depressed area at the center (Fig. 2P-Q). The prominent apical localization of DE-cadherin and Dlg seen in wildtype cells was lost from most of these mutant cells (Fig. 2B, C, E and F), but occasionally, a mutant cell at the border of the clones would still show apical DE-cadherin localization (Suplementary fig. 3D). Other epithelial polarity markers including Crumbs and Coracle were also not localized (data

not shown). The results were in contrast to the observations by Lee and Treisman (2004), who have described that morphological aberrations and changes in DE-cadherin distribution were not observed in the wing imaginal discs of *DMBS*/*Mbs* mutant clones. So we obtained the same *Mbs* alleles to compare with our alleles. In our hands, those *Mbs* alleles showed similar phenotypes to ours when care was taken not to flatten out the discs and observations were made at high magnification (Supplementary fig. 3). One possibility for this discrepancy could be that Lee and Treisman (2004) use the non-ubiquitous protein Nubbin as a nuclear marker. The Nubbin staining that they show appears as if it is only staining the nuclei of the peripodial membrane. Therefore we judge that the loss of normal apical organization of cells in imaginal disc epithelium is observed in multiple *DMBS* mutant alleles, suggesting that *DMBS* could be essential for the integrity of epithelial cells in general.

DMBS regulates the reorganization of cytoskeleton through

down-regulation of Myosin II

It has been shown that the major role of *MBS*/*DMBS* is to regulate the reorganization of the actin cytoskeleton through the inactivation of Myosin II both in mammalian cultured cells and in *Drosophila* embryos (Xia et al., 2005; Mizuno et al., 2002). We stained wing discs containing *DMBS* mutant clones with an anti-phospho-MRLC antibody or phalloidin, and detected stronger signals of phospho-MRLC than those observed in wild-type clones (Fig. 2G-I), indicating an up-regulation of Myosin II in *DMBS* mutant cells as described previously (Lee and Treisman, 2004). Phalloidin staining of the cytoskeleton in wild-type tissues shows a honeycomb pattern. However, such a pattern is lost in the *DMBS* mutant clones, with F-actin accumulated in the central region of the clones (Fig. 2M-O). Perpendicular views of the mutant clones demonstrate a heavy accumulation of phospho-MRLC in the apical region of the *DMBS* mutant cells (Fig. 2J-L). F-actin is localized mainly in the apical lateral region in the wild-type cells, whereas a strong accumulation of F-actin is seen at the

apical cortex of cells in the *DMBS* mutant clones (Fig. 2P-R). Thus, although the apical organization of DMBS mutant cells is perturbed, the overall apical-basal polarity seems to be retained.

To detect the localization of DMBS within the imaginal disc epithelium, we raised antibodies against a DMBS fusion protein. The specificity of the antibody was verified by non-staining of the mutant clones, whereas significant staining could be seen in the wild-type sister clones and in surrounding heterozygous cells (Fig. 3A-C). DMBS colocalizes with the apical junction marker, DE-cadherin, and localizes more apically than Dlg (Fig. 3D-F and G-I). These results suggest that DMBS has the potential to locally modulate Myosin II near the apical surface of the epithelium.

Up-regulation of Myosin II promotes reorganization of cytoskeleton

To examine whether *DMBS* mutant phenotypes such as the disorganization of disc morphology and the aberrant apical-basal polarity depend on excessive

Myosin II activation, we overexpressed transgenes that are known to activate Myosin II. Using the *MS1096*-GAL4 driver, which induces strong expression of UAS-regulated transgenes in the wing disc, we overexpressed an activated D20D21 allele of *spaghetti squash* (*sqh*) which encodes the regulatory light chain of Myosin II. The residues Thr-20 and Ser-21 of Sqh (equivalent to Thr-18 and Ser-19 in the vertebrate MRLC) have been changed to Asp to mimic the constitutive phosphorylated form. Wing discs overexpressing Sqh^{D20D21} were multilayered and showed malformed disc shapes (Fig. 4A). Similar results were obtained when Drok, which phosphorylates Sqh directly (Winter et al., 2001; Mizuno et al., 2002), was overexpressed (Fig. 4C), whereas no such abnormality was observed when wild-type *sqh* or the kinase negative form of *Drok* ($Drok^{KA}$) were overexpressed (Fig. 4B and D, respectively).

To further investigate the effects of an up-regulation of Myosin II on cells within epithelial sheets, we used the flip-out UAS-GAL4 system (Ito et al., 1997) to overexpress sqh^{D20D21} or *Drok* clonally. Cells in the clones

overexpressing Sqh^{D20D21} or Drok shrank basally in contrast to the columnar wild-type cells (Fig. 4E, H and Fig. 4K, N) Furthermore, the strong localizations of apical junction markers, such as DE-cadherin, are not visible in these cells (Fig. 4G, M, J and P). In contrast, overexpression of wild type *sqh* or $Drok^{KA}$ affected neither the cell shape nor apical-basal polarity (Fig. 4Q-S and data not shown). These results suggest that the *DMBS* mutant phenotype in wing discs is manifested via Myosin II activity and that its proper regulation is essential for the maintenance of normal morphology of imaginal disc epithelial tissues.

Phenotypes similar to those of *DMBS* have been observed in the mutants of *Moesin* (Speck et al., 2003) and Moesin is known to bind to MBS and to be a potential substrate for MLCP (Fukata et al., 1998). We therefore tested whether phospho-Moesin is increased in the *DMBS* mutant clones in the wing disc using specific anti-serum. Although changes in morphology made precise comparisons of the apical regions of the cells problematic, no major differences in the levels of phospho-Moesin was observed in the *DMBS* mutant clones,

compared to their wildtype sister clones or the surrounding heterozygous cells of the epithelia (Supplementary fig. 4). Similarly negative observations have been reported for the eye disc (Lee and Treisman, 2004). Thus there is no indication that DMBS functions as a Moesin phosphatase in these tissues.

Up-regulation of Myosin II results in mislocation of cells

It has been reported that MBS affects cell migration by regulating myosin phosphorylation (Xia et al., 2005). We marked a subset of cells within the imaginal epithelium by expressing GFP under the control of the region-specific *patched*-GAL4 driver as previously described (Speck et al., 2003). In the wild-type wing imaginal disc, marked cells were observed within a straight stripe along the anterior-posterior boundary (Fig. 5A and B). On the other hand, in the *DMBS* mutant discs, the borders of cells expressing GFP were diffuse, and a number of cells were mislocated out from the stripe (Fig. 5C and D).

Since the tissue surrounding the mislocated cells is also mutant, it is not clear whether the marked cells actively migrated or became passively misplaced by disorganization of their tissue environment. Therefore we studied the behavior of cells overexpressing sqh^{D20D21} or *Drok* and found that these were also moved into the surrounding wildtype tissue. Although the overall morphology of the imaginal discs including areas of wildtype cells was affected in the experiments, it showed that cells having up-regulated Myosin II became widely mislocated within wildtype tissue with normal adhesive properties and this could be due to the cells having acquired invasive properties (Fig. 5E and F and Fig. 5G and H, respectively).

Discussion

We show here that DMBS is essential for maintaining the integrity of imaginal disc epithelium. Imaginal discs of *Drosophila* are characterized by a monolayer

of tall columnar epithelial cells with an apparent apical-basal polarity and defined morphologies. However, the shapes of imaginal discs in *DMBS* mutants are disorganized and the cells multilayered (Fig. 1B, E, H, and K). In addition, those imaginal discs are fused with adjacent tissues (Fig. 1M and N). The results suggest that DMBS is essential for maintaining the proper morphology and organization of epithelial cells. In *DMBS* null mutant clones, cells lose normal apical organization as indicated by a loss of localization of apical junction markers such as DE-cadherin seen in wildtype cells (Fig. 2A-F). However, the effects on apical markers due to loss of DMBS differed slightly with those reported with photorecepter cells by Lee and Treisman (2004). Whereas they reported the retainment of apical localization of DE-cadherin in basally retracting *Mbs* mutant photoreceptor cells, we found that most mutant clones cells of the wing imaginal disc appeared to lose localization of DE-cadherin and Dlg (Fig. 2) when they basally retracted or changed shape. However, as the apically exposed area in our mutants clones induced in wing disc epithelia were very small, and the resolution in our vertical confocal

sections of epithelia was insufficient, we can not conclude whether apical markers merely lose their localizations or are completely lost in mutant clone cells.

Phenotypes similar to DMBS have been observed in the mutants of *Moesin*, and it has been suggested that Moesin facilitates epithelial morphology by antagonizing the activity of Rho GTPase/Rho1 which activates Myosin II via Rho-associated kinase/Drok (Speck et al., 2003, Supplentary fig. 1). Because Moesin binds to MBS and is a potential substrate for MLCP (Fukata et al., 1998) and DMBS also acts antagonistically toward the Rho/Rho-associated kinase signaling cascade (Mizuno et al., 2002), we considered the possibility that DMBS could be dephosphorylating Moesin, as well as dephosphorylating MRLC directly. Although this seemed rather unlikely since dephoshorylation of Moesin is reported to lead to its inactivation, it was tested by immnuostaining of phosphorylated Moesin in DMBS mutant clones to make certain. As changes in the levels of phosphorylated Moesin were not detected, increased phosphorylation levels of MRLC that were observed in DMBS mutant cells is

likely to be due to loss of direct dephosphorylation of MRLC by DMBS. This interpretation is also supported by findings that apical F-actin appears to increase or become more concentrated in *DMBS* mutant clones whereas loss of Moesin activity causes loss of apical F-actin (Speck et al., 2003).

Immunostaining of DMBS revealed that it specifically localizes at the apical domain of the columnar epithelial cells (Fig. 3). The results suggest that the MRLC is locally phospho-regulated in the apical region of epithelial cells and that this dynamic regulation of Myosin II is important for the organization of the actin cytoskeleton and for the maintenance of epithelial cell integrity. Overexpression of constitutive active Sqh and Drok (Fig. 4), which up-regulates Myosin II, showed results identical to *DMBS* mutations supporting the conclusion that the DMBS mutant phenotype occurs via the activation of Myosin II.

Hyperactivation of Myosin II by the loss of DMBS or overexpression of the constitutive active Sqh or Drok also caused the gross mislocation of marked epithelial cells (Fig. 5). It has been reported that the mutations in *DMBS* cause

photoreceptor cells to drop out of the eye disc epithelium and move toward and through the optic stalk (Lee and Treisman, 2004). In that case also, the highest levels of phospho-MRLC have been detected in the apical region of the mutant cells suggesting dependency on Myosin II activity.

The importance of DMBS in maintaining epithelial integrity has been previously demonstrated in cells participating in dynamic processes, such as the leading edge cells of embryonic dorsal closure, the photoreceptor cells extending axons from the retinal epithelia, and the nurse cells with growing ring canals during oogenesis. All of these cells are known for specialization of cytoskeletal actin structure corresponding to their morphological changes in normal development. In this study we have shown requirement for the maintenance of the integrity of undifferentiated epithelial cells of the imaginal disc at a developmental stage when no dynamic morphological events other than cell proliferation occur. This suggests that the dynamic regulation of Myosin II in the apical region by DMBS has a more general role in epithelial cells than has been previously thought.

The spatiotemporal regulation of the actomyosin cytoskeleton is important for epithelial morphogenesis, and MBS/DMBS plays an essential role in this process by negatively regulating Myosin II. In this study, defects in DMBS activity were found to cause a loss of the apical cellular architecture typical of epithelial cells, and resulted in reduced adhesiveness, in tissue overgrowth, tissue fusion, and extreme mislocation of cells. Thus, *DMBS* fits many of the criteria for a potential neoplastic type tumor suppressor gene, which are genes in which mutant cells are theorized to become neoplastic as a secondary effect of polarity alterations (Hariharan and Bilder, 2006).

Acknowledgements

We are grateful to Dr. R. G. Fehon, J. E. Treisman, the Developmental Studies Hybridoma Bank and the Bloomington Drosophila Stock Center for the antibodies and fly stocks, and to Ms. M. Wada for her technical assistance. This

work is supported by grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan and the Japan Science and Technology Agency.

References

Alessi, D., MacDougall, L. K., Sola, M. M., Ikebe, M. and Cohen, P. 1992. The control of protein phosphatase-1 by targetting subunits. The major myosin phosphatase in avian smooth muscle is a novel form of protein phosphatase-1. Eur. J. Biochem. 210, 1023-35.

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. and Kaibuchi, K. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 271, 20246-9.

Bertet, C., Sulak, L. and Lecuit, T. 2004. Myosin-dependent junction remodelling controls planar cell intercalation and axis elongation. Nature

429, 667-71.

Bilder, D. 2004. Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. Genes Dev. 18, 1909-25.

Bilder, D. and Perrimon, N. 2000. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. Nature 403, 676-80.

Brand, A. H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401-15.

Capdevila, J. and Guerrero, I. 1994. Targeted expression of the signaling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. EMBO J. 13, 4459-4468.

Fehon, R. G., Dawson, I. A. and Artavanis-Tsakonas, S. 1994. A *Drosophila* homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the coracle gene. Development 120, 545-57.

Fukata, Y., Kimura, K., Oshiro, N., Saya, H., Matsuura, Y. and Kaibuchi, K. 1998. Association of the myosin-binding subunit of myosin phosphatase

and moesin: dual regulation of moesin phosphorylation by Rho-associated kinase and myosin phosphatase. J. Cell Biol. 141, 409-18.

Gibson, M. C. and Perrimon, N. 2005. Extrusion and death of DPP/BMP-compromised epithelial cells in the developing *Drosophila* wing. Science 307, 1785-9.

Hariharan, I. K. and Bilder, D. 2006. Regulation of imaginal disc growth by tumor-suppressor genes in *Drosophila*. Annu. Rev. Genet. 40, 335-61.

Hartshorne, D. J., Ito, M. and Erdodi, F. 1998. Myosin light chain phosphatase: subunit composition, interactions and regulation. J. Muscle Res. Cell Motil.19, 325-41.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y. and Yamamoto, D. 1997. The *Drosophila* mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. Development 124, 761-71.

Kaibuchi, K., Kuroda, S. and Amano, M. 1999. Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu.

Rev. Biochem. 68, 459-86.

Kawano, Y., Fukata, Y., Oshiro, N., Amano, M., Nakamura, T., Ito, M., Matsumura, F., Inagaki, M. and Kaibuchi, K. 1999. Phosphorylation of myosin-binding subunit (MBS) of myosin phosphatase by Rho-kinase in vivo. J. Cell Biol. 147, 1023-38.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K. et al. 1996. Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science 273, 245-8.

Klebes, A. and Knust, E. 2000. A conserved motif in Crumbs is required for E-cadherin localisation and zonula adherens formation in *Drosophila*. Curr. Biol. 10, 76-85.

Lee, A. and Treisman, J. E. 2004. Excessive Myosin activity in mbs mutants causes photoreceptor movement out of the *Drosophila* eye disc epithelium. Mol. Biol. Cell 15, 3285-95.

Mizuno, T., Tsutsui, K. and Nishida, Y. 2002. *Drosophila* myosin phosphatase

and its role in dorsal closure. Development 129, 1215-23.

Muller, H. A. and Bossinger, O. 2003. Molecular networks controlling epithelial cell polarity in development. Mech. Dev. 120, 1231-56.

Muller, H. A. and Wieschaus, E. 1996. *armadillo*, *bazooka*, and *stardust* are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in *Drosophila*. J. Cell Biol. 134, 149-63.

Schock, F. and Perrimon, N. 2002. Molecular mechanisms of epithelial morphogenesis. Annu. Rev. Cell. Dev. Biol. 18, 463-93.

Speck, O., Hughes, S. C., Noren, N. K., Kulikauskas, R. M. and Fehon, R. G. 2003. Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. Nature 421, 83-7.

Tan, C., Stronach, B. and Perrimon, N. 2003. Roles of myosin phosphatase during *Drosophila* development. Development 130, 671-81.

Tan, J. L., Ravid, S. and Spudich, J. A. 1992. Control of nonmuscle myosins by phosphorylation. Annu. Rev. Biochem. 61, 721-59.

Tepass, U., Tanentzapf, G., Ward, R. and Fehon, R. 2001. Epithelial cell polarity and cell junctions in *Drosophila*. Annu. Rev. Genet. 35, 747-84.

Winter, C. G., Wang, B., Ballew, A., Royou, A., Karess, R., Axelrod, J. D. and Luo, L. 2001. *Drosophila* Rho-associated kinase (Drok) links Frizzled-mediated planar cell polarity signaling to the actin cytoskeleton. Cell 105, 81-91.

Xia, D., Stull, J. T. and Kamm, K. E. 2005. Myosin phosphatase targeting subunit 1 affects cell migration by regulating myosin phosphorylation and actin assembly. Exp. Cell Res. 304, 506-17.

Xu, T. and Rubin, G. M. 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development 117, 1223-37.

Figure legends

Fig. 1. Imaginal discs from *DMBS* mutants are malformed and adjacent discs

are fused. Wild type (A, D and G) and *DMBS*^{E1}/*DMBS*^{P2r31} (B, E and H) imaginal discs are stained with phalloidin to show their overall morphology. (A-C) Eye-antenna discs; (D-F) leg discs; (G-I) wing discs. (C, F and I) Aberrant morphologies of *DMBS* mutant imaginal discs are rescued by expression of wild-type *DMBS* transgene. (J-L) Perpendicular sections of imaginal discs. The imaginal discs were stained with phalloidin (green), and nuclei were stained with TOPRO-3 (red). In the wild type, the imaginal disc is constituted of a monolayer of tall columnar cells (K), while in the *DMBS* mutant, the disc is multilayered (L). The phenotype is rescued by expression of the wild type transgene (L). Examples in *DMBS*^{E1}/*DMBS*^{P2r31} mutants, showing fusion of halter disc and wing disc, and of adjacent leg discs (M and N respectively). Scale bars in (A) and (M) are 100 μm, and that in (J) is 10 μm.

Fig. 2. Loss of *DMBS* causes cell shape changes, loss of localization of the apical markers DE-Cadherin and Discs Large, and increased accumulations of phospho-MRLC and F-actin. (A-F) Perpendicular views of third-instar wing

discs containing *DMBS*^{P1} mutant clones. Apical is up, basal is down, and *DMBS*^{P1} mutant cells are identified by loss of GFP.nls (green in A and D). Nuclei are marked with TOTO-3 (blue in B and E) and those of mutant cells are displaced basally. The apical markers DE-cadherin (red in A-C), and Dlg (red in D-F) were detected immunohistologically and are greatly reduced or absent in mutant clone cells. Arrows indicate the presumed apical surface of *DMBS* mutant clones and asterisks denote peripodial membrane nuclei which are round and are evenly spaced, differing from the apico-basally elongated epithelial nuclei of the disc proper. (G-R) Wing discs containing *DMBS*^{P1} mutant clones are stained with anti-phospho-MRLC (red in H, I, K and L) or phalloidin (red in N, O, Q and R). Confocal xy sections (G-I and M-O) and zx optical cross sections (J-L and P-R) are shown. Apical is up and basal is down (J-L and P-R). *DMBS*$^{-/-}$ clones lacking GFP (green in G, I, J, L, M, O, P and R) are circled with dotted lines, and their wild type sibling clones with solid lines (G-I and M-O). (I), (L), (O), and (R) are merged images of the two panels to their left. Scale bars in (A, D, G, J, M, and P) are 10 μm. The strong apical

distribution of actin is retained in the mutant clone cells and suggests that they have retracted basally and maintain their apico-basal polarity. Note that the GFP.nls-expressing cells surrounding the basally-retracted mutant clone extend over and cover much of it leaving only a small region exposed on the apical side of the imaginal disc epithelium.

Fig. 3. DMBS localizes in the apical lateral membrane of wing imaginal disc epithelium. (A-C) *DMBS*^{P1} mutant clones indicated by loss of GFP (green in A) are circled with dotted lines and did not stain with anti-DMBS antibodies (red in B), demonstrating the specificity of the antibody. (C) is a merged image. Wild-type sibling clones are circled with solid lines. (D-I) Wild-type wing discs were double stained with anti-DMBS antibodies (red in E and H), and anti-DE-cadherin antibodies (green in D) or anti-Dlg antibodies (green in G). (F) and (I) are merged images of those to their left. Overlap of green and red in (F) indicates that DMBS colocalizes with DE-cadherin in the area of the

adherens junctions. Scale bar in (A) is 20 μm, and those in (D) and (G) are 10 μm.

Fig. 4. Excessive Myosin II activity causes malformation of imaginal disc shapes and loss of localization of DE-Cadherin and Discs Large in imaginal epithelium. (A-D) Wing discs overexpressing sqh^{DD} (A), sqh^{WT} (B), *Drok* (C), and $Drok^{KA}$ (D) are stained with phalloidin. Genotypes are as follows: (A) *MS1096-GAL4*/+; *UAS-*sqh^{DD}/+. (B) *MS1096-GAL4*/+; *UAS-*sqh^{WT} /+. (C) *MS1096-GAL4*/+; *UAS-Drok*, and (D) *MS1096-GAL4*/+; *UAS-*$Drok^{KA}$. (E-P) Perpendicular views of wing discs containing flip-out clones expressing sqh^{DD} (E-J) or *Drok* (K-P) stained either with anti-DE-cadherin antibodies (E-G and K-M) or anti-Dlg antibodies (H-J and N-O). Discs containing control flip-out clones expressing wildype *sqh* were also stained with anti-Dlg antibodies (Q-S). Nuclei are stained with TOTO-3 (blue in F, I, L, O, and R) and flip-out clone cells expressing transgenes are marked by GFP (green in E, H, K, N, and Q). Genotypes are as follows: (E-G and H-J) *y,f,hs-flp*/+; *P{w[+mC]=AyGAL4}25*

P{w[+mC]=UAS-GFP.S65T}T2/*UAS-sqh*^{DD}, (K-M and N-P) *y,f,hs-flp*/+; *P{w[+mC]=AyGAL4}25* *P{w[+mC]=UAS-GFP.S65T}T2/UAS-Drok*, and (Q-S) *y,f,hs-flp*/+; *P{w[+mC]=AyGAL4}25* *P{w[+mC]=UAS-GFP.S65T}T2*/ *UAS-sqh*^{DD}. Scale bar in (A) is 100 μm, and those in (E), (H), (K), (N) and (Q) are 10 μm.

Fig. 5. Loss of *DMBS* or excessive Myosin II activity induce cell mislocalization in imaginal epithelium. Wing imaginal discs were stained with phalloidin (red in A, C, E, and G). GFP in merged images (green in A, C, E, and G) is also shown separately (B, D, F, and H). (A and B) *patched-GAL4*, *UAS-GFP*/+. All GFP-marked cells remain within the GFP-expressing stripe of the *patched* domain in wild-type disc. (C and D) *patched-GAL4*, *UAS-GFP*/+; *DMBS*^{E1}/*DMBS*^{P2r31}. (E and F) *patched-GAL4*, *UAS-GFP*/*UAS-sqh*^{DD}. (G and H) *patched-GAL4*, *UAS-GFP*/*UAS-Drok*. In contrast to wild-type, *DMBS*^{E1}/*DMBS*^{P2r31} cells are mislocated and cells overexpressing *sqh*^{DD} or *Drok* appear to migrate out from the *patched* expression domain (arrowheads

in D, F, and H). Scale bar in (A) is 20 μm.

Legends to Supplementary figures

Supplementary Figure 1. Schematic diagram of Myosin II contraction signaling in Drosophila. Myosin Regulatory Light Chain (MRLC), also known as Spagetti-Squash is the main regulatory subunit of the Myosin II hexamer. It is negatively regulated by the Myosin Light Chain Phospatase (MLCP) hetero-trimer, which is composed of Protein Phosphatase 1c (PP1c), M20, and the Drosophila Myosin-Binding Subunit (DMBS). MLCP is in turn negatively regulated through the phosphorylation of its MBS subunit by Rho-Associated Kinase (Rho-kinase). In addition, Rho-kinase positively regulates MRLC by directly phosphorylating it. Rho-kinase itself is negatively regulated via Rho-GTPase when the actin-binding ERM protein Moesin is phosphorylated by the Sterile 20 kinase Slik. Abnormal activation of MRLC in the apical regions

of imaginal disc cells led to instability of epithelial cell organization.

Supplementary Figure 2 . Perpendicular views of the third-instar wing discs containing mutant clones of the *DMBS*^{P1} allele. Wild-type cells are labeled with GFP.nls (green in A, E, I) and mutant cells are marked by loss of GFP.nls. Nuclei are marked with DAPI (blue in B, F, J), and apical surface is stained with anti-DE-cadherin (red in C, G, K). Note that the evenly-spaced cells with round nuclei above the clone are of the peripodial membrane, and that the GFP.nls-expressing cells surrounding the basally-retracted mutant clone cover much of it leaving only a small region exposed on the apical side of the imaginal disc epithelium which shows loss of or highly reduced expression of apical DE-cadherin in most cells. Scale bars are 10 μm.

Supplementary Figure 3. Perpendicular views of the third-instar wing discs containing mutant clones of the *Mbs*^{T666} allele described by Lee and Treismann (2004). Wild-type cells are labeled with GFP.nls (green in A, E, I) and mutant

cells are marked by loss of GFP.nls. Nuclei are marked with DAPI (blue in B, F, J), and apical surface is stained with anti-DE-cadherin (red in C, G, K). Note that the evenly-spaced cells with round nuclei above the clone are of the peripodial membrane, and that the GFP.nls-expressing cells surrounding the basally-retracted mutant clone cover much of it leaving only a small region exposed on the apical side of the imaginal disc epithelium which shows loss of or highly reduced expression of apical DE-cadherin in most cells and thus shows the same phenotype as our *DMBS*^{P1} allele. Note that a mutant clone cell (asterisk in D) at the border with wildtype cells appears to express apical DE-cadherin. Scale bars are 10 μm.

Supplementary Fig. 4. Phosphorylation levels of Moesin are not increased in *DMBS* mutant clone cells. Perpendicular views of third-instar wing discs containing *Mbs*^{T666} mutant clones. Wild-type cells are labeled with GFP.nls (green in A) and mutant cells are identified by loss of GFP.nls. Nuclei are marked with DAPI (blue in B), and the cytoskeleton is stained with

anti-phospho-Moesin (red in C). (D) is a merged image of A-C. The phosphorylated Moesin level in *DMBS* mutant clones did not increase in comparison with neighboring wildtype cells. The results were the same in another *DMBS* mutant allele ($DMBS^{P1}$). Scale bar is 10μm.

Fig. 1

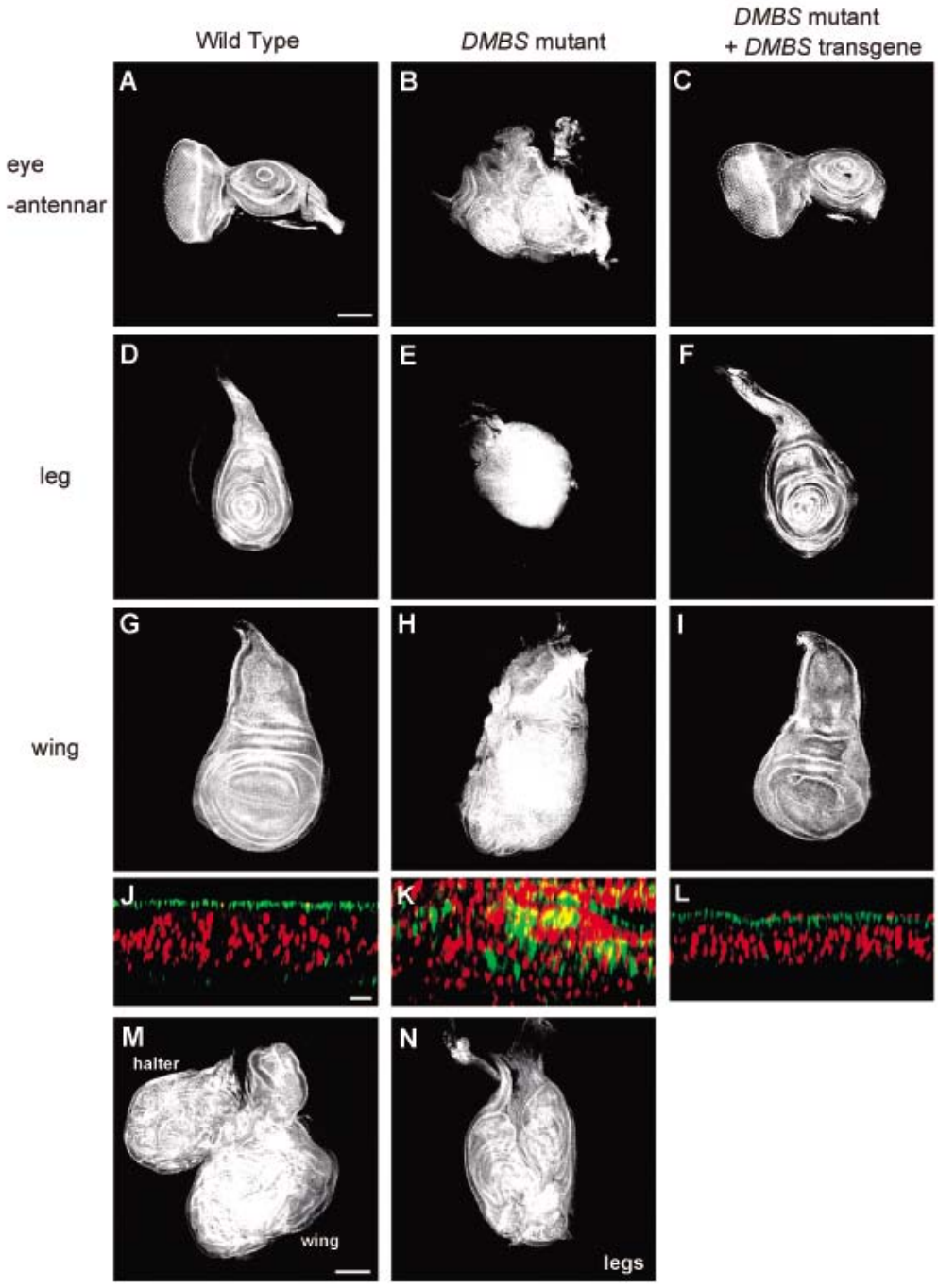

Wild Type
DMBS mutant
DMBS mutant
+ DMBS transgene
A
B
C
eye
-antennar
D
E
F
leg
G
H
I
wing
J
K
L
M
halter
wing
N
legs

Fig. 2

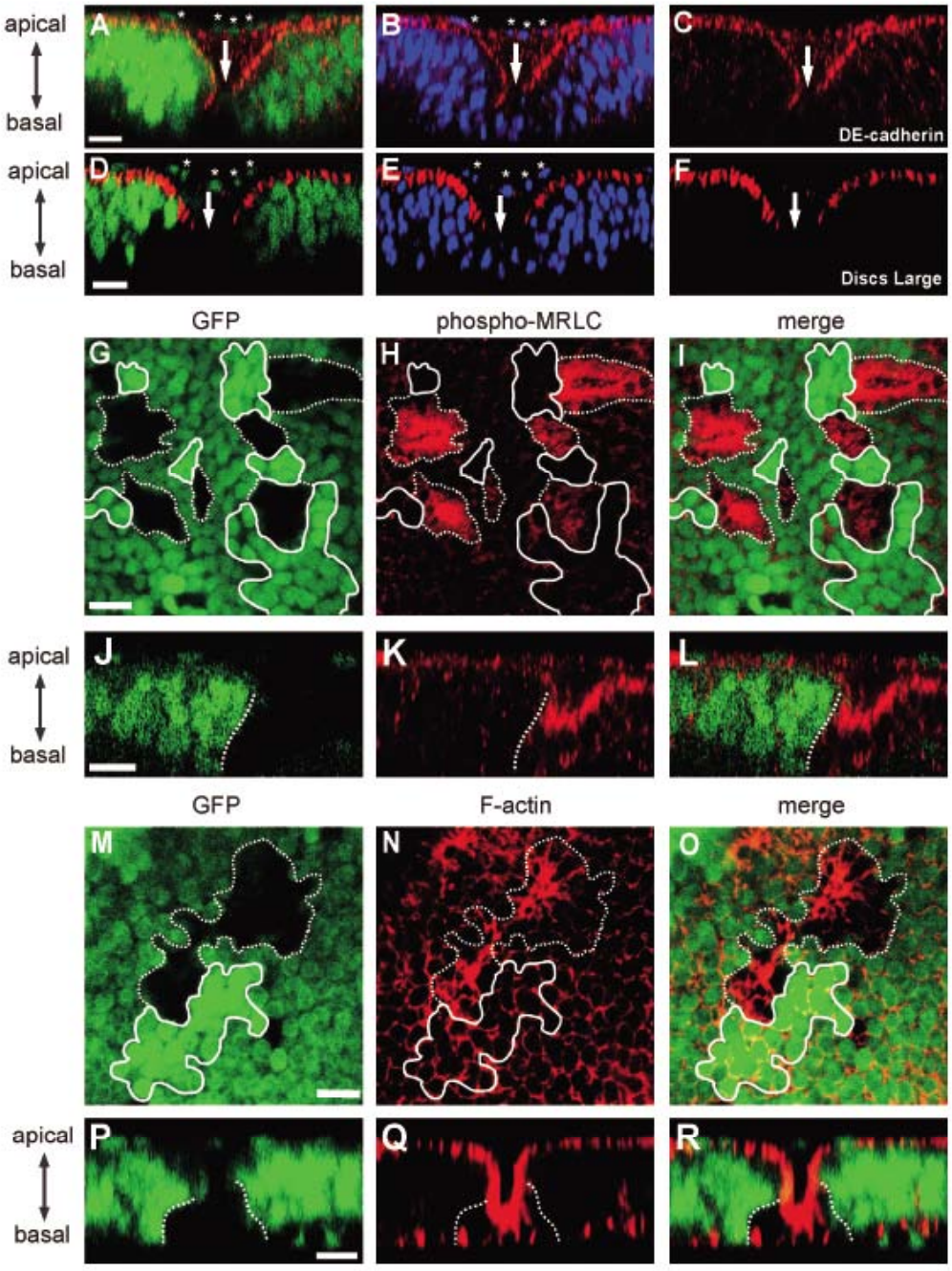

apical
basal
A
B
C
DE-cadherin
apical
basal
D
E
F
Discs Large
GFP
phospho-MRLC
merge
G
H
I
apical
basal
J
K
L
GFP
F-actin
merge
M
N
O
apical
basal
P
Q
R

Fig. 3

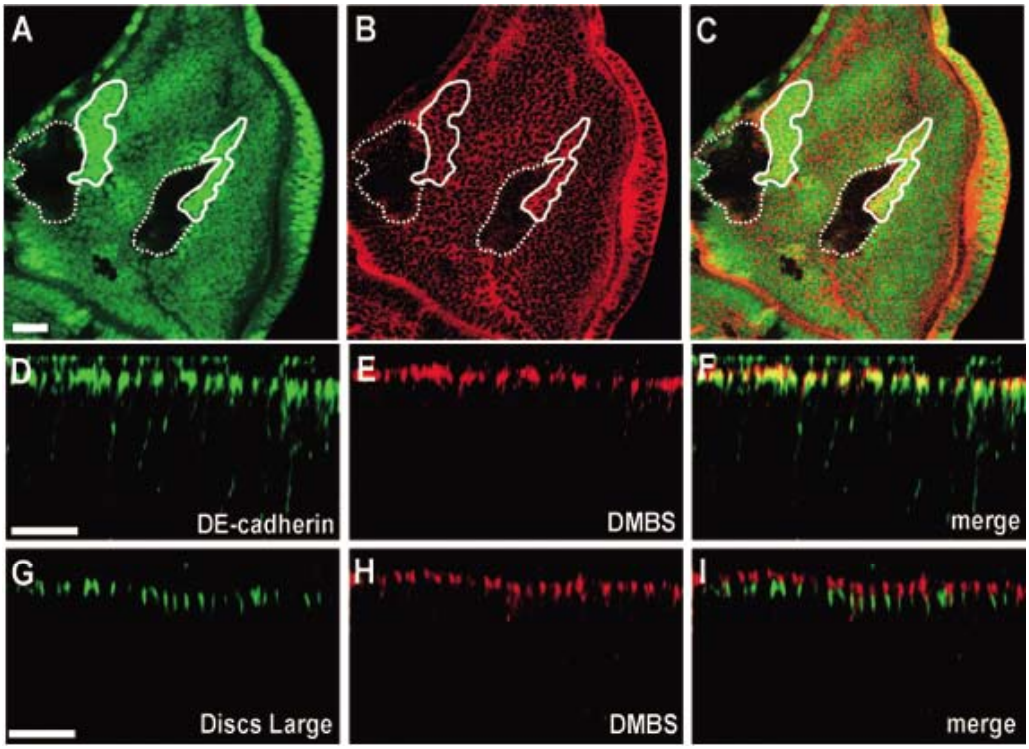


Fig. 4

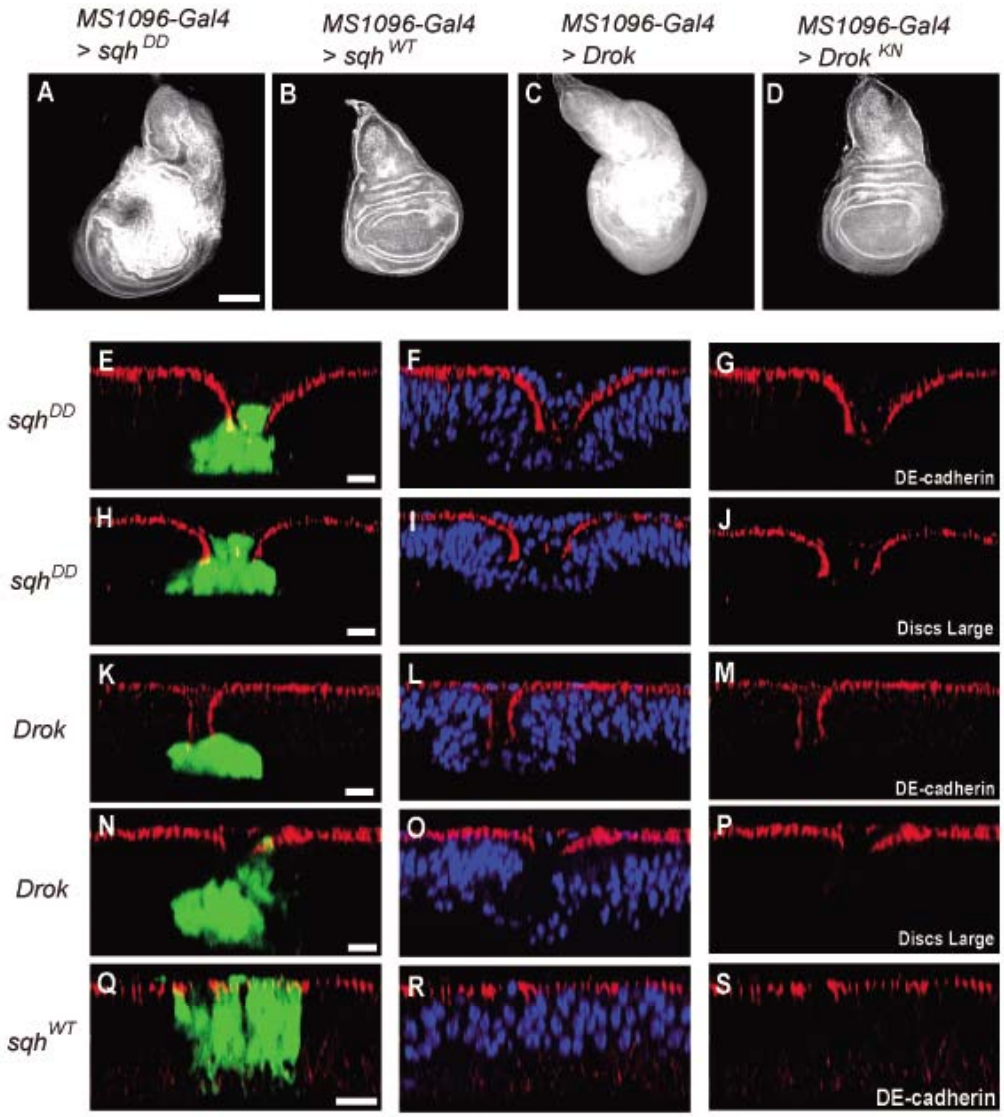
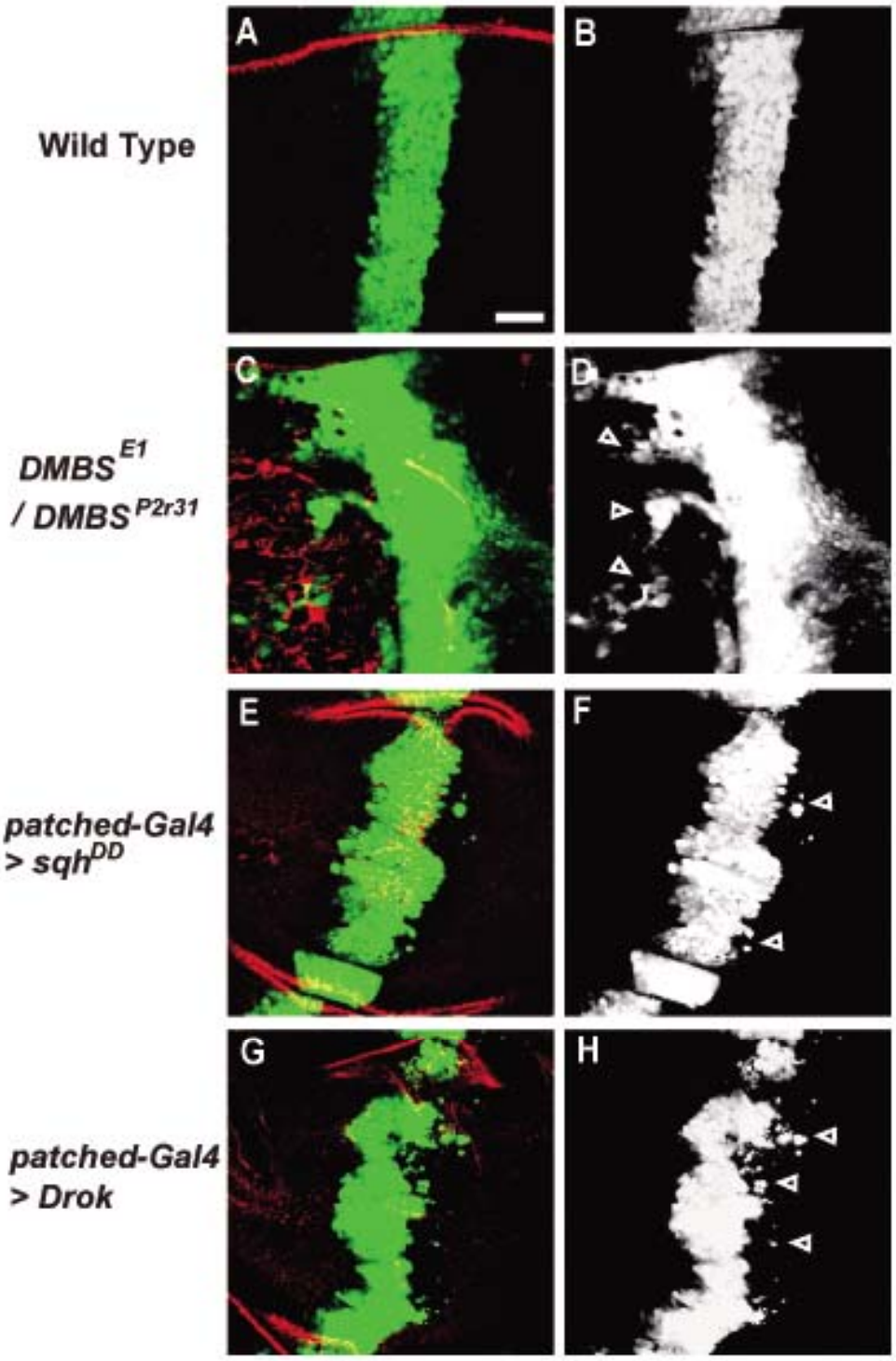
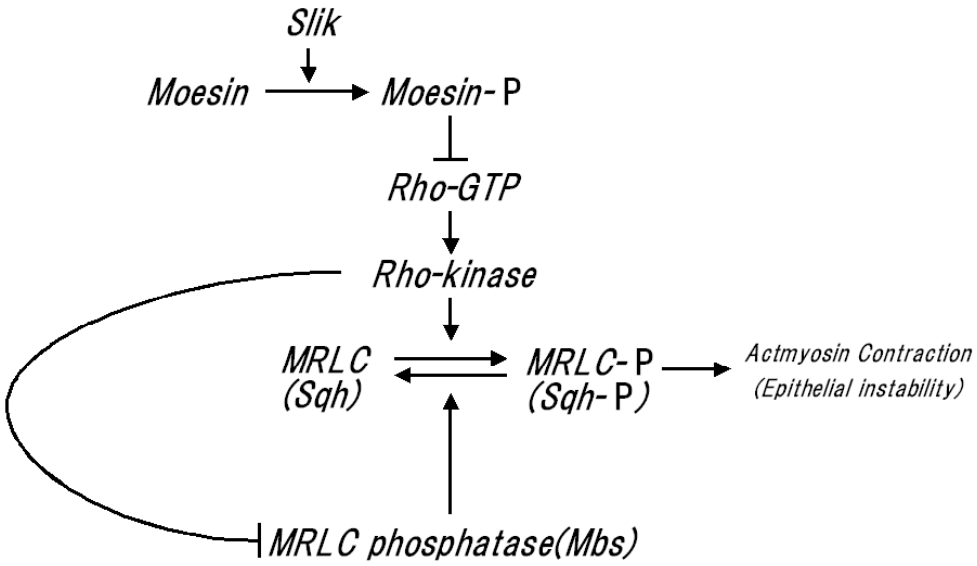

MS1096-Gal4 > sqh DD
MS1096-Gal4 > sqh WT
MS1096-Gal4 > Drok
MS1096-Gal4 > Drok KN
A
B
C
D
sqhDD
E
F
G
DE-cadherin
sqhDD
H
I
J
Discs Large
Drok
K
L
M
DE-cadherin
Drok
N
O
P
Discs Large
sqhWT
Q
R
S
DE-cadherin

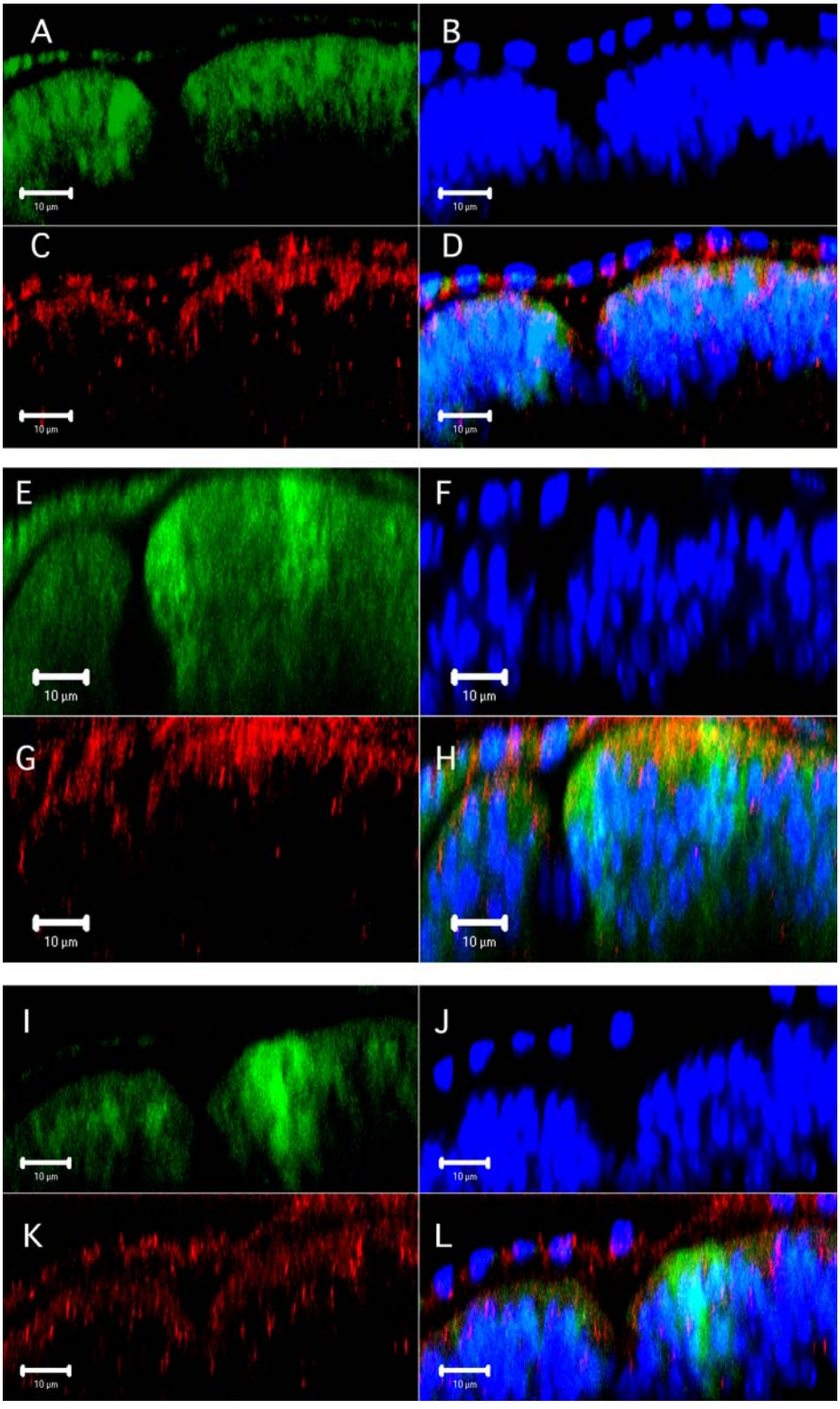
Fig. 5


A
B
Wild Type
C
D
DMBS E1
/ DMBS P2r31
E
F
patched-Gal4
> sqh DD
G
H
patched-Gal4
> Drok

Supple. Fig. 1


Slik
Moesin
Moesin-P
Rho-GTP
Rho-kinase
MRLC
(Sqh)
MRLC-P
(Sqh-P)
Actmyosin Contraction
(Epithelial instability)
MRLC phosphatase(Mbs)

Supple. Fig. 2



Supple. Fig. 3