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Article

Regulated and Polarized PtdIns(3,4,5)P3 Accumulation Is Essential for Apical Membrane Morphogenesis in Photoreceptor Epithelial Cells

Noelia Pinal,[1] Deborah C.I. Goberdhan,[3] Lucy Collinson,[1] Yasuyuki Fujita,[1,2] Iain M. Cox,[3] Clive Wilson,[3] and Franck Pichaud[1,3,*]

[1] Medical Research Council Laboratory for Molecular Cell Biology and
Cell Biology Unit
Department of Anatomy and Developmental Biology
[2] Department of Biology
University College London
Gower Street
WC1E 6BT London
United Kingdom
[3] Department of Human Anatomy and Genetics
University of Oxford
South Parks Road
OX1 3QX Oxford
United Kingdom

Summary

Background: In a specialized epithelial cell such as the *Drosophila* photoreceptor, a conserved set of proteins is essential for the establishment of polarity, its maintenance, or both—in *Drosophila,* these proteins include the apical factors Bazooka, *D*-atypical protein kinase C, and *D*-Par6 together with *D*-Ecadherin. However, little is known about the mechanisms by which such apical factors might regulate the differentiation of the apical membrane into functional domains such as an apical-most stack of microvilli or more lateral sub-apical membrane.

Results: We show that in photoreceptors Bazooka (*D*-Par3) recruits the tumor suppressor lipid phosphatase PTEN to developing cell-cell junctions (*Zonula Adherens*, *za*). *za*-localized PTEN controls the spatially restricted accumulation of optimum levels of the lipid PtdIns(3,4,5)P3 within the apical membrane domain. This in turn finely tunes activation of Akt1, a process essential for proper morphogenesis of the light-gathering organelle, consisting of a stack of F-actin rich microvilli within the apical membrane.

Conclusions: Spatially localized PtdIns(3,4,5)P3 mediates directional sensing during neutrophil and *Dictyostelium* chemotaxis. We conclude that a conserved mechanism also operates during photoreceptor epithelial cell morphogenesis in order to achieve normal differentiation of the apical membrane.

Introduction

The appearance of epithelia represents a crucial step during evolution in that it permits the elaboration of organs with an inside compartment (lumen) separated from the outside. Epithelial cells in the gut or kidney are capable of directionally transporting solutes. To perform this function, these cells have evolved a specialized and extended apical membrane domain. This domain, the brush border, faces the lumen and consists of a stack of microvilli. Other epithelial cells, such as the fly photoreceptor, are specialized in light detection. According to the same principle, they are endowed with an apical organelle, which also consists of a stack of microvilli, the rhabdomere, and which in this case is involved in collecting photons [1].

The fruit fly embryonic epithelium has been successfully used to study the specification of cell polarity, and factors that are crucial for generating functional polarization have been identified. Although Bazooka (Baz, *D*-Par3) is detected in the *D*-Ecadherin-containing adherens junction (AJ) early during cellularization [2, 3], it is later found in the sub-apical region, apical to the AJ, together with *D*-Par6 and *D*-aPKC (*Drosophila* Partitioning defective 6/*Drosophila* atypical protein kinase C) [3]. These conserved factors, together with Crumbs (Crb), *D*-Pals1-associated tight junction protein (*D*-PATJ), and Stardust (Sdt), have been shown to be crucial for defining apico-basal polarity and for the formation of junctional complexes between neighboring cells not only in invertebrates but also in vertebrates [4–7]. In turn, AJs play a crucial part in preserving the integrity of epithelial sheets. Indeed, loss of cell-cell adhesion is a major factor in carcinogenesis [5].

Fly photoreceptors are specialized epithelial neurons that differentiate from a columnar epithelium, and their morphogenesis requires appropriated specification and maintenance of their apico-basal polarity [8, 9]. Just like an epithelial-cell brush border, the microvilli-containing rhabdomere is restricted to the apical-most membrane. Similar to the situation found in the fly embryo during cellularization, Baz is localized in the developing photoreceptor AJs, basal to *D*-Par6, *D*-aPKC, and the Crb complex (Crb/*D*-PATJ/Sdt) [10]. Only later during development, Baz relocalizes more apically to the nascent rhabdomere. In this context, Crb, *D*-PATJ, and Sdt are important for the differentiation of the sub-apical membrane (stalk membrane, found directly apical to the *za*) and to some extent for the morphogenesis of the rhabdomere [8–10]. However, it is unclear how the apical factors mentioned above are coupled to the production of the specialized apical membrane domain.

Among the mutations that have been reported to alter the morphology of the photoreceptor rhabdomere are mutations in the gene encoding the tumor suppressor and lipid phosphatase PTEN (Phosphatase and Tensin homolog) [11]. *PTEN* is frequently inactivated in a wide range of sporadic human tumors, especially in glioblastomas and endometrial carcinomas [12, 13]. Although PTEN has been placed in the family of dual-specificity phosphatases (able to phosphorylate tyrosine, threonine, and serine), in vitro experiments have directly demonstrated that its major substrates are 3-phosphoinositides,

*Correspondence: f.pichaud@ucl.ac.uk

particularly phosphatidylinositol-3,4,5-*tris*-phosphate (PtdIns(3,4,5)P3), a major product of PI3-kinase. Thus, PTEN is antagonistic to PI3-Kinases activity [14–17] by preventing the accumulation of PtdIns(3,4,5)P3 at the plasma membrane.

The analysis of directed cell migration has provided some major insights into the molecular mechanisms by which cells can establish polarity. In migrating *Dictyostelium*, cell polarity is achieved through the accumulation of phosphatidylinositol-3,4,5-*tris*-phosphate (PtdIns(3,4,5)P3) at the leading edge [18]. Neutrophils [19] and macrophages [20] also employ similar mechanisms for chemotaxis. Interestingly, several PH-domain-containing proteins, such as protein kinase B (PKB/AKT), are found to associate preferentially with the plasma membrane at the leading edge in mammalian neutrophils and fibroblasts [21, 22]. In this system, the action of two antagonistic enzymes, PI3-Kinase and PTEN phosphatase, lead to a localized accumulation of the phosphoinositol lipid PtdIns(3,4,5)P3 at the leading edge of cells responding to chemotatic stimuli [18–20], which plays a major role in polarizing the F-actin cytoskeleton. We therefore explored whether PtdIns(3,4,5)P3 might regulate polarized epithelial-cell morphogenesis by using the photoreceptor as a model system.

Results

PtdIns(3,4,5)P3 Is Localized within the Developing Photoreceptor Apical Membrane

We first examined the distribution of PtdIns(3,4,5)P3 in the developing photoreceptor by using a PH-GFP transgene containing GFP fused to the PtdIns(3,4,5)P3-specific pleckstrin homology domain from cytohesin/GRP1 [23]. A major accumulation of PtdIns(3,4,5)P3 was detected in the apical membrane of the developing photoreceptor at the onset of microvilli morphogenesis (37% through pupal development [pd]; compare Figures 1A and 1D; Figure 4A shows that at this stage only a few membrane ruffles are observed, indicating that PH-GFP accumulation is not due to a general increase in membrane area). At this time, the apical domain also contains the Crb/*D*-PATJ/Sdt polarity complex as well as *D*-Par6 (Figures 1E and 1F; data not shown), whereas Baz is found in the *za* (Figures 1B and 1E). Levels of PtdIns(3,4,5)P3 reporter continued to increase throughout the rest of pupal development (Figures 1G and 1J). By mid pupation, the PtdIns(3,4,5)P3 reporter was detected in the whole apical membrane, whereas the Crb/*D*-PATJ/Sdt complex became restricted to the subapical stalk membrane immediately apical to the *za* (Figures 1G–1I; location marked in Figures 4B–4C). During the last third of pupal development, although the length of the microvilli increases, the accumulation of PH:GFP was predominantly detected in the developing microvilli and not in the Crb/*D*-PATJ/Sdt-containing stalk membrane (Figures 1J–1L). Interestingly, no major overlap was seen between the PH-GFP-positive apical membrane and the cell-cell junctions (*za*) visualized either with Baz (Figures 1D–1I) or Arm (*Drosophila* β-catenin; data not shown) during any stage of pupal photoreceptor morphogenesis.

PTEN Is Recruited to the Developing *za* through the Apical Determinant Bazooka

In polarized migrating cells, spatially localized PtdIns(3,4,5)P3 is generated through the activity of two antagonistic enzymes, PI3 kinase at the leading edge and the PtdIns(3,4,5)P3 3-phosphatase PTEN [24], which is largely excluded from this edge. *Drosophila* PTEN is found as three splice variants (PTEN1–3), with only PTEN2 containing a C-terminal PDZ binding domain like mammalian PTEN does. Using specific antibodies that we and others [25] raised against PTEN, we could not detect endogenous PTEN expression in fly tissues, including the eye. To circumvent this problem, we generated N-terminal GFP fusions for the two most common isoforms, PTEN2 and PTEN3. Both transgenes were less active in growth-inhibitory assays than non-fusion PTEN proteins, but like these latter proteins [11], they were able to rescue the lethal and cell-growth phenotypes produced by *PTEN* loss of function. Furthermore, in early embryos, overexpressed PTEN2:GFP, but not PTEN3:GFP, was enriched in the apical cortex of the epidermis, as was the case with overexpressed PTEN2 [25]. These results indicate that the fusion did not affect this protein's localization. When expressed in the developing fly eye via the pan-retinal GMRGal4 driver, PTEN2:GFP and PTEN3:GFP localized to photoreceptor nuclei and more weakly in the cytosol (Figures 2A–2F). However, unlike PTEN3:GFP, PTEN2:GFP, the isoform containing a PDZ binding domain, was first clearly enriched together with Baz within the apical membrane of the newly specified photoreceptors (Figures 2A–2C). More importantly, as pupal development proceeds, PTEN2:GFP was then found at the developing *za* (Figures 2D–2I). Finally, toward the end of pupal development (60% pupation), after microvilli have been established in the apical membrane [26], both PTEN2:GFP and Baz started to be lost from the *za* (Figures 2J–2L). At this stage PTEN2:GFP was enriched in the apical nascent rhabdomere, and its localization only partially overlapped with that of Baz, also found in the apical cortex (see also Figure 1K).

High levels of *za*-localized PTEN2 probably explain why PtdIns(3,4,5)P3 is apparently excluded from this region during the first half of pupal development. However, PtdIns(4,5)P2, the product of PTEN, might be expected to be present or even enriched at *za*. To test this hypothesis, we used a PtdIns(4,5)P2-specific PH-GFP fusion protein that we generated by using the PH domain of PLCγ and expressed under Gal4/UAS control (a similar construct has previously been used by von Stein et al. [24] for the same purpose in embryos). As expected, before the onset of microvilli induction within the apical membrane, at stages when *za* are first forming, we found that elevated PLCγPH-GFP colocalized with markers of the developing *za* (Figures 1M–1O), where PTEN2 is already enriched. In addition, PtdIns(4,5)P2 visualized by PLCγPH-GFP could also be detected at lower levels within the basolateral membrane. Slightly later, during photoreceptor morphogenesis, *za*-produced PLCγPH-GFP staining continued to be observed at the *za*, but it also accumulated within the differentiating apical membrane (Figures 1P–1R and data not shown), perhaps partly through diffusion from the *za* source of PtdIns(4,5)P2.

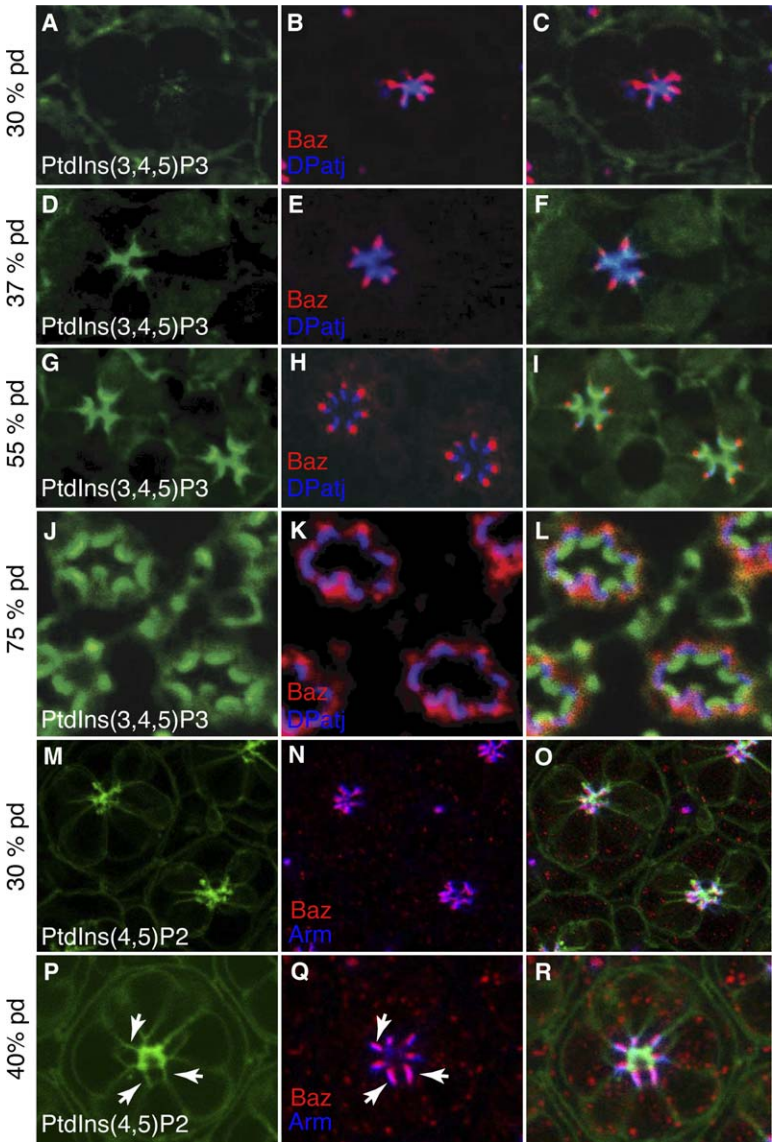

Figure 1. PtdIns(3,4,5)P3 Accumulates in the Developing Rhabdomere

(A–L) Localization and time course of PtdIns(3,4,5)P3 (A–L) and PtdIns(4,5)P2 (M–R) biosynthesis revealed with PH-GFP transgenes (green). (A) Before the onset of microvilli morphogenesis, little PtdIns(3,4,5)P3-specific PH:GFP fusion protein is detected in the apical membrane of the developing photoreceptor costained in blue with *D*-PATJ. Baz (red) labels the *za* (B and C). At the onset of microvilli morphogenesis (D–F), the PH:GFP fusion protein is detected in the apical membrane, where it is found together with *D*-PATJ (blue), apical to the *za* labeled with Baz (red). By mid-pupal life (G–I), staining is localized in the nascent rhabdomere and in the stalk membrane, costained with *D*-PATJ. No significant levels of the PH:GFP fusion protein are detected in the *za* (F and I). In late pupae (J–L), the PtdIns(3,4,5)P3 marker is concentrated in the rhabdomere, whereas Baz (red) is mostly within the cortical cytoskeleton, below the developing rhabdomere. The PtdIns(3,4,5)P3 reporter is largely excluded from the *D*-PATJ-labeled stalk membrane at this stage. Before the onset of microvilli induction (M–O), the PtdIns(4,5)P2 marker is detected at the developing *za*, stained with Baz (red) and Arm (blue). Later in development, it is enriched in the developing *za* (arrows) when compared to the basolateral membrane. In addition, PtdIns(4,5)P2-specific PH:GFP fusion protein is also detected at elevated levels in the apical membrane.

Targeting of mammalian PTEN to the plasma membrane is important for its function in decreasing membrane PtdIns(3,4,5)P3. Its PDZ binding domain is able to interact with a number of PDZ domain proteins, including the Membrane-Associated Guanylate Kinase hDlg [27], MAGI-1, 2, 3 [28], and the Microtubule-Associated Serine/Threonine protein kinase MAST-205 [29], but the in vivo relevance of these interactions is unclear. In addition, a recent study reported that overexpressed *Drosophila* PTEN2 localizes within the apical cortex of embryonic epithelia and neuroblasts [25], where it interacts directly with the PDZ-containing protein Baz [25]. As Baz localizes to the developing *za* of the developing photoreceptor ([30]; Figures 1B, 1E, and 1H), this factor represents a good candidate for recruiting PTEN2 to that compartment. The interaction between PTEN2 and Baz was confirmed in pull-down experiments performed with embryonic extracts and a PTEN2:GST fusion protein [31] (data not shown). In addition, overexpression of Baz was sufficient to recruit PTEN2:GFP to ectopic regions of the photoreceptor membrane where Baz was mislocalized (Figure 3A–3C) together with Arm (data not shown). In photoreceptors mutant for *baz*, no accumulation of PTEN2:GFP was detected at the developing *za* in the first half of pupal development (Figures 3D and 3E). Loss of *baz* function ultimately leads to disruption of the *za* [30], so in the mutant, failure to localize PTEN2 normally could be an indirect result of defective junctions. However, in combination with our Baz overexpression data, our results are consistent with a model in which Baz plays a direct role in localizing PTEN2 at the *za* of the developing photoreceptor.

PTEN Function Is Required for Polarized Assembly of the apical, F-Actin-Rich Light-Gathering Organelle

Analysis of the *PTEN* loss-of-function phenotype in the fly embryo revealed a role for this dual phosphatase in regulating F-actin organization during oogenesis [25]. *PTEN* mutant rhabdomeres are known to have defective morphology, and loss of *PTEN* function in the wing epithelial cells leads to occasional hair duplications [11]. Both types of defect are also observed under conditions

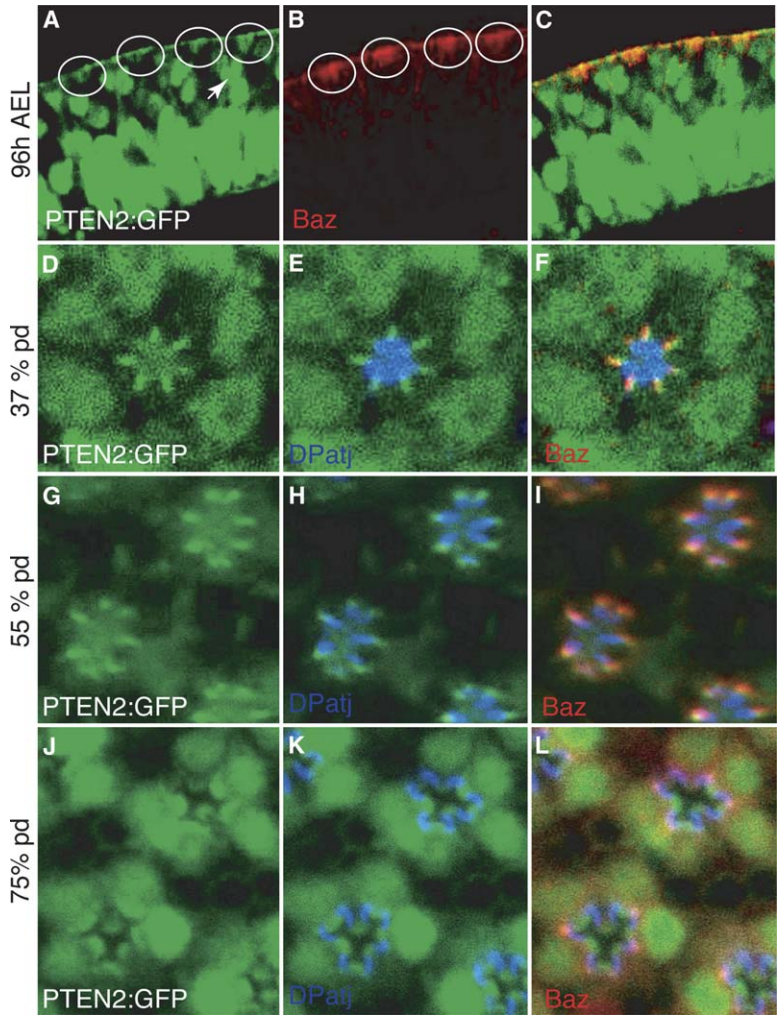

Figure 2. PTEN2 Localizes at the Developing *za*

(A–C) Like PTEN3:GFP (data not shown), PTEN2:GFP shows a persistent staining in photoreceptor nuclei and shows weaker staining in the cytoplasm. However, the PTEN2:GFP fusion protein also accumulates and colocalizes with Baz (red) within the apical membrane of the early developing retinal epithelium (96 hr after egg laying; AEL), including that of the developing photoreceptor (circled in white). (D–F) At the onset of microvilli morphogenesis, high levels of PTEN2:GFP colocalize with Baz (red) at the *za*, whereas *D*-PATJ is in the apical membrane. (G–I) By mid-pupation PTEN2:GFP is still concentrated at the *za* with Baz (red) while *D*-PATJ is restricted to the stalk membrane. (J–L) By 65% pd, PTEN2:GFP starts localizing to the nascent rhabdomere apical to the stalk membrane, labeled by *D*-PATJ, in a region that only partially overlaps with Baz.

in which the F-actin cytoskeleton is perturbed in these cells. To explore in detail the functional role of PTEN at the photoreceptor *za*, we eliminated *PTEN* function from these cells by using the eyFLP/FRT system [32]. Using both electron microscopy and phalloidin staining of the F-actin cytoskeleton, we observed major defects in apical membrane morphogenesis. Strikingly, in the distal part of the *PTEN*^{1} *and PTEN*^{3} retinas, representing more than one third (35 μm) of the total photoreceptor cell length, rhabdomere morphology was severely

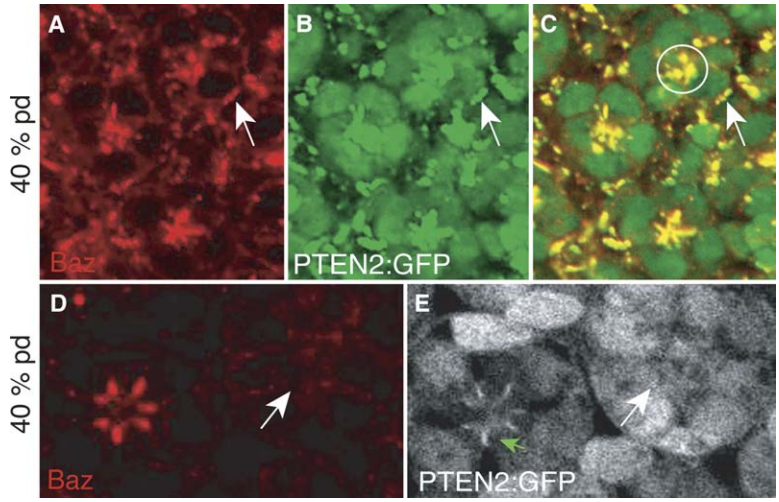

Figure 3. Baz Is Required for PTEN *za* Localization

(A–C) Ectopic expression of Baz via the pan-retina GMRGal4 driver. Both the endogenous apical membranes (circled), which are severely disorganized, and the ectopic lateral membrane domains containing Baz (red) are able to recruit PTEN2:GFP (arrow indicates an example of the latter). (D and E) *baz*^{xi106} mutant ommatidium (on right; arrow), identified by the lack of Baz staining (red in [D]) at the *za*. Note the presence of PTEN2:GFP (green arrowhead in [E]) in the wild-type photoreceptor *za* and its absence in the mutant ommatidium (arrow).

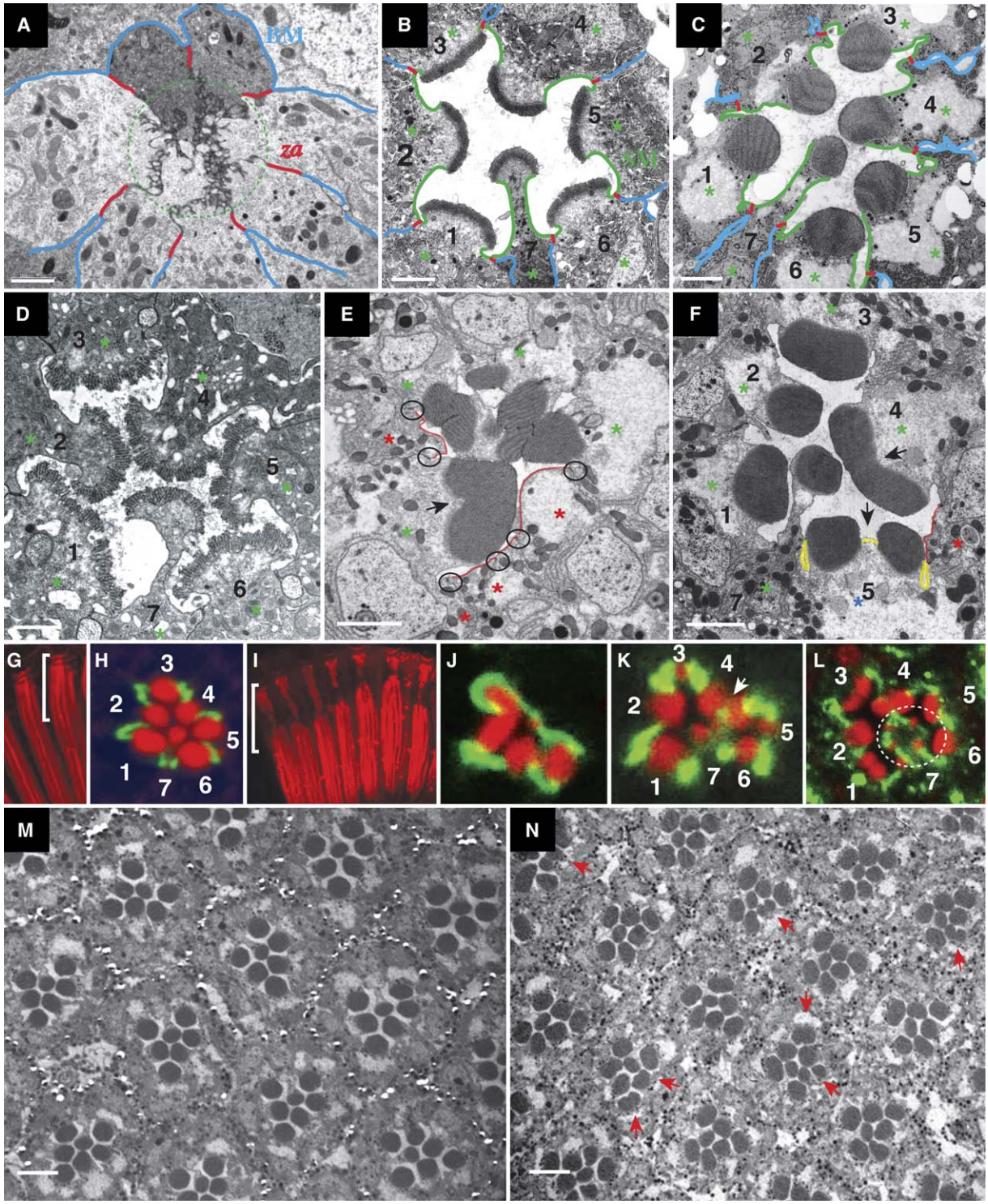

Figure 4. PTEN Regulates Rhabdomere Morphogenesis

(A) Electron micrograph of a cross-section through a wild-type ommatidium at 37% pupal development. Apical microvilli (circled in green) are readily visible; the *za*(s), which connect adjacent cells, have been highlighted in red, and the basolateral membranes (BM) are in blue.

(B) In the second half of pupal life, more microvilli are detected in the apical membrane. More laterally, the apical membrane, referred to as stalk membrane (SM), is marked in green.

(C) In the adult animal, the stalk membrane (green) supports the apical stack of microvilli bearing the visual pigment. R1–R7 are numbered; asterisks mark the photoreceptor cell bodies (B–F).

(D) A 55% ommatidium homozygous for the strong hypomorphic allele *PTEN*^{1} reveals the highly disorganized and compromised apical stack of microvilli (c.f., [B]).

(E and F) Distal and proximal sections through a single retina showing different ommatidia homozygous for *PTEN*^{1}. (E) Apical membranes (orange) of four photoreceptors (marked with red asterisks) exert a total failure of rhabdomere morphogenesis. Inter-photoreceptor *za* have

compromised, with approximately 50% of the rhabdomeres (n: 1037) absent (Figures 4E, 4I, and 4J). In the more proximal part of the retina (Figures 4F, 4K, and 4L), rhabdomeric structures were generally present, but as previously reported, they were characterized by an elliptical or misshapen morphology. About 20% of rhabdomeres were split (n: 1037). Absent or misshapen rhabdomeres (Figures 4E and 4F) are characteristic of retinas mutant for factors affecting either the cytoskeleton [33] or vesicle trafficking and membrane fusion [34], and our observations suggest that *PTEN* might be involved in one of these two processes. Related defects can also be seen in mutants associated with retinal degeneration [35]. However, we did not observe any obvious signs of photoreceptor degeneration in *PTEN* mutant retinas in a series of aged adult flies. Furthermore, consistent with a role for PTEN during photoreceptor morphogenesis, apical membrane defects could be readily detected from mid-pupal development onward (compare Figure 4D, in which the mid-pupal ommatidium already shows discontinuities in the microvilli arrangement in some photoreceptors, with Figure 4B).

In order to better understand the nature of the rhabdomeric *PTEN* phenotypes, we examined the expression pattern of factors previously shown to present similar rhabdomeric defects when mutated. In the absence of *PTEN*, we were not able to detect any gross defects in the onset and expression pattern of markers of the *za* (Arm, Baz), the terminal web that supports the rhabdomere in the cell cytosol (Phospho-Moesin [33], Myosin II [36]), or the developing microvilli (Wiskott-Aldrich syndrome protein, WASp [37]) (Figure S2 in the Supplemental Data available with this article online). However, although the apical stalk membrane markers Crb and *D*-PATJ [8, 9] remained localized within the apical membrane, we found that the split rhabdomeres, which we had previously detected by electron microscopy, were interrupted by segments of Crb/*D*-PATJ-positive (stalk-like) membrane (Figures 4K and 4L). Therefore, these epithelial sub-apical (photoreceptor stalk membrane) markers are mislocalized within the photoreceptor apical membrane. From this analysis, we conclude that *PTEN* is a key regulator of apical membrane morphogenesis in the epithelial photoreceptor. In particular, *PTEN* function is important for rhabdomere morphogenesis and the proper patterning of rhabdomeric and stalk membrane structures.

Bazooka-Dependent Localization of PTEN to the *za* Is Crucial for PTEN Function in Rhabdomere Morphogenesis

To assay the relative contribution of PTEN2 and PTEN3 to photoreceptor morphogenesis, we performed rescue experiments in whole eyes mutant for $PTEN^1$. These experiments were performed under conditions in which overexpression of the respective GFP-tagged transgenes did not produce detectable gain-of-function rhabdomeric phenotypes in normal photoreceptors. Both the PTEN2 and PTEN3 GFP-tagged isoforms were equivalent in their ability to suppress photoreceptor growth (Figure S1). However, PTEN3 failed to rescue the numerous split and misshapen apical organelles (Figure 4N), and similar to the case with $PTEN^1$ mutant retinas, at least one split rhabdomere *per* ommatidium (approximately 20% of total rhabdomeres examined; n = 350) could still be detected. Interestingly, there was a significant rescue of rhabdomeric structures at the distal portion of the *PTEN* mutant retina, suggesting that a global attenuation of PtdIns(3,4,5)P3 levels is sufficient to restore this aspect of rhabdomere morphogenesis. In contrast, PTEN2 was able to fully suppress all rhabdomeric phenotypes (Figure 4M), and the corresponding retina looked virtually wild-type.

A correct balance of PtdIns(3,4,5)P3 and PtdIns(4,5)P2 seems to be required to control morphogenesis of the apical membrane; overexpression of the more active non-GFP fusion forms of either PTEN2 or PTEN3 led to gain-of-function phenotypes in mature rhabdomeres. These were similar to those observed with *PTEN* loss of function except there were no missing rhabdomeres in the distal part of the retina (Figure S2). In this case, we also observed a significant number of missing photoreceptors (20%; n = 200), suggesting that high level of PtdIns(4,5)P2 might be toxic for these cells and could lead to apoptosis. From these data, we conclude that appropriate control of the PtdIns(3,4,5)P3 levels throughout the plasma membrane is required to optimally regulate the morphogenesis of the photoreceptor apical membrane.

The S/T Kinase AKT Acts Downstream of PtdIns(3,4,5)P3 in Regulating Microvilli Morphogenesis

Several molecules linked to cytoskeletal regulation, such as members of the Rho family of monomeric G

been circled in black. (F) An ommatidium, presenting a range of phenotypes. Most photoreceptors have misshapen elliptical rhabdomeres with a deformed rhabdomere terminal web (black arrow in [E] and [F]), a phenotype seen in all mutant *PTEN* ommatidia. The arrow in (F) points to a single photoreceptor (marked with a blue asterisk) with a split rhabdomere [approximately 20%; n = 1037] (the stalk membrane of this cell is highlighted in yellow). The apical membrane of a photoreceptor (marked with a red asterisk) where rhabdomere morphogenesis has totally failed is highlighted in orange. The scale bars (A–F) represent 2 μm.

(G) Proximo-distal confocal section of a wild-type ommatidium stained for F-actin (phalloidin, red), reveals the rhabdomeres. The distal part of the retina is indicated by a white bracket.

(H) Cross-section through a wild-type ommatidium stained for F-actin (Phalloidin, red), with *D*-PATJ (green) labeling the stalk membrane apical to the *za*. The cell bodies are numbered (1 to 7).

(I) Proximo-distal confocal section of $PTEN^1$ ommatidia stained with phalloidin. The severe rhabdomeric phenotype in the distal part of the retina is systematically detected over 35–40 μm, indicated by a white bracket.

(J–L) Confocal cross-sections of $PTEN^1$ mutant ommatidia with similar phenotypes to those seen in (E) (distal part of the retina, [J]) and (F) (more proximal section [K and L]). Phalloidin-red labels the F-actin-rich rhabdomeres, and *D*-PATJ labels the apical stalk membranes in green. Note the general disorganisation of rhabdomeric actin and stalk membrane, and note also in (K) and (L) the presence of significant *D*-PATJ staining in the apical membrane of a split rhabdomere ([K], arrow, photoreceptor R4; and [L], circled R7 rhabdomere split in four pieces).

(M and N) $PTEN^1$ mutant ommatidia expressing PTEN2:GFP (M) and PTEN3:GFP (N) using the pan-retinal Gal4 driver GMR-Gal4. Note that only PTEN2:GFP restores normal rhabdomeric morphology, whereas the rhabdomeres in (N) are abnormally elongated, deformed, or split. Scale bars represent 5 μm. Red arrows are pointing to some split rhabdomeres.

proteins, for example, have been shown to be regulated by levels of PtdIns(3,4,5)P3 [38]. However, previous work has reported that loss of *PTEN* function in *Drosophila* could be fully rescued by reducing the coupling of the downstream kinase Akt1 to its activator PtdIns(3,4,5)P3 through *Akt1* mutation [39]. We therefore examined flies in which PTEN had been fully removed (with a combination of null alleles, $PTEN^{117}$/$PTEN^{i89}$) and the amount of active Akt1 had been decreased (using a specific combination of *Akt1* alleles, $Akt1^{3}$/$Akt1^{1}$), thus rescuing the lethality [39]. Electron and confocal microscopy analysis revealed that in this genetic background, photoreceptor morphology was close to that of the wild-type and showed a low frequency of deformed rhabdomeres (compare Figures 4K and 4L with Figure 5K). These data imply that Akt1 is a major downstream PtdIns(3,4,5)P3 effector, regulating the morphogenesis of the photoreceptor apical membrane. The continuing presence of some elongated rhabdomeres (approximately 7%; n = 400) in these rescued flies suggests that the precise control of Akt activation is an important parameter or that additional effectors might also contribute to this process.

Akt1 activation is promoted by PtdIns(3,4,5)P3 and requires two successive phosphorylations, one at Thr423 and the second at Ser505 [40]. We examined the distribution of Akt1 during photoreceptor morphogenesis with an antibody that detects both activated and nonactivated protein and found that it accumulates in the *za* (Figures 5A and 5B). By contrast, at approximately 55% pupal development, the activated Ser-505 phosphorylated species was readily detected in the nascent rhabdomere of normal photoreceptors (Figure 5E, arrow). As expected, in the absence of *PTEN* function, PtdIns(3,4,5)P3 levels were dramatically increased, and in these imaging conditions the wild-type neighboring photoreceptors showed a relatively dim staining. In addition, this lipid distributed throughout the whole cell membrane, including the *za*, where PTEN2 is normally enriched (Figures 5G and 5H). Concomitantly, a massive and precocious accumulation of P-Ser505-Akt1 was observed in the photoreceptor apical membrane (and to a lesser extent in the basolateral membranes; Figures 5C–5F) and reached levels much higher than in normal photoreceptors at any stage of development. This defect could be rescued by PTEN2:GFP expression (Figures 5I and 5J). At least in pupae, PTEN3:GFP could also significantly reduce the high levels of Akt1 activation in the developing apical membrane as well as in the basolateral membrane, although ultimately this effect is not sufficiently regulated to fully rescue the *PTEN* mutant defects in adults (Figure 4N). We conclude that PTEN2 not only prevents accumulation of PtdIns(3,4,5)P3 at the developing *za* but also plays a major role in precisely controlling the level of PtdIns(3,4,5)P3-dependent activation of Akt1 within the entire photoreceptor apical membrane.

Discussion

Although localized accumulation of PtdIns(3,4,5)P3 is thought to be an essential player in generating the polarity required for directed cell migration, Par proteins have been shown to play a key role in the establishment of polarity in many other biological contexts. We report a direct connection between these two pathways in the retinal epithelium and show that, in photoreceptors, Baz recruits PTEN to the developing *za* and thus promotes PtdIns(3,4,5)P3 degradation and PtdIns(4,5)P2 biosynthesis in that membrane domain. Importantly, *za*-localized PTEN is required for precise regulation of the accumulation of PtdIns(3,4,5)P3 in the entire apical membrane (Figure 6). Although localized rhabdomeric PtdIns(3,4,5)P3 that we observe is likely to involve localized activation of PI3-Kinase, the juxtaposition of PTEN2 to the source of PtdIns(3,4,5)P3 biosynthesis appears essential for achieving the optimal fine tuning of the PtdIns(3,4,5)P3-dependent Akt1 activation. Activated Akt1 is in turn important for controlling the precise localization of Crb and *D*-PATJ within the photoreceptor apical membrane and for achieving proper microvilli morphogenesis. This could be due to a direct regulation of the Crb complex by Akt1 or to a more general role for Akt1 in apical membrane differentiation. Interestingly, Akt1 activation occurs precociously within the photoreceptor apical membrane in the absence of PTEN (Figures 5C and 5D). This is consistent with a previous report that photoreceptor differentiation is accelerated in the absence of PTEN function [41], but it may also merely reflect the fact that normal levels of Akt activation at these early stages are too low to be detected in our system. Correlating with the early onset and over-activation of Akt 1 detected in the absence of PTEN function, disruption of the microvilli is readily observed early during photoreceptor differentiation (Figure 4D).

Later, during photoreceptor morphogenesis, both Baz and PTEN2 progressively localize to the nascent rhabdomere, at a time when microvilli are extending to reach their mature length. Although the arrangement of microvilli is already defective at this stage, part of the *PTEN* mutant phenotype (i.e., deformed rhabdomeres with short microvilli) might be due to a role for PTEN during this late phase of morphogenesis. Although PtdIns(4,5)P2 can be detected in the developing *za* before the onset of microvilli induction within the apical membrane, correlating with the location of PTEN2:GFP, this phosphatidylinositol species is also found in the developing rhabdomere later in development (Figures 1M–1R). Some PtdIns(4,5)P2 may diffuse into the apical membrane from the *za*, but it is also likely to be produced by PTEN2 in the apical domain itself from 60% pupal development onward (Figures 2J–2L), perhaps maintaining a precise balance between PtdIns(3,4,5)P3 and PtdIns(4,5)P2.

Our data and those of others indicate that precise regulation of phophoinositide levels in a range of polarized cells is critical for regulating appropriate cytoskeleton-dependent responses such as microvilli morphogenesis or protruding activity in migrating cells. We have demonstrated an important role for Akt in the process of apical membrane differentiation and, in particular, rhabdomere morphogenesis. It will be interesting to test whether this molecule is also important for the directed migration of neutrophils and macrophages or for defining neuronal polarity, for which localized PtdIns(3,4,5)P3 and Par-3 are also crucial [42]. *PTEN* and *Akt* are both major effectors of the insulin signaling pathway regulating cell growth, and it is likely that this pathway is coupled to

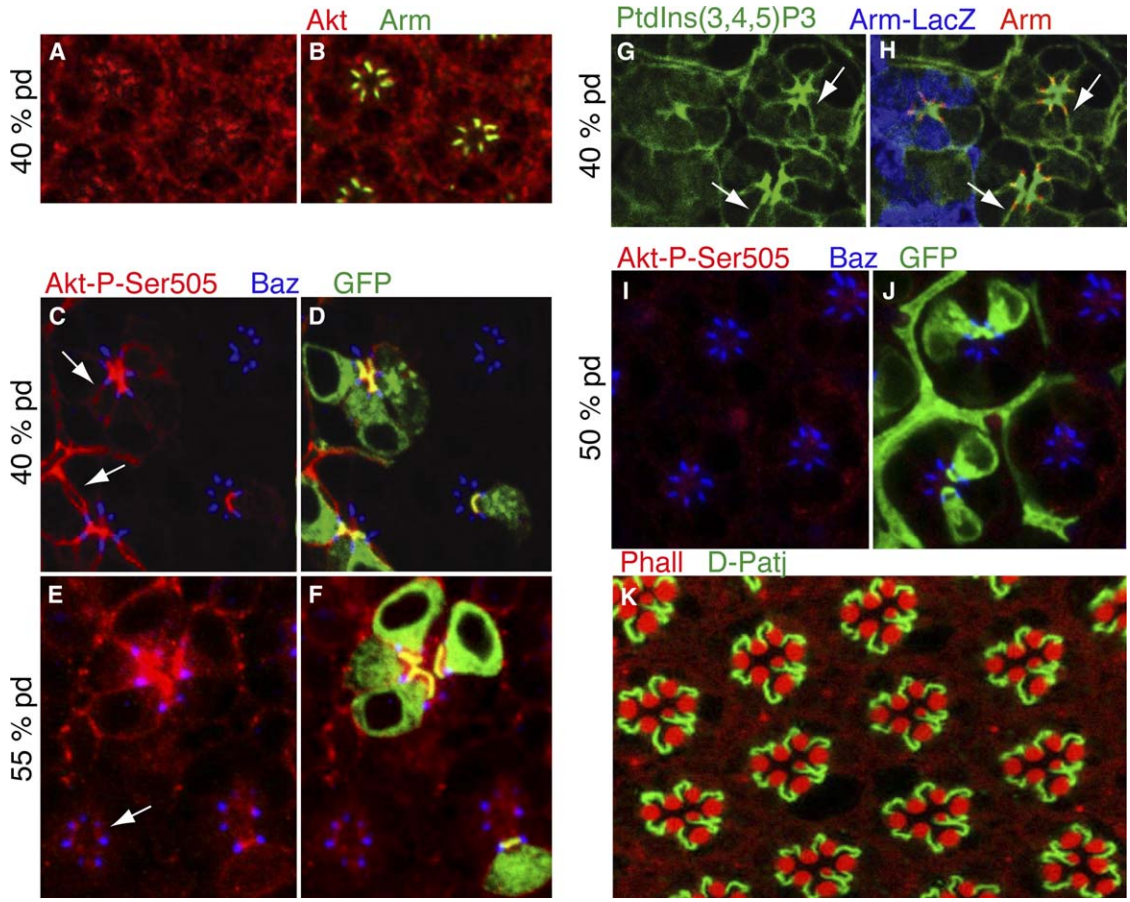

Figure 5. Ectopic Activation of Akt in the Absence of PTEN

(A and B) Akt1 (red) localizes unevenly in the cytosol but accumulates most strongly in the *za*, which is labeled with Arm (green).

(C–F) In *PTEN*^{1} mutant cells (green), P-Ser505 Akt1 (red) prematurely accumulates (C and D) in the apical and basolateral membrane ([C], arrows) at the onset of microvilli morphogenesis. By 55% pd (E and F), levels of P-Ser505 Akt1 are massively upregulated, particularly in the apical membrane of mutant cells. Wild-type photoreceptors also start accumulating P-Ser505 Akt1 at a much lower level in their apical membrane ([F], white arrow), but not in the *za*.

(G–H) Compared those of wild-type cells stained by the reporter gene *Armadillo-LacZ* (blue), *PTEN*^{1} mutant photoreceptors accumulate high levels of PtdIns(3,4,5)P3 reporter (green), particularly in the apical-most membrane. Note the presence of the PtdIns(3,4,5)P3 staining (arrows) in the *za* marked by Arm (red), and lateral membrane in the *PTEN*^{1} mutant photoreceptors.

(I and J) Cells mutant for *PTEN*^{1} (green), expressing PTEN2:GFP and a UAS-GFP marker, no longer show hyperactivation of Akt1 in the apical membrane. These transgenes are expressed in *PTEN* mutant cells via the source of Gal4 provided by the MARCM system [44]; Baz (blue) labels the *za*.

(K) A *PTEN*^{117}/*PTEN*^{i89} retina rescued by decreasing signaling to Akt1 with a specific combination of *Akt1* alleles (*Akt1*^{3}/*Akt1*^{1}). The phalloidin staining (red) reveals wild-type rhabdomeres, and *D*-PATJ (green) stains the highly organized stalk membranes (c.f., Figures 4H, 4K, and 4L).

effectors of the cell cytoskeleton to accommodate modulation of cell size. Interestingly, loss of function of TSC1 or TSC2 (tuberous sclerosis complex), important downstream targets of Akt, leads to rhabdomeric phenotypes that are very similar to that of *PTEN* and, in particular, to split rhabdomeres interrupted by segments of Crb/*D*-PATJ stalk-like membrane (F.P., unpublished data). This observation raises the possibility that the cytoskeletal or membrane effector(s), or both, of the pathway described here lie downstream of the TSC1/2 complex.

PTEN is one of the most frequently mutated genes in human cancer [12, 13]. Based on the results presented here, we propose that in addition to modulating growth and promoting cell survival, the increase in Akt activity could lead to instabilities within the apical membrane of mutant epithelial cells and that these instabilities might contribute to metastatic and invasive phenotypes by facilitating PtdIns(3,4,5)P3-dependent migratory activity.

Experimental Procedures

Fly Strains and Genetics

w^{1118} was used as the wild-type. Flies were raised on a standard cornmeal diet at 22ºC. Pupae were staged at 20ºC as previously described [33]. To generate FLP/FRT homozygous whole mutant eyes for *PTEN*, we used previously described homozygous lethal *PTEN*^{1}, *PTEN*^{3} and *PTEN*^{117} *FRT40* chromosomes [11] with an *FRT [ry, neo]40A, GMRhid, cl* chromosome [43] in the presence of an eye-specific source of recombinase (*w, eyflp*) [32]. *baz* mosaic eyes were obtained with the *baz*^{xi106} and *baz*$^{B18\text{-}9}$ alleles [30], and pUAS-Baz [5] was used for overexpression. Pupal mosaic analyses were performed via the MARCM system [44] or with an FRT40 chromosome labeled with arm-lacZ. The *PTEN*/*Akt1* rescue assay was performed as described in [39] .

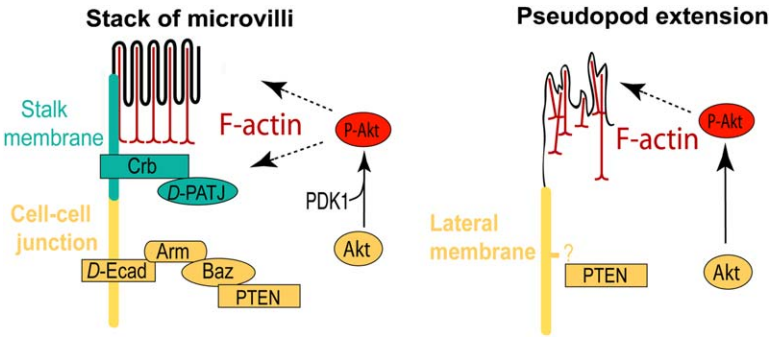

Figure 6. A Conserved Role for PI3-Kinase/PTEN in Promoting Functional Polarity

Schematic comparison of a wild-type epithelial cell such as the fly photoreceptor (left panel) and a migrating *Dictyostelium* cell (right panel). The photoreceptor cell-cell junction and the lateral membrane in *Dictyostelium* are in yellow. The apical photoreceptor membrane producing microvilli and the protruding membrane of *Dictyostelium* are in black, and the F-actin cytoskeleton is in red. In the photoreceptor, Baz requires Arm for proper localization to the *za* [47]. In turn, it is able to directly recruit an isoform of PTEN containing a PDZ binding domain (this work and [25]). The level of Akt activation in the apical membrane is restricted by *za*-localized PTEN, and this precise regulation is critical for controlling apical membrane differentiation, including the positioning of Crb/*D*-PATJ in the stalk membrane in blue (dashed arrows). This could be achieved through modulating the cortical F-actin cytoskeleton organization. By contrast, PTEN is completely excluded from the leading-edge membrane of migrating *Dictyostelium*, leading to a major accumulation of PtdIns(3,4,5)P3 and activated Akt in this domain. These effects are correlated with F-actin remodeling and are essential for promoting directional movement.

PTEN-GFP and PLCγPH-GFP Chimeras

PTEN2 and PTEN3 cDNAs carrying a NotI site at their 3′ ends were subcloned into a modified form of pEGFP-C1 (from Clontech; containing a NotI recognition sequence inserted at the 5′ NheI site), so that the final N-terminal fusion sequence from the BamHI site of pEGFP-C1 was 5′-GGATCCGCTTCAGGAGCGGCCAAC-3′ and all but the N-terminal methionine of the PTEN isoforms was included in the fusions. The fusion constructs were excised with NotI and subcloned into pUAST. A fusion between the coding sequence of the PLCγ PH domain and EGFP was also subcloned into pUAST. Transformants were generated by standard techniques. Multiple independent transgenic lines produced similar overexpression phenotypes and GFP expression patterns.

Immunostaining

Immunostaining on developing retinas and early embryos was performed as previously described [2, 33]. The following antibodies were used: rabbit anti-Akt 1/200 (Cell signaling), mouse anti-Armadillo (4C5, 1/100, Hybridoma bank), rat and rabbit anti-Bazooka 1/2000 [5], rabbit anti-βgalactosidase (Cappel, 1/4000), rat anti-Crumbs 1/500 [45], mouse anti-Crumbs (Cq4, 1/50, Hybridoma bank), rabbit anti-*D*-PATJ 1/1000 [45], rabbit anti-non muscle Myosin 1/400 [46], mouse anti-Phospho Akt 1/200 (Cell signaling), rabbit anti-Phospho Moesin 1/500 [33], rabbit anti-PTEN 1/200 [25], and rabbit anti-WASp 1/200 (gift from B. Baum), with the appropriate combination of mouse, rabbit, and rat secondary antibodies at 1/200 each (Jackson). Imaging was performed with a Biorad radiance 2100 MP confocal lens, and the images were edited with Adobe Photoshop 7.0.

Electron Microscopy

Electron microscopy was typically performed on one-day-old flies as in [18] with a transmission electron microscope Philips model EM240 (FEI, UK limited, Cambridge, UK). Films were scanned at 1000 dpi with the scanner Flexlight, Precision II, and images were edited with Adobe Photoshop 7.0.

Supplemental Data

Supplemental Data include three figures and are available with this article online at http://www.current-biology.com/cgi/content/full/16/2/140/DC1/.

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