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The F-box protein Slmb restricts activity of atypical PKC to polarize epithelial cells

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Running title: Slmb regulates basolateral polarity

ABSTRACT
The Par-3/Par-6/aPKC complex is the primary determinant of apical polarity in epithelia across animal species, but how activity of this complex is restricted to allow polarization of the basolateral domain is less well-understood. In Drosophila, several multiprotein modules antagonize the Par complex through a variety of means. Here we identify a new mechanism involving regulated protein degradation. Strong mutations in *supernumerary limbs (slmb)*, which encodes the substrate adaptor of an SCF-class E3 ubiquitin ligase, cause dramatic loss of polarity in imaginal discs, accompanied by tumorous proliferation defects. Slmb function is required to restrain apical aPKC activity, in a manner independent of endolysosomal trafficking and parallel to the Scribble module of junctional scaffolding proteins. The implication of the Slmb E3 ligase in epithelial polarity, specifically limiting Par complex activity to distinguish the basolateral domain, points to parallels with polarization of the *C. Elegans* zygote.

INTRODUCTION

Polarization is a fundamental feature of animal cells, from newly fertilized zygotes to dividing stem cells to homeostatic epithelia. This common feature is controlled by a conserved set of regulators, which segregate the single plasma membrane into several discrete domains. The most broadly used polarity regulators are the 'Par complex', consisting of the PDZ-containing scaffolds Par-3 and Par-6, which associate with Cdc42-GTP and the atypical protein kinase aPKC (Goldstein and Macara, 2007; St Johnston and Ahringer, 2010). In the *C. elegans* zygote and the Drosophila oocyte, these proteins localize to and specify the anterior cortex. In most epithelial cells and neural stem cells, they localize to and specify the apical plasma membrane, and in migrating cells they define and act at the leading edge. The Par complex thus serves as a 'master regulator' for many types of cell polarity.

To achieve and maintain polarity, the Par complex must be restrained to distinguish a complementary membrane domain. In contrast to the preeminent role of the Par complex, multiple protein modules that limit Par activity have been identified in different contexts (Tepass, 2012). In the *C. elegans* zygote, the protein kinases Par-1 and Par-4 act downstream of the RING finger protein Par-2 to antagonize Par localization and define the posterior cortex (St Johnston and Ahringer, 2010; Zonies et al., 2010). Par-1 and Par-4 are also key regulators of fly oocyte polarization, but often have less central roles in other polarized cell types (Haack et al., 2013; Partanen et al., 2013). Instead, in many of these tissues a second group of proteins, the Scribble (Scrib) module, acts to restrict the Par complex. In the Scrib module, Scrib and Dlg are basolaterally localized PDZ-containing scaffolds that regulate Lgl, a syntaxin- and myosin-binding protein that can directly antagonize aPKC (Bilder, 2004; Elsum et al., 2012). Yet another module, the Yurt/Coracle complex, specifies the basolateral domain in mid-stage Drosophila embryos and zebrafish photoreceptors (Laprise and Tepass, 2011). Rac and PI3 kinase also play a role at this stage (Chartier et al., 2011). Further, in fly epithelia but not neuroblasts, AP-2-mediated endocytosis restricts apical polarity regulators to their appropriate surface; endocytosis also plays a critical role in polarization of the worm zygote (Halbsgut et al., 2011; Shivas et al., 2010). The mechanisms by which this diverse set of proteins – which we will call 'Par or apical antagonists'—negatively regulate the Par complex are an active field of investigation. In none of these cases is the mechanism well-understood, and how they coordinate with each other remains a mystery.

Our incomplete knowledge of the mechanisms of the Par antagonists raises the possibility that additional regulators of basolateral polarity remain to be identified. Here we report that strong mutations in the F-box protein Slmb, a substrate adaptor for SCF E3 ubiquitin ligases, result in excess Par complex activity in Drosophila imaginal discs, thereby expanding the apical membrane domain. Our results indicate that Slmb-mediated protein degradation acts in parallel to the Scrib module to oppose aPKC activity and thus specify the epithelial basolateral membrane.

RESULTS AND DISCUSSION

Slmb is a novel Drosophila neoplastic TSG

To identify new regulators of basolateral polarity, we analyzed mutants isolated in a genetic screen for Drosophila tumor suppressor genes (TSGs). The screen utilized mitotic recombination to generate imaginal discs predominantly populated by homozygous mutant cells, growing in an otherwise heterozygous larva. Mutations in a small set of genes cause larval or pupal lethality in this context; many of these show a set of tumor-like phenotypes collectively called 'neoplastic' (Menut et al., 2007). Discs mutant for one uncharacterized complementation group, *MENE(3R)-B,* show multiple hallmarks of neoplastic transformation. Monolayered organization is lost, disc size is deregulated, F-actin levels are elevated and differentiation is prevented (**Fig. 1A-F).** In addition, Matrix metalloproteinase 1 (Mmp1), a mediator of tissue invasion, is upregulated (**Fig. 1A, B)**. These phenotypes closely resemble those of the Scrib module, suggesting that *MENE(3R)-B* identifies a gene with similar function.

MENE(3R)-B alleles fail to complement mutants in *slmb. Slmb* encodes an F-box and WD40-repeat protein homologous to vertebrate β-TrCP that functions as a specificity factor in a Skp-Cullin-F-box (SCF) E3 ubiquitin ligase complex (Frescas and Pagano, 2008; Jiang and Struhl, 1998; Theodosiou et al., 1998). Sequencing identified coding region lesions, including early and late truncations as well as missense mutations, in all six alleles (**Fig. 1G**), and a *slmb* transgene rescues the neoplastic phenotype (**Fig. S1**). Previously existing alleles $slmb^{1}$ and $slmb^{2}$ have been widely used, and epithelial organization phenotypes have not been reported. Sequencing revealed that these contain missense mutations in the 5th and 7th WD40 domains (**Fig. 1G**), indicating that both may be hypomorphic. We confirmed that $slmb^{1}$ and $slmb^{2}$ mutant discs show no and only a limited degree of neoplastic transformation respectively (**Fig. S1**). However, discs predominantly mutant for the deletion allele $slmb^{8}$ (Milétich and Limbourg-Bouchon, 2000) show neoplasia, confirming that this phenotype is induced only by strong alleles. Null mutations in *Roc1a*, a frequent component of SCF^{Slmb} (Noureddine et al., 2002), also show neoplasia (**Fig. S1**). These data demonstrate that *slmb* functions as a new neoplastic TSG, and suggest that it does so via its role in the SCF^{Slmb} E3 ligase.

Slmb restricts apical polarity

Known neoplastic TSGs regulate epithelial polarity. We therefore analyzed *slmb* tissue with markers for polarized membrane domains. In WT imaginal epithelia, the transmembrane proteins Cadherin 87 (Cad87) and Fasciclin III (FasIII) occupy complementary apical and basolateral membrane domains. In *slmb* cells, Cad87 is distributed ectopically around the cell circumference, in discontinuous domains that sometimes overlap with FasIII (**Fig. 2A,B**). Similar effects are seen with polarized peripheral membrane proteins Baz and Cor (**Fig. 2C,D).** Expanded apical domains of *slmb* tissue resemble those of *scrib* module mutants (**Fig. 2E**), and indicate that Slmb also acts as an apical antagonist.

To explore the breadth of Slmb function in cell polarity, we attempted to generate clones of strong alleles in other tissues. We were generally unable to recover clones in the follicle cell epithelium, and females carrying germline clones failed to produce eggs, preventing analysis of embryonic epithelia (data not shown). Clones in the larval central nervous system showed defective optic lobe organization. Intriguingly, clones derived from type I neuroblasts frequently contained multiple neuroblast-like cells, suggesting that Slmb may also be required for asymmetric cell division (**Fig. S2**) (Li et al., 2014).

Slmb does not regulate endolysosomal trafficking

Of the known Par antagonists, only the Scrib module and endocytic components have been shown to strongly regulate imaginal basolateral polarity, raising the possibility that *slmb* might act primarily via one of these pathways. We first asked whether either Scrib or endocytic components promote Slmb-mediated protein degradation. However, Arm levels (Jiang and Struhl, 1998), are not increased in *scrib* or *AP2σ* depleted tissue (**Fig. 3A-C**), nor was there evidence for misregulation of other Slmb targets (**Fig. S3**). We next asked whether Slmb might act through either the Scrib module or endocytic regulators to restrain apical polarity. To test whether Slmb interferes with AP-2-mediated endocytosis, we analyzed Notch trafficking and used a lysosomal inhibitor to monitor accumulation in the endolysosomal pathway (Windler and Bilder, 2010). Endocytic mutants prevent this accumulation **(Fig. 3E**), but in *slmb,* as in WT and Scrib module mutant discs, Notch is internalized and trafficked appropriately to endolysosomal compartments (**Fig. 3D, F, G)**. Consistent with a lack of endocytic regulation, heterozygosity for *slmb* does not enhance a weak *avalanche-RNAi* phenotype, which is sensitive to dosage of endocytic regulators of polarity (Morrison et al., 2008). Taken together, these data fail to support a general endocytic role for Slmb.

Slmb and Scrib regulate polarity via distinct but parallel pathways

To test whether Slmb might directly influence Scrib module activity, we examined protein localization. A distinctive feature of *scrib* and *dlg* mutants is that, while many proteins are mispolarized, Dlg is specifically lost from the plasma membrane of *scrib* mutants, as is Scrib in *dlg* mutants (Bilder et al., 2000). By contrast, examination of *slmb* mutant cells reveals that both Scrib and Dlg retain tight, albeit deregulated, cortical localization (**Fig. 3H-M**). Additionally, heterozygosity for *slmb* does not enhance weak *lgl-RNAi* nor *lgl* or *dlg* hypomorphic phenotypes, all of which are sensitive to dosage of Scrib module components (Morrison, 2010). Thus, despite the many phenotypic similarities, the failure to control Scrib and Dlg membrane recruitment and the lack of genetic interaction suggest that *slmb* regulates polarity in parallel to the Scrib module.

Misregulation of known substrates cannot account for the Slmb null phenotype.

The strong neoplastic phenotype seen in *slmb* tissue points to the existence of a polarity-regulating substrate whose levels must be controlled. We

therefore examined known Slmb substrates to see whether any could account for this phenotype (**Fig. S4**). Both Armadillo (Arm) and Cubitus Interruptus (Ci) are subject to proteolytic regulation by Slmb. Cells individually or co-expressing active, non-degradable forms of Arm and Ci displayed a degree of hyperplastic overgrowth, consistent with known roles in the imaginal disc, but retain normal polarity, tissue architecture and do not upregulate MMP1. Overexpression of stabilized Plk4 and Cap-H2 caused no growth or polarity phenotypes (Buster et al., 2013; Rogers et al., 2009). Overexpression of a stabilized form of the polarity kinase Par-1, recently shown to be a Slmb substrate at Drosophila synapses, also failed to phenocopy *slmb* loss (Lee et al., 2012). This suggests that an unidentified Slmb substrate normally regulates epithelial organization in imaginal discs.

aPKC is required for *slmb*-mediated neoplastic transformation

One attractive candidate for a target of Slmb-mediated polarity regulation is the Par complex component aPKC. Overexpression of activated aPKC is sufficient to expand the apical domain and confer neoplastic phenotypes similar to those of *slmb* tissue **(Fig. 4O)** (Eder et al., 2005). Intriguingly, one predicted isoform of aPKC (aPKC-G) contains two Slmb binding degrons and, when expressed in S2 cells, is degraded in a *slmb*-dependent manner (**Fig. S4**). However, *aPKC-G* transcripts in L3 discs are very low, and expression of a degron-lacking aPKC-G had no effect on disc polarity or growth (**Fig. S4**). We then used an antibody directed against a shared protein region to analyze total aPKC and found that it is mispolarized in *slmb* tissue and more widely distributed around the plasma membrane (**Fig. 4A,B**). However, aPKC levels are not obviously elevated by immunohistochemistry when compared to neighboring WT cells, in contrast to the evident elevation seen with Arm (**Fig. 3A).** We quantitated western blots of disc lysates which indicated a modest but not significant elevation of aPKC in *slmb* versus WT (**Fig. 4C)**. These data suggest that, while aPKC is mislocalized in *slmb* cells, it is unlikely to be a target of *slmb*-mediated degradation.

Despite the absence of elevated levels of aPKC, we found multiple signs of increased aPKC activity in *slmb* discs. Excessive aPKC activity drives neoplastic overgrowth in part through upregulation of JAK-STAT pathway ligands, mediated by the Yorkie transcription factor (Doggett et al., 2011; Robinson and Moberg, 2011; Sun and Irvine, 2011). *slmb* discs show robust activation of a STAT signaling reporter, and their neoplastic phenotype is sensitive to the dosage of *yorkie* (**Figs. 4D-G)**. A second aPKC-regulated process is seen upon overexpression of the Crb intracellular domain in photoreceptors (**Fig. 4H, I)** (Tanentzapf and Tepass, 2003); the resultant morphogenetic defects are dependent on aPKC (**Fig. 4J)**. While heterozygosity for *slmb* does not enhance endocytic or Scrib module phenotypes, it does robustly enhance Crb overexpression (**Fig. 4K)**. Finally, elevated aPKC activity is sufficient to induce trafficking defects of the retromer-dependent transmembrane cargo Wntless (Eaton, 2008), leading to a distinctive subcortical trapping phenotype (**Fig. 4L, N**; PM localization=73+/-12% for WT vs. 52+/-13%

for aPKC^{Act}**)** (GdV and D.B., unpublished); *slmb* mutant tissue phenocopies this trapping (**Fig. 4M;** PM localization=44+/-25%; p<2x10^{-5} vs WT, 0.14 vs aPKC^{Act}**)**.

To directly test the functional involvement of hyperactive aPKC, we reduced aPKC activity in *slmb* cells using a weak dominant negative construct (aPKC^{DN}) that has no effect on WT cells but can suppress phenotypes driven by elevated aPKC activity (Sotillos et al., 2004). Strikingly, expression of aPKC^{DN} in *slmb* clones strongly suppressed tumorous growth (**Fig. 4P-S**). The resultant clones were smaller than WT clones, suggesting that aPKC activity also promotes *slmb* survival in this context, perhaps because tumorous *slmb* cells are 'addicted' to oncogenic aPKC, whose excess activity allows them to survive in the presence of other misregulated *slmb* substrates. This reliance demonstrates a specific requirement for aPKC in *slmb* tissue, and, along with the above results, reveal that Slmb acts as a negative regulator of aPKC activity.

CONCLUSIONS

Here we extend the mechanisms involved in epithelial polarity to include a new function: targeted protein degradation. Targeted degradation can create spatial asymmetries in protein distributions, and there is precedent for roles of E3 ubiquitin ligases, including SCF^{Slmb} (Li et al., 2014; Morais-de-Sá et al., 2013), in polarizing different aspects of cells. The involvement of Slmb in Drosophila apicobasal polarity has gone unnoticed due to the previous use of hypomorphic alleles. The strong alleles described here display potent expansion of the apical pole of imaginal epithelia, demonstrating that Slmb is a new polarity regulator that functions to restrict the apical domain.

Loss of Slmb phenocopies the polarity defects associated with mutations in two classes of 'apical antagonists': the Scrib module of core polarity regulators, and endocytic regulators that control trafficking through the early endosome. Despite the similar polarity defects, *slmb* mutations do not alter endolysosomal cargo traffic, nor do they display protein recruitment defects characteristic of Scrib module mutants; furthermore, genetic interactions with either pathway are not seen. Nevertheless, the downstream consequences of polarity misregulation –including tumor-like transformation and upregulation of specific target genes— are again shared between *slmb* and the other apical antagonists, and moreover *slmb* and Scrib module mutant cells share a distinctive trafficking defect associated with elevated aPKC activity. We therefore suggest that Slmb acts in parallel to the Scrib module to antagonize the Par complex and other apical regulators.

The role for Slmb defined here points to the existence of an apical polarity-regulating protein substrate whose levels must be controlled. We have ruled out a number of validated Slmb substrates as the relevant target. Bioinformatic scans of Drosophila proteins for Slmb degron sequences suggest other candidates including Expanded (Ex), but overexpression of Ex is not sufficient to induce polarity defects resembling those of *slmb* (Blaumueller and Mlodzik, 2000; Fernández et al., 2011). Although we cannot rule out that elevation of multiple substrates contributes, *slmb*-like polarity phenotypes are capable of being induced by elevated activity of several single proteins, including Crb and aPKC.

Despite evidence that aPKC undergoes ubiquitin-mediated degradation in embryos (Colosimo et al., 2009), neither aPKC nor Crb levels appear to be controlled by Slmb-mediated degradation in imaginal discs. Nevertheless, our data together suggest that whatever the substrate of Slmb in polarity regulation is, it will function as a positive regulator of aPKC-driven outcomes.

Our demonstration that Slmb limits aPKC activity to distinguish the epithelial basolateral domain reveals intriguing parallels to polarization of the worm zygote. In this context, Par-2 is the primary antagonist that restricts aPKC/Par activity, while Lgl homologs function in a parallel redundant role. Par-2 contains a RING finger domain characteristic of single-subunit E3 ligases, but Par-2 homologs have not been identified outside of nematodes, Par-2 does not affect aPKC/Par levels and a degraded substrate in polarity regulation has yet to be identified (St Johnston and Ahringer, 2010; Zonies et al., 2010). The discovery of a Drosophila E3 ligase with a similar function to Par-2 raises the possibility of a conserved molecular logic to polarity in these two paradigmatic systems; determination of the relevant substrate will shed further light on this question.

MATERIALS AND METHODS

Predominantly mutant imaginal discs were generated as in (Menut et al., 2007). *Slmb* mutant images represent *slmb*^{UU11} unless otherwise specified. Western blots loaded with equivalent protein concentrations were probed with anti-βtubulin and anti-aPKC. Two biological replicates for each of six WT and four mutant technical replicates were quantitated. Image quantitation used Fiji (Schindelin 2012): cortical fluorescence intensity plots measured gray values along single cell tracings, while Wls cortical localization measured correlation coefficients with phalloidin staining. See **Supplementary Material** for more details.

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FOOTNOTES

Competing interests statement

The authors declare no competing financial interests.

Author contributions

LCS, SLW, and DB designed the research; LCS, GdV, GCR and SLW performed the experiments; LCS, GdV, SLW, GCR and DB analyzed the data; LCS, GdV, SLW, and DB wrote the manuscript.

FIGURE LEGENDS

Figure 1. *slmb* is a novel neoplastic tumor suppressor gene.
(**A-D**) *slmb* wing discs show upregulation of Mmp1 (green) and loss of epithelial organization (DNA, blue; F-actin is red in all figures). (**E, F**) *slmb* eye discs fail to differentiate (Elav, cyan). (**G**) Schematic of Slmb and identified lesions (*MENE(3R)-B* alleles in violet). Scale: A, E=50 μm, C=10 μm.

Figure 2. Slmb represses apical polarity. WT and *slmb* wing discs stained for TM proteins Cad87 (apical, green) and FasIII (basolateral, magenta) **(A, B)**, or peripheral proteins Baz (apical, green) and Cor (basolateral, red) **(C, D)**. In the absence of Slmb, distinct domains are lost and apical proteins expand around the cell cortex. Quantitation of Baz and FasIII staining along PM profiles of representative WT, *slmb* and *dlg* cells (**E**) documents apical expansion. Scale=5 μm.

Figure 3. Slmb is not a regulator of endolysosomal traffic and acts in parallel to the Scribble module. (**A-C**) *slmb* cells exhibit increased Arm, but *dlg* and *AP-2σ* do not. Inset shows clonal boundaries. (**D-G**) Wing discs cultured with lysosomal inhibitor and stained for Notch. Notch is trapped prior to the lysosomal accumulation in *shibire* tissue, but in *slmb* or *dlg* tissue is trafficked similar to WT. (**H-M**) Scrib and Dlg remain cortical in *slmb* mutants, but are lost from the plasma membrane in *dlg* or *scrib* mutants. Scale: A=25 μm; D, H=10 μm.

Figure 4. Slmb limits aPKC activity to prevent neoplasia. (**A, B**) aPKC localization is expanded in *slmb* cells. (**C**) Western blots of WT and *slmb* wing discs. aPKC levels are normalized to β-tubulin and quantitated. (**D, E**) A JAK/STAT pathway reporter (blue) is elevated in *slmb*. (**F, G**) The *slmb* neoplastic phenotype is sensitive to levels of *yki*. (**H-K**) Crb^{intra} expression in developing photoreceptors causes eye defects. This phenotype reflects excess aPKC activity, and is enhanced by loss of one copy of *slmb*. PL=degree of pupal lethality. (**L-N**) Wntless (Wls, cyan) is cortical in WT cells but accumulates subcortically in *slmb* mutant and activated aPKC-expressing cells. **(O)** Neoplastic transformation driven by excess aPKC activity in the wing pouch. (**P-S**) *slmb* MARCM clones (GFP positive) grow larger than WT clones but are strongly reduced when $aPKC^{DN}$ is expressed. $aPKC^{DN}$ alone does not affect growth or survival. Scale: A, L=10 μm; D=100 μm, H=400 μm.

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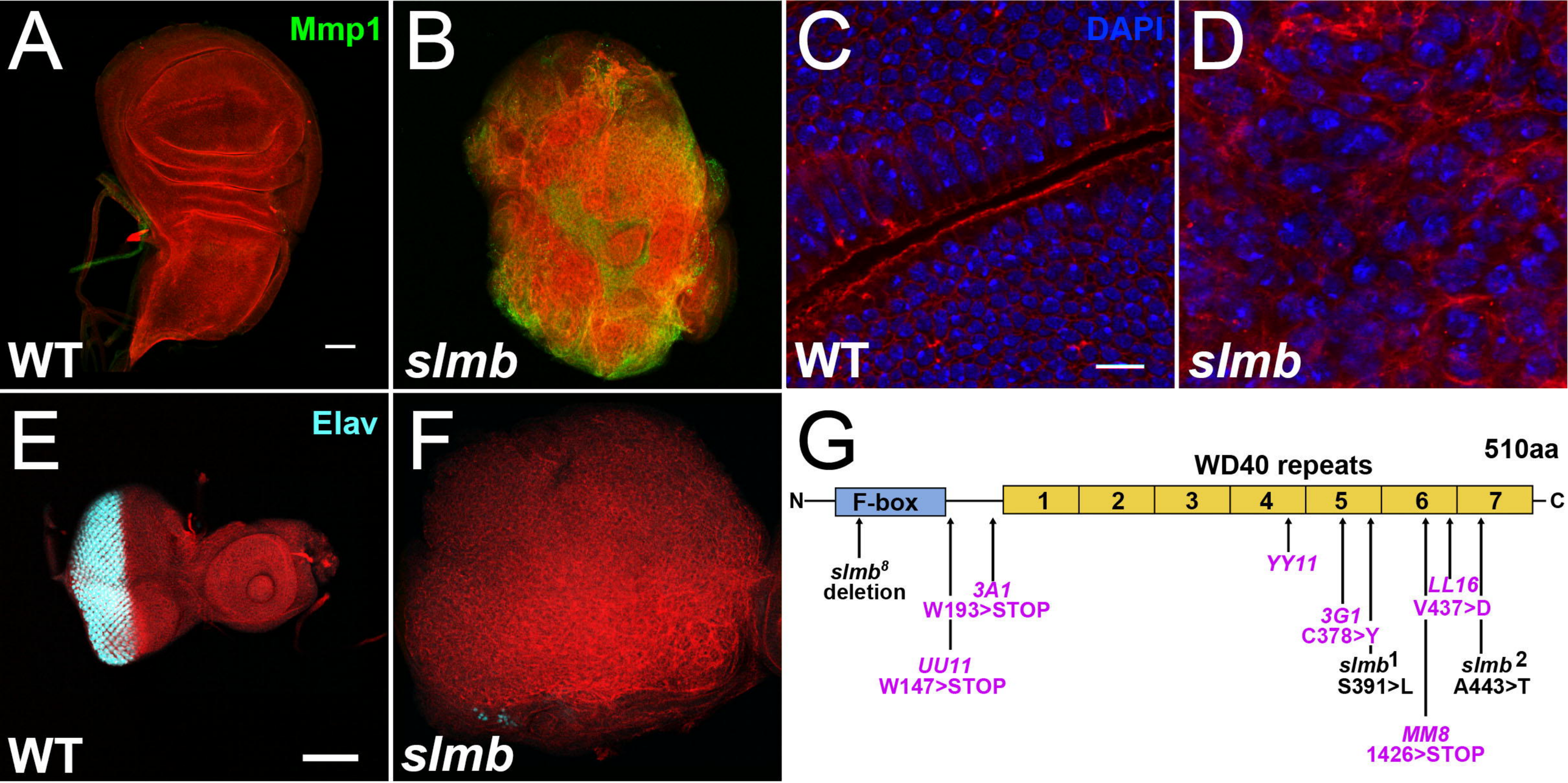
A
Mmp1
WT
B
slmb
C
DAPI
WT
D
slmb
E
Elav
WT
F
slmb
G
510aa
WD40 repeats
N
F-box
1
2
3
4
5
6
7
C
slmb8
deletion
3A1
W193>STOP
UU11
W147>STOP
YY11
3G1
C378>Y
slmb1
S391>L
LL16
V437>D
slmb2
A443>T
MM8
1426>STOP

A
Cad87
FasIII
WT
A’
B
Cad87
FasIII
slmb
B’
C
Baz
Cor
WT
C’
D
Baz
Cor
slmb
D’
E
WT
FasIII
Baz
Fluorescence intensity
Cell perimeter (μm)
slmb
FasIII
Baz
Fluorescence intensity
Cell perimeter (μm)
dlg
FasIII
Baz
Fluorescence intensity
Cell perimeter (μm)

A
WT clone
Arm
slmb
B
scrib
C
AP2-σ
D
Notch
WT
E
shibire
F
slmb
G
dlg
D’
Notch
E’
F’
G’
H
Scrib
WT
I
slmb
J
dlg
K
Dlg
WT
L
slmb
M
scrib

A
slmb clone
aPKC
WT
B
aPKC
slmb
C
WT
slmb
WT
slmb
75kDa
aPKC
50kDa
β-tubulin
Normalized aPKC levels
p = 0.34
1.00
1.29
WT
slmb
D
STAT reporter
WT
E
slmb
F
Mmp1
slmb
G
yki/+;slmb
H
PL=0%
I
PL=13%
J
PL=3%
K
PL=41%
WT
GMR>Crb^{intra};UAS GFP
GMR>Crb^{intra};UAS aPKC$^{D/N}$
GMR>Crb^{intra};slmb/+
L
Wls
WT
M
slmb
N
aPKC^{Act}
O
aPKC^{Act}
P
clones
WT
Q
slmb
R
aPKC$^{D/N}$;slmb
S
aPKC$^{D/N}$