Regulation of somatic myosin activity by Protein Phosphatase 1β controls Drosophila oocyte polarization

Yi Sun, Yan Yan*, Natalie Denef and Trudi Schüpbach†

SUMMARY

The Drosophila body axes are established in the oocyte during oogenesis. Oocyte polarization is initiated by Gurken, which signals from the germline through the epidermal growth factor receptor (Egfr) to the posterior follicle cells (PFCs). In response the PFCs generate an unidentified polarizing signal that regulates oocyte polarity. We have identified a loss-of-function mutation of flapwing, which encodes the catalytic subunit of Protein Phosphatase 1β (PP1β) that disrupts oocyte polarization. We show that PP1β, by regulating myosin activity, controls the generation of the polarizing signal. Excessive myosin activity in the PFCs causes oocyte mispolarization and defective Notch signaling and endocytosis in the PFCs. The integrated activation of JAK/STAT and Egfr signaling results in the sensitivity of PFCs to defective Notch. Interestingly, our results also demonstrate a role of PP1β in generating the polarizing signal independently of Notch, indicating a direct involvement of somatic myosin activity in axis formation.

KEY WORDS: PP1β, Myosin, Oocyte polarity, Drosophila

INTRODUCTION

Cell-cell interactions between somatic and germline cells are crucial to the formation of body axes in Drosophila. During oogenesis, the anterior-posterior (AP) and dorsal-ventral (DV) axes are established in the oocyte, which, together with the nurse cells, is surrounded by a monolayer epithelium of somatically derived follicle cells. During early oogenesis, the oocyte nucleus and a microtubule organizing center (MTOC) are localized at the posterior pole of the oocyte, with microtubule (MT) plus ends extending through the ring canals into the connected nurse cells (Theurkauf et al., 1992). The TGFα-like ligand Gurken (Grk) is localized to the posterior cortex of the oocyte and signals to adjacent follicle cells through the epidermal growth factor receptor (Egfr), also known as Torpedo (Top). In response, these follicle cells adopt a posterior cell fate (Gonzalez-Reyes et al., 1995; Roth et al., 1995). At around stage 6/7 of oogenesis, these posterior follicle cells (PFCs) send an unknown signal back to the oocyte to repolarize the oocyte, resulting in the final determination of the AP axis (Gonzalez-Reyes et al., 1995; Roth et al., 1995). This polarizing signal from the PFCs causes the disassembly of the posterior MTOC and the formation of a gradient of MTs from the anterior to the posterior of the oocyte with a concentration of minus ends now emanating from the anterior and lateral cortex of the oocyte (Clark et al., 1994; Doerflinger et al., 2010; Theurkauf et al., 1995). The TGFα-β receptor (Smad2) also controls the generation of the polarizing signal independently of Notch, indicating a direct involvement of somatic myosin activity in axis formation.

Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA.

*Present address: Institute of Neuroscience, University of Oregon, Eugene, OR 97403, USA
†Author for correspondence (schupbac@princeton.edu)

Accepted 14 February 2011
Serine/Threonine Phosphatase 1β (PP1β). We show that PP1β is required in the PFCs for axis specification in the oocyte through its function of regulating the activity of non-muscle myosin II. Strikingly, PP1β acts specifically in the PFCs to regulate endocytosis of Notch and other transmembrane proteins and, in addition, it operates as a separate and direct factor in generating the posterior polarizing signal to the oocyte. Furthermore, our results demonstrate that the particular sensitivity of the PFCs to defective Notch signaling is not caused merely by their position at the termini of the egg chamber, but results from the coordinated activation of both the JAK/STAT and the Egfr pathways. Our study establishes a novel connection between the mechanical force-generating machinery and the crucial signaling pathways controlling cell differentiation and organism development.

MATERIALS AND METHODS
Fly stocks and genetics
The βwP{P} mutation was isolated in a mosaic genetic screen in which mutations were induced by ethylmethanesulfonate (EMS) mutagenesis in y w FRT19A flies (Denef et al., 2008). Duplication and P-element lines used for mapping were obtained from the Bloomington Stock Center. Follicle cell clones were generated by e22c-Flp using the FRT/UAS-Flp/GAL4 system (Duffy et al., 1998). Eye disc clones were generated using FRT/eyFlp. Wing disc clones were generated using FRT/hsFlp. Flip-out clones were generated using y Act > FRT yellow > FRT/Gal4; hsFlp. UAS-GFP (Ito et al., 1997). UAS-Notch (Struhl et al., 1993), UAS-NEXT and UAS-NICD (Rebay et al., 1993) were expressed in follicle cell clones generated by the mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo, 1999). The Mhs alleles Mhs{P} and Mhs{799} were gifts from J. E. Treisman (Lee and Treisman, 2004).

The following reporter lines were used: kinesin-lacZ (Clark et al., 1994), pointed-lacZ (Gonzalez-Reyes and St Johnston, 1998), mirror-lacZ (Xi et al., 2003), expanded-lacZ (Hamaratoglu et al., 2006), cyclinE-lacZ (Jones et al., 2000) and diap1-lacZ (Hay et al., 1995). The following lines were obtained from the Bloomington Stock Center: flapping-HA, zip1, Rho3.10, RhoGEF204291, UAS-Rok-CAT, UAS-Rok-CAT-KG, UAS-MYPT75D and UAS-MYPT75DFI17A. Rab5-GFP (Wucherpfennig et al., 2003), Rab6-GFP (Entchev et al., 2000) and Rab11-GFP (Emery et al., 2005) were used to mark endocytic compartments. UAS-Unpaired (Harrison et al., 1998) and UAS-tdTomato (Queenan et al., 1997) were recombined to generate UAS-Unpaired, UAS-tdTomato.

Mapping of FP41
The lethal mutation FP41 was mapped by meiotic recombination with visible recessive markers to the regions between cut (7B4) and vermilion (9F11) and between P-elements PBac(WH)CG34408 (9B7) and PBac(WH)Neb-c-GFP2152 (9C4). The genomic DNA from single FP41/Y larvae was isolated. PCR products covering the flapping gene region were sequenced and compared with the sequences of the y w FRT19A control. Sequencing was repeated with three independent genomic isolates.

Immunostaining
Ovaries, eye discs and wing discs were dissected in PBS followed by fixation in PBS with 4% paraformaldehyde for 20 minutes at room temperature and staining using standard procedures (Denef et al., 2008). Samples were mounted in Aqua-polymount (Polysciences) and imaged using a Zeiss LSM510 microscope.

Primary antibodies used were: mouse anti-Grk (1:10), rabbit anti-Stau (1:2000 (St Johnston et al., 1991)), rabbit anti-beta-galactosidase (1:1000, Millipore), rabbit anti-phospho-Histone H3 (Ser28) (1:500, Millipore), mouse anti-Cut (2B10, 1:20, Developmental Studies Hybridoma Bank (DSHB)), mouse anti-Hdin (1G9, 1:20, DSHB), rabbit anti-phospho-myosin light chain II (Ser19) (1:10, Cell Signaling; Ser19 in human RMLC corresponds to Ser21 in Drosophila (Jordan and Kares, 1997)), rabbit anti-Zip (1:1000 (Foe et al., 2000)), mouse anti-NICD (C17.9C6, 1:100, DSHB), rat anti-NECD (1:50 (Klueg et al., 1998)), rabbit anti-Lava (1:250 (Sisson et al., 2000)), goat anti-Egfr (dc-20, 1:100, Santa Cruz), rabbit anti-Dome [1:200 (Devergne et al., 2007)], rabbit anti-aPKC (1:1000, Santa Cruz), rabbit anti-Baz (1:500 (Wodarz et al., 1999)), rabbit anti-Patj (1:500, (Bhat et al., 1999)), mouse anti-Arm (N2 7A1, 1:50, DSHB), rat anti-DE-Cad (DCAD2, 1:20, DSHB) and mouse anti-Dlg (1:500 (Woods and Bryant, 1991)). Secondary antibodies used were Alexa Fluor 488-, 568- or 647-conjugated (1:1000, Invitrogen). F-actin and DNA were stained with Phalloidin conjugates and Hoechst (1:1000, Invitrogen), respectively.

RESULTS
Oocyte polarity is disrupted when FP41 mutant follicle cells are present at the posterior of the egg chamber
In a genetic mosaic screen performed to identify genes regulating the morphogenesis of follicle cells (Denef et al., 2008), a lethal mutation named FP41 was isolated in which oocyte mispolarization was frequently observed in the presence of posterior mutant follicle cell clones. In wild-type egg chambers after stage 7, the oocyte nucleus and Gurken (Grk) RNA and protein relocalize from the posterior pole to a dorsal-anterior corner of the oocyte in response to the polarizing signal from the PFCs (Fig. 1A). When the PFCs are homozygous mutant for FP41 (as identified by the absence of GFP), the oocyte nucleus and Grk often failed to relocalize and remained at the posterior pole of the oocyte (Fig. 1B; 50%, n = 105). To characterize further the oocyte polarity defect in FP41, we examined the position of Staufen (Stau), an RNA-binding protein required for the localization of oskar (osk) mRNA to the posterior pole of the oocyte (St Johnston et al., 1991). In stage 9 wild-type egg chambers, Stau is localized as a posterior crescent in the oocyte (Fig. 1C). When all PFCs were mutant for FP41, Stau was dispersed or mislocalized at the center of the oocyte (Fig. 1D; data not shown; 76%, n = 153). Interestingly, when only a portion of the PFCs were mutant for FP41, Stau was localized as a crescent precisely adjacent to the wild-type PFCs, but was missing in the region underneath the mutant cells (Fig. 1E–E’), suggesting a very local effect of FP41 in disrupting oocyte polarity.

We also examined the position of Kinesin-β-galactosidase (Kinβgal) (Clark et al., 1994), an MT plus-end marker, which is normally localized at the posterior pole of the oocyte at stage 9 of oogenesis (Fig. 1F). Similar to observations of Stau, Kinβgal accumulated at the center of the oocyte or in a diffuse pattern when all PFCs were mutant for FP41 (Fig. 1G; data not shown), and was missing from the region in the oocyte beneath the clones when only some of PFCs were mutant for FP41 (data not shown). Therefore, the MT polarity in the oocyte was clearly perturbed by the presence of FP41 mutant PFCs.

Notably, in egg chambers with wild-type PFCs but anterior or lateral FP41 mutant clones, the oocyte polarity was unaffected (data not shown). In addition, polar cells were not sufficient for oocyte polarization, because the oocyte was still mispolarized in cases where mutant PFCs surrounded wild-type polar cells (Fig. 1B). Taken together, our data demonstrate that the gene product disrupted by FP41 is required specifically in the PFCs to generate the polarizing signal for MT-dependent axis determination of the oocyte.

Proliferation and differentiation of the posterior follicle cells are affected by FP41
The ability of PFCs to repolarize the oocyte requires the correct specification of PFC fate as well as the production of the polarizing signal induced by Grk/Egfr signaling. When examining the morphology of FP41 mutant follicle cells, we found that although most mutant cells formed a monolayered epithelium as observed in
Notch signaling is indeed disrupted (Fig. 2H). However, we did not observe defective FP41 occurrence in the entire follicular region, which is upregulated by Notch at stage 6. In wild-type egg chambers, the role of Notch signaling to the endocycle switch (Fig. 2C) was still present at a high level in which is normally downregulated in the posterior upon the mitotic-to-endocycle switch. Such an overproliferation phenotype is very rare in mutant clones even when the oocyte nucleus was mislocalized at the posterior (see Fig. S1B in the supplementary material). The defects are restricted to the PFCs, which are normally promoted this switch as well as a change in the transcriptional program of all follicle cells (Deng et al., 2001; Lopez-Schier and St Johnston, 2001; Ruohola et al., 1991). We found that the misexpression of Cut and Hnt, which normally occurs at stage 6, is completely restricted to the PFCs but did not occur in lateral or anterior follicle cell clones. This is different from mutant clones involving components in the Notch pathway, where defects are observed in all follicle cells (Sun and Deng, 2005; Sun and Deng, 2007; Yan et al., 2009).

In conclusion, FP41 mutant PFCs are defective specifically in Notch signaling. The defects are restricted to the PFCs, which are nevertheless still responsive to Egfr, JAK/STAT and Hippo signaling.
FP41 is a loss-of-function mutation of PP1β

We mapped the lethal mutation of FP41 to the genomic region between 9B7 and 9C4 using P-elements. Sequencing the candidate genes in this region identified a point mutation in the coding region of the gene flapwing (flw), which encodes the catalytic subunit of the beta isoform of type 1 serine/threonine protein phosphatase (PP1β) (Raghavan et al., 2000).

PP1β belongs to a highly conserved family of serine/threonine phosphatases (Fig. 3A) (Shi, 2009). The gene flw (PP1β) encodes two isoforms of the catalytic subunit of Drosophila PP1β: flw-PA (461 aa) and flw-PB (330 aa) (Fig. 3A). Both isoforms contain the same catalytic domain, but flw-PA has a longer N terminus than flw-PB owing to alternative splicing of an extra exon. FP41 is a miss-sense mutation changing one of the six highly conserved residues in the catalytic domain, D222N (flw-PA) and D222N (flw-PB) (Fig. 3A). Earlier biochemical studies on λ phosphatase, a homolog of PP1β, showed that substitution of the corresponding Asp residue to Asn leads to a dramatic decrease in catalytic activity (Zhuo et al., 1994), predicting that the FP41 mutation should be a strong allele of PP1β (Zhuo et al., 1994), predicting that the FP41 mutation should be a strong allele of PP1β, showing that cell differentiation is affected. (E,F) In wild-type egg chambers (E), Cut is normally downregulated in follicle cells at stage 7 by Notch signaling and only expressed in polar cells afterwards. In egg chambers containing FP41 mutant follicle cells at stage 9 (F), Cut (red) is still expressed but is restricted to the posterior mutant clones. (G,H) In wild-type chambers, expression of Cut is induced in all the follicle cells when Notch signaling is activated at stage 7 (Hnt (red) fails to be upregulated exclusively in FP41 mutant follicle cells (arrow) whereas other mutant follicle cells express Hnt normally. (I,J) A wing disc containing FP41 mutant clones marked by the absence of GFP (green) and stained for Cut (red). The expression pattern of Cut is not affected in FP41 mutant cells in wing discs. (K, L) Stage 9 egg chambers expressing the posterior cell fate marker pnt-lacZ, and stained for β-galactosidase (red). Upon activation of the Egr pathway, β-galactosidase staining reveals that pnt-lacZ is expressed in the induced PFCs in wild-type controls (I). FP41 mutant PFCs, marked by the absence of GFP (green), are able to express pnt-lacZ and respond to Egr signaling (K). Scale bars: 10 μm. Egg chambers are oriented with the posterior side to the right.

Oocyte polarity is disrupted by myosin hyperactivation in flwFP41 mutant PFCs

PP1β is a crucial regulator of a wide range of cellular processes, consists of a catalytic subunit and a regulatory subunit that targets its cellular function to specific substrates (Shi, 2009). Drosophila
myosin, and in the gene RhoGEF2, which facilitates the function of Rho1, also suppressed the oocyte mislocalization caused by PFCs mutant for flwFP41, with proper Stau localization in 54% (n=108) and 58% (n=137) of the egg chambers in these backgrounds, respectively.

Conversely, if myosin regulation is indeed crucial for generation of the polarizing signaling from the PFCs, enhancing the activity of the activating components of the pathway, or blocking the catalytic capability of PP1β in wild-type PFCs, both of which result in myosin hyperactivation, should produce an oocyte polarity defect similar to that observed in flwFP41. Indeed, when a constitutively active form of Rok, Rok.CAT (Winter et al., 2001), was expressed in the wild-type PFC by the flip-out technique (Ito et al., 1997), we observed elevated levels of MLC phosphorylation and moderate disruption of oocyte polarity. This was not observed when expressing a kinase-dead form of Rok, Rok.CAT KG (Winter et al., 2001) (Fig. 4A-D′; data not shown). Furthermore, there are two MYPTs in Drosophila that mediate the function of PP1β in regulating myosin: Drosophila Myosin binding subunit (Mbs), which binds to both PP1α and PP1β, and MYPT-75D, the PP1β-specific myosin regulatory subunit (Vereshchagina et al., 2004). We tested three nonsense mutants of Mbs (Lee and Treisman, 2004) and observed mislocalization of Stau protein and the oocyte nucleus with the presence of posterior Mbs mutant clones (Fig. 4G-I). Moreover, expression of a mutant form of the PP1β-specific subunit MYPT-75D F117A (Vereshchagina et al., 2004) that fails to bind to PP1β causes mild oocyte polarity defects represented by a partial mislocalization of Stau (Fig. 4E-F′). Compared with flwFP41, the oocyte polarity defects generated by Rok.CAT and MYPT-75D F117A are weaker in terms of both the penetrance and the expressivity of the phenotype. As for Rok, it is possible that the overexpressed, constitutively active Rok cannot...
fully antagonize wild-type PP1β. Similarly, the mutant form of MYPT-75D might only mildly disrupt myosin dephosphorylation, owing to the presence of wild-type protein and Mbs, another myosin targeting subunit. Interestingly, in these genetic backgrounds, in spite of the oocyte polarity defects, we did not observe a significant Cut or Hnt misregulation in the PFCs expressing these constructs (data not shown). There are two possibilities that could account for this result. PP1β might regulate Notch signaling in the follicle cells by a different pathway and not through its effects on myosin activity. Alternatively, constitutively active Rok or mutant MYPT-75D might not disrupt the myosin organization severely enough to produce an obvious Notch phenotype. To distinguish between these two scenarios, we measured the occurrence of Cut upregulation in flwFP41 mutant PFCs in combination with heterozygous mutations of zip, Rho1 and RhoGEF2. We found a significant reduction of penetrance from ~70% in flwFP41 to 40-50% after loss of a single copy of the factors that activate myosin (Table 1). Therefore, the presence of specific mutations in the myosin pathway is able to suppress Cut upregulation caused by loss of PP1β function, suggesting that PP1β interacts with the Notch pathway at least partially through its function in regulating myosin activity. In addition, our results also suggest that the oocyte polarity defect can be produced in the absence of defective Notch signaling by mutations that affect myosin activation in the PFCs.

In summary, these data demonstrate that correct regulation of myosin activity is crucial for the production of the polarizing signal in the PFCs. In addition, increasing myosin phosphorylation can result in oocyte mispolarization without overtly disrupting the Notch pathway.

The Notch intracellular domain (NICD) can rescue the Notch signaling phenotype, but not the oocyte polarity phenotype of flwFP41 mutant PFCs

Our results left us with the open question of whether the oocyte polarity phenotype observed in flwFP41 is simply a secondary consequence of disrupted Notch signaling or whether it is also a direct result of myosin hyperactivation. In addition, we wanted to determine how the regulation of myosin activity affects Notch signaling. Therefore, we analyzed whether overexpression of various Notch constructs in flwFP41 mutant PFCs using the MARCM system (Lee and Luo, 1999) would be able to rescue the Notch and the oocyte polarity defects. In wild-type egg chambers, in response to Delta binding Notch undergoes a set of sequential cleavages, in particular an extracellular (S2) cleavage by ADAM-family metalloproteases and an intramembranous (S3) cleavage by γ-secretase to produce the membrane-bound NEXT (Notch extracellular truncation) and the intracellular domain NICD, respectively (Fortini, 2009). NICD is then released and translocates into the nucleus to regulate the transcription of downstream genes (Rebay et al., 1993; Struhl et al., 1993). When posterior flwFP41 mutant clones were generated by the MARCM system, as expected we observed Cut upregulation in 74% of the mutant PFCs (n = 69) (Fig. 5A). Cut upregulation was also observed in 77% of posterior flwFP41 clones overexpressing full-length Notch (n = 71) and in 78% overexpressing NEXT (n = 59). By contrast, only 19% of clones with NICD overexpression showed upregulation of Cut (n = 57) (Fig. 5B-D), suggesting that only NICD, and not the full-length or the S2 cleavage product NEXT, was able to restore normal Notch signaling. However, none of these constructs fully rescued the oocyte polarity defect. We observed Stau mislocalization in 74% of the cases with posterior flwFP41 MARCM clones (n = 84), 70% with full-length Notch co-expressed (n = 92), 74% with NEXT (n = 96) and 53% with NICD (n = 99). Whereas Cut was properly downregulated with NICD expressed in the PFCs, Stau was still observed to be mislocalized at the center of the oocyte (Fig. 5E’). The oocyte polarity phenotype was not caused by NICD on its own, because Stau mislocalization was not observed when NICD was overexpressed in posterior MARCM clones in a wild-type background (n = 21, data not shown). Therefore, although NICD expression can mildly suppress the oocyte polarity phenotype, it is not as effective as its rescue of the transcriptional output of Notch signaling. Considered together with the effects of constitutively active Rok and mutant MYPT-75D, our results strongly suggest that the observed oocyte polarity defect in flwFP41 is not merely a side effect of Notch disruption, but that PP1β might additionally play an independent role in the generation of the polarizing signal through its regulation of myosin activity.

The Notch receptor and other transmembrane proteins accumulate in flwFP41 mutant PFCs

Although the Notch protein is downregulated after stage 7 in wild-type PFCs (Fig. 5F), we observed ectopic accumulation of Notch both on the apical surface and in cytoplasmic punctae of flwFP41 mutant PFCs, but not in anterior or lateral clones (Fig. 5G,G’). Using appropriate antibodies we found that both the intracellular and the extracellular domain of Notch are present in these punctae (data not shown). Consistent with the restriction of the Notch defect to the PFCs, we did not observe a similar Notch accumulation in flwFP41 mutant cells in eye discs (data not shown).

When we analyzed the colocalization of the ectopic Notch with various endocytic markers, we observed no significant overlap between the Notch punctae and the Golgi marker Lava Lamp (Lva) (Sisson et al., 2000) or the recycling endosomal marker Rab11-GFP (Emery et al., 2005) (data not shown). However, we observed colocalization of Notch in flwFP41 mutant PFCs with the late

---

Table 1. Suppression of flwFP41 by heterozygous mutations of the myosin regulatory pathway

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Percentage Staufen mislocalization (n)</th>
<th>Percentage Cut upregulation (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>75.8 (153)</td>
<td>73.2 (138)</td>
</tr>
<tr>
<td>+/-Balancer</td>
<td>76.3 (93)</td>
<td>70.0 (168)</td>
</tr>
<tr>
<td>e22C&gt;flw-HA</td>
<td>12.4 (242)</td>
<td>22.9 (157)</td>
</tr>
<tr>
<td>zip1/+</td>
<td>15.2 (165)</td>
<td>40.0 (190)</td>
</tr>
<tr>
<td>Rho1^E1010G/+</td>
<td>45.7 (108)</td>
<td>42.5 (155)</td>
</tr>
<tr>
<td>RhoGEF2^P2029/+</td>
<td>42.3 (137)</td>
<td>53.4 (131)</td>
</tr>
</tbody>
</table>

*Egg chambers containing posterior flwFP41 clones and heterozygous for various mutant alleles were scored for the occurrence of Stau mislocalization in the oocyte or Cut upregulation in the PFCs. Expression of a wild-type flw-HA transgene using the GALA-UAS system rescues the phenotypes. Loss of one copy of zip, Rho1 or RhoGEF2 also significantly suppresses. Significance of difference between all mutants and the +/-Balancer control: P < 0.001.
protein trafficking. Rather causes a general problem in the apical membrane of PP1 function does not specifically disrupt Notch protein localization but rather causes a general problem of membrane protein trafficking.

endosomal marker Rab7-GFP (Fig. 5I) and the early endosomal marker Rab5-GFP (Wucherpfennig et al., 2003) (Fig. 5K–K’), but not in the wild-type cells (Fig. 5H, J), suggesting that defective Notch endocytosis accounts for the observed ectopic accumulation of Notch protein in the mutant PFCs. In addition, we observed a similar accumulation of other transmembrane proteins such as Egfr and Domeless (Dome) as cytoplasmic punctae in the PFCs (Fig. 5L–O), indicating that loss of PP1β function does not specifically disrupt Notch protein localization but rather causes a general problem of membrane protein trafficking.

**Fig. 4. Excessive myosin activity in the PFCs causes the oocyte polarity defect.** (A–D’) Egg chambers containing flip-out follicle cell clones positively marked by GFP (green) and stained for Stau (red), with the expression of a kinase-dead form of Rok, Rok.CAT KG (B) or a constitutively active Rho kinase, Rok.CAT (C–D’) in the clones. With the posterior flip-out clones alone (A), or with the posterior clones expressing Rok.CAT KG (B), the oocyte polarity manifested by Stau localization is normal. By contrast, with the posterior clones expressing active Rok.CAT (C), Stau is mislocalized in the middle of the oocyte. The level of PMLC (red) is elevated in the clones expressing Rok.CAT (D–D’), showing excessive myosin activity. (E–F’) Egg chambers containing flip-out follicle cell clones positively marked by GFP (green) and stained for Stau (red), with the expression of a wild-type construct of the PP1-specific myosin targeting subunit MYPT-75D (E), or a mutant form MYPT-75D<sup>ΔII117A</sup> (F–F’) in the clones. With the posterior clones expressing wild-type MYPT-75D, Stau is correctly localized, whereas the posterior clones expressing MYPT-75D<sup>ΔII117A</sup> result in a partial mislocalization of Stau. (G–I) Egg chambers containing follicle cells clones mutant for Mbo<sup>79E1</sup> (G), Mbo<sup>766E</sup> (H) and Mbo<sup>79F1</sup> (I) marked by the absence of GFP (green) and stained for Stau (red). Stau is mislocalized in the middle of the oocyte when the PFCs lose the function of the general myosin targeting subunit Mbo. Scale bars: 10 μm. Egg chambers are oriented with the posterior side to the right.

**PP1β regulates the membrane levels of apical complexes in the PFCs**

To probe further the role of PP1β in regulating the polarizing signal from the PFCs, we examined the apical-basal polarity of the flw<sup>PP41</sup> mutant cells. By analyzing the distribution of major components of epithelial polarity complexes, we found that the overall apical-basal polarity of the flw<sup>PP41</sup> mutant cells is normal (Fig. 6). However, we detected significantly increased levels of the apical complexes, such as Atypical Protein Kinase C (aPKC), Bazooka (Baz) and Patj on the apical membrane of the flw<sup>PP41</sup> mutant PFCs (Fig. 6A–F). The levels of adherens junction components DE-cadherin (DE-cad; Shg – FlyBase) and Armadillo (Arm) were also elevated at the apical apex of the mutant PFCs (Fig. 6G–J). Notably, these increases were only observed in the posterior mutant clones. However, we found that the level of cortical F-actin in the mutant PFCs, which is also present at the apical membrane, remained the same as observed in the neighboring wild-type cells (Fig. 6B, H’), suggesting that the concentrated apical markers in the mutant PFCs were not simply due to apical cortex contraction caused by excessive myosin activity. By contrast, the basolateral component Discs Large (Dlg) maintained its lateral distribution and protein level as in the wild-type cells (Fig. 6K, L). Therefore, our results suggest that PP1β regulates the membrane levels of the apical proteins in the PFCs. As this phenotype is not observed in mutants of the Notch pathway, it might account for one aspect of the Notch-independent function of PP1β in the regulation of the posterior polarizing signal.

**Integration of multiple signaling pathways renders PFCs sensitive to myosin misregulation**

A striking feature of the ovarian phenotypes of flw<sup>PP41</sup> is that the Notch defects represented by target gene misregulation and ectopic accumulation of Notch protein can only be detected in the PFCs. To determine what renders the PFC especially sensitive to myosin hyperactivation, we attempted to transform the anterior and the lateral follicle cells into PFCs and to test whether such a cell fate transformation would be able to generate the PFC restricted phenotypes in other locations in the follicular epithelium. We expressed a constitutively active form of Egr, α-torpedo (α-top) (Queenan et al., 1997), in clones of flw<sup>PP41</sup> using the MARCM system, and observed a significant overproliferation and Cut upregulation in the transformed cells at the anterior but not in the lateral clones (Fig. 7C, C’). Expression of α-top also elevated the level of cell overproliferation in the mutant PFCs compared with flw<sup>PP41</sup> clones alone (Fig. 7A, A’). Expression of α-top in a wild-type background did not have such an effect (Fig. 7B, B’), indicating that the observed phenotypes at the anterior do not merely result from constitutively active Egr signaling but arise in combination with loss of PP1β function. By contrast, expression of a ligand of the JAK/STAT pathway, Unpaired (Upd) (Harrison et al., 1998), did not produce the Notch defects either in the anterior or in the lateral clones of flw<sup>PP41</sup> (Fig. 7D, D’). However, when α-top and Upd were co-expressed, a combination that is able to transform all the follicle cells into the posterior fate (Xi et al., 2003), Cut upregulation and mild overproliferation were found even in the lateral flw<sup>PP41</sup> clones (Fig. 7F, F’).

In summary, our results demonstrate that the integrated activity of multiple signaling pathways is responsible for the sensitivity of Notch signaling in the PFCs to loss of PP1β function.
DISCUSSION

The AP body axis of Drosophila is established during oogenesis through intracellular communication between the oocyte and the somatic follicle cells (Poulton and Deng, 2007). Correct oocyte polarity requires a polarizing signal generated by the PFCs, in response to an earlier signal (Gurken) that is secreted from the oocyte and received by the PFCs via Egfr. Previous studies have shown that genes regulating PFC proliferation, differentiation and epithelial polarity must function normally to render the PFC competent to signal back to the oocyte; however, the nature of this polarizing signal is still unknown, neither is it clear how the signal is produced or transmitted from the PFCs to the germline. Here, we report a direct role of Drosophila PP1β in the production of the polarizing signal. We found that loss of PP1β in the PFCs due to the \textit{flw}^{F41} mutation causes a disruption of the oocyte MT polarity and the mislocalization of determinants of embryonic AP polarity indicative of a defect in the polarizing signal. This oocyte polarity defect was not observed with anterior or lateral follicle cell clones mutant for \textit{flw}^{F41}, demonstrating that the activity of PP1β is required in the PFCs to repolarize the oocyte. We have also shown that heterozygous mutants of positive regulators of myosin activity suppress the oocyte polarity defect, whereas constitutive activation of Rok or expression of a mutant myosin targeting subunit in the PFCs induces a similar oocyte polarity phenotype. This supports our conclusion that myosin activity controls the polarizing signal in the PFCs.

The fact that elevated myosin activity in the PFCs interferes with the production of the polarizing signal raises the question of the specific function of myosin in this process. We found that there are two separable effects of elevated myosin activity in the PFCs: an effect on Notch signaling and a Notch-independent effect. Loss of Notch signaling in the follicle cells inhibits the developmental progress of the PFCs and results in the disruption of the formation of the AP polarity in the oocyte (Gonzalez-Reyes and St Johnston, 1998; Lopez-Schier and St Johnston, 2001). In \textit{flw}^{F41} PFC clones, the cells are still responsive to the patterning signals of Egfr and the JAK/STAT pathway and the mutant PFCs are able to adopt the posterior fate as indicated by the expression of \textit{pnt}-\textit{lacZ}. Therefore, the major problem in the generation of the polarizing event by loss of PP1β is not cell specification or cell survival. Instead, we propose that loss of Notch signaling directly affects the production of the polarizing signal, and that myosin activity is further required for the proper generation of this signal independently of its effects on Notch signaling, as discussed below.
We have shown that defective Notch signaling in flwFP41 mutant PFCs can be rescued by expression of NICD, but not by full-length Notch or NEXT. This indicates that myosin hyperactivation through loss of PP1β disrupts Notch signaling probably at the level of the final Notch cleavage. This cleavage, which is γ-secretase dependent and generates the functional NICD, is subject to regulation at the level of endosomal trafficking (Fortini and Bilder, 2009). In mutants that disrupt entry of the receptor into early endosomes, Notch accumulates at the cell surface or below the plasma membrane with significantly reduced signaling activity (Lu and Bilder, 2005; Vaccari et al., 2008). In mutants affecting the function of the Vacuolar ATPase, Notch signaling is also blocked at the step of the third cleavage, indicating that this cleavage requires an endosomal environment (Yan et al., 2009; Vaccari et al., 2010). We observed an elevated level of Notch protein at the cell surface and in early and late endosomal compartments in the subapical cell cortex in the flwFP41 mutant PFCs. It is therefore likely that the defective Notch activity in flwFP41 is caused by a failure of the receptor to efficiently enter early endosomes and subsequent sorting compartments. Such a defect in endosomal trafficking might be a direct consequence of abnormal myosin activity. The regulation of the actin cytoskeleton and of actin motor

Fig. 6. PP1β regulates the membrane levels of apical complexes in the PFCs. (A-L) Egg chambers stained for aPKC (A,B), Baz (C,D), Patj (E,F), DE-Cad (G,H), Arm (I,J), Dlg (K,L) or F-actin (B,H). flwFP41 mutant PFCs, marked by the absence of GFP (green), contain increased membrane levels of the apical proteins aPKC, Baz, Patj and the adherens junction components Arm and DE-Cad, whereas the level of apical F-actin stained by Phalloidin in the mutant PFCs remains the same as in the neighboring wild-type cells. The distribution and protein levels of the basolateral protein Dlg are indistinguishable from wild type. Scale bars: 10 μm. Egg chambers are oriented with the posterior side to the right.

Fig. 7. The PFC restricted Notch defects in flwFP41 are caused by the integrated activity of JAK/STAT and Egfr Signaling. (A-F) Egg chambers containing wild-type follicle cell clones or flwFP41 mutant clones positively marked by GFP (green), expressing a constitutively active form of Egfr, A-top (B-C′), or the ligand of the JAK/STAT pathway Upd (D-D′), or both (E-F′), and stained for Cut (red). In flwFP41 clones alone (A,A′) or in flwFP41 clones expressing Upd (D,D′), Cut is upregulated only in the mutant PFCs. In flwFP41 clones expressing A-top (C,C′), Cut is upregulated in both the anterior and the posterior mutant clones and severe cell overproliferation is observed. In flwFP41 clones expressing both A-top and Upd (F,F′), Cut staining is visible in all the mutant cells regardless of clone positions. Expression of these constructs in wild-type follicle cells does not produce abnormal Notch signaling (B,B′,E,E′). Scale bars: 10 μm. Egg chambers are oriented with the posterior side to the right.
proteins plays an important role in the endocytic pathway in yeast and mammalian cells (Girao et al., 2008). In *Drosophila* embryos, cortical actin regulates endocytic dynamics at early celluarization (Sokac and Wieschaus, 2008a; Sokac and Wieschaus, 2008b). In addition, studies in mammalian cell culture have shown that Rho, Rok and myosin II directly regulate phagocytosis (Araki, 2006; Olazabal et al., 2002), revealing important roles of myosin II in the process of endocytosis. However, loss of PP1β does not cause a significant block in endocytosis in all cell types. We found that *flwPP41* clones in the eye discs allow apparently normal Notch signaling to occur and do not show ectopic Notch accumulation. We also did not detect an overt endocytic defect in mutant eye disc cells by performing a trafficking assay (data not shown). In addition, mutant clones in anterior and lateral follicle cells did not show a defect in Notch signaling. This indicates a particular sensitivity of the PFCs to problems in Notch endocytosis and Notch activation, which is due to the coordinated activities of JAK/STAT and Egfr signaling, as shown by our results.

Our data strongly suggest that PP1β has an independent role in axis formation apart from its effects on regulating Notch cleavage and activation. Excessive myosin activity resulting from constitutive Rok activity, or from expression of a mutant myosin targeting subunit in the PFCs, disrupts Stau localization without inducing a measurable Notch phenotype. Additionally, expression of NICD only marginally suppresses Stau mislocalization caused in the *flwPP41* mutant cells, whereas it strongly rescues the Notch signaling defect. Therefore, we observe oocyte polarity defects by myosin misregulation even in the presence of normal Notch signaling.

The effects of excessive myosin activity are also different from those of the Hippo pathway, which is also specifically required in the PFCs for axis formation (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). Similar to *flwPP41*, hippo mutant PFCs are defective in Notch signaling and result in oocyte mispolarization, and these defects are restricted to PFCs. However, previous studies demonstrate that the effects of the Hippo pathway are mediated solely by its effects on Notch (Polesello and Tapon, 2007; Yu et al., 2008). Hippo signaling itself appears to occur normally in the *flwPP41* mutant follicle cells.

The abnormal accumulation of membrane proteins suggests a general membrane trafficking problem associated with myosin hyperactivation. It raises the possibility that PP1β regulates the polarizing signal, which might be a membrane associated protein, by controlling its intracellular trafficking as it is trafficked to the cell surface. However, hyperactive myosin caused by loss of PP1β function might also directly impede the interaction between the PFCs and the oocyte, possibly by affecting the function of cellular structures, such as microvilli, required for the presentation of the polarizing signal on the apical surface of the PFCs to the oocyte. We observed higher levels of components of apical membrane complexes as well as of the adherens junction proteins on the apical surface, which might result from changes in the underlying actin cytoskeleton caused by excessive myosin activity. Consequently, changes in the membrane properties, especially on the apical side that contacts the germline, might also change cell surface protein interactions between the PFCs and the oocyte, which might then affect the transmission of the polarizing signal. We observe a very local effect on oocyte polarity when a subset of PFCs are mutant for *flwPP41*, where Staufen protein is still localized correctly in the oocyte underneath the wild-type cells, but is absent from the region underneath the mutant cells. This strongly suggests that the polarizing signal is not freely diffusing over longer distances, and points to local interactions between the PFCs and the oocyte.

One very puzzling aspect of the *flwPP41* phenotype is the fact that the phenotypes of defective Notch signaling and cell overproliferation are restricted to the PFCs. Position-dependent phenotypes have been observed in mutants disrupting the epithelial integrity of the follicle cells, such as *dlg1* and *crb* mutants (Goode and Perrimon, 1997; Tanentzapf et al., 2000). There, defects of the epithelial architecture, such as multilayering, are mostly observed at the poles of the egg chamber. In mutants of the Hippo pathway, dramatic Notch defects are observed in PFC clones but only modest ones in clones at other sites of the epithelium (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). Such position-dependent responses might be due to the special terminal positions of the cells at the poles where they could experience more mechanical stress than the lateral cells. Excessive myosin activity caused by loss of PP1β function might exacerbate the mechanical forces experienced by the PFCs, leading to posterior-restricted phenotypes. Alternatively, signaling events specific to subpopulations of follicle cells might cause the cells to react differentially to the loss of common gene products. Strikingly, we found that the hyperactive myosin can lead to loss of Notch signaling and overproliferation when the Egfr pathway is activated in anterior follicle cells where JAK/STAT activity is normally present. Even the lateral cells produced these phenotypes when subject to the combined activity of JAK/STAT and Egfr signaling. Therefore, whereas loss of PP1β function elevates myosin activity in all the mutant cells independent of cell position, the coordinated activation of JAK/STAT and Egfr signaling creates a sensitized intracellular environment in the PFCs and renders them particularly susceptible to phenotypes such as defects in protein trafficking due to myosin misregulation. It is likely that particular targets of the combined activity of Egfr and JAK/STAT enhance the defects generated by the elevated myosin activity; however, it is presently unknown what these target proteins might be.

Overall our study has shown that the regulation of myosin activity by PP1β is crucial in the posterior follicle cells where overactive myosin interferes with intracellular trafficking and with the generation of the posterior polarizing signal. This demonstrates the importance of the general cellular physiology in both signal transduction as well as signal generation, and adds a layer of complexity to the analysis of developmental signals important for cell specification.

Acknowledgements
We thank S. Artavanis-Tsakonas, E. Bach, H. Bellen, D. Harrison, K. Irvine, V. Riechmann, R. Karess, G. Struhl, J. E. Treisman, J. Zallen, the Developmental Studies Hybridoma Bank and the Bloomington Stock Center for providing flies and antibodies; G. Barcelo for technical help; J. Goodhouse for assisting with confocal microscopy; and members of the Schüpbach and Wieschaus laboratories for feedback and advice. We also thank E. Gavis, O. Grimm and A. Martin for helpful comments on the manuscript. This work was supported by the Howard Hughes Medical Institute and US Public Health Service Grant R01 GM077620. Deposited in PMC for release after 6 months.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.062190/-/DC1

References


