Protein phosphatase 2A negatively regulates aPKC signaling by modulating phosphorylation of Par-6 in Drosophila neuroblast asymmetric divisions

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Summary

Drosophila neural stem cells or neuroblasts undergo typical asymmetric cell division. An evolutionally conserved protein complex, comprising atypical protein kinase C (aPKC), Bazooka (Par-3) and Par-6, organizes cell polarity to direct these asymmetric divisions. Aurora-A (AurA) is a key molecule that links the divisions to the cell cycle. Upon its activation in metaphase, AurA phosphorylates Par-6 and activates aPKC signaling, triggering the asymmetric organization of neuroblasts. Little is known, however, about how such a positive regulatory cue is counteracted to coordinate aPKC signaling with other cellular processes. During a mutational screen using the Drosophila compound eye, we identified microtubule star (mts), which encodes a catalytic subunit of protein phosphatase 2A (PP2A), as a negative regulator for aPKC signaling. Impairment of mts function causes defects in neuroblast divisions, as observed in lethal (2) giant larvae (lgl) mutants. mts genetically interacts with par-6 and lgl in a cooperative manner in asymmetric neuroblast division. Furthermore, Mts tightly associates with Par-6 and dephosphorylates AurA-phosphorylated Par-6. Our genetic and biochemical evidence indicates that PP2A suppresses aPKC signaling by promoting Par-6 dephosphorylation in neuroblasts, which uncovers a novel balancing mechanism for aPKC signaling in the regulation of asymmetric cell division.

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Key words: Drosophila, Mts, PP2A, aPKC, Asymmetric cell division, Neuroblast

Introduction

Polarity is a fundamental characteristic of cells and underlies a variety of cellular processes involved in the development and homeostasis of living organisms. In epithelial cells, which consist of the apical and basolateral membrane domains, cell polarity creates distinct subcellular compartments to arrange the cells into a well-ordered structure. In asymmetric cell division, cell polarity is coupled with mitosis. Cell polarity creates two subcellular domains with distinct characteristics in the mitotic mother cell and coordinates the mitotic spindle with the polarity axis to allow the two daughter cells to be distinct. Because these cell polarity events are tightly linked to other elementary processes such as the cell cycle and mitotic events, cell polarity is finely controlled to coordinate with those cellular processes.

Drosophila neural-stem-like cells, or neuroblasts, undergo typical asymmetric divisions, providing an excellent model for the study of how cell polarity is controlled (Knoblach, 2008). Neuroblasts repeatedly divide into a large, self-renewing daughter (the neuroblast itself) and a smaller, differentiating daughter [the ganglion mother cell (GMC)]. Cell fate determinants, such as Prospero (Doe et al., 1991), Brain tumor (Brat) (Bello et al., 2006; Betschinger et al., 2006; Lee et al., 2006) and Numb (Uemura et al., 1989), are segregated to the GMC. The localization of these determinants and the coordination with mitotic spindle orientation are controlled by the apically localized protein complexes—the aPKC-Par complex and the Pins complex—which are mutually linked by Insuteable (Insc) (Kraut and Campos-Ortega, 1996; Kraut et al., 1996). The aPKC-Par complex consists of atypical protein kinase C (aPKC), Bazooka (Baz) and Par-6 and is primarily involved in organizing cell polarity and the asymmetric distribution of the cell fate determinants along the axis of polarity (Petronczki and Knoblach, 2001; Rolls et al., 2003; Schober et al., 1999; Wodarz et al., 2000; Wodarz et al., 1999). The Pins complex, which consists of Partner Insuteable (Pins), Locomotion defects (Loco) and Gozi, determines the orientation of the mitotic spindle relative to the cell polarity axis (Schafer et al., 2001; Yu et al., 2000; Yu et al., 2005).

aPKC is a key enzyme involved in establishment of neuroblast polarity and definition of the apical cortex. A tumor suppressor protein, Lethal (2) giant larvae (Lgl), is thought to antagonize aPKC as an inhibitory substrate. Although aPKC binds to non-phosphorylated Lgl, Lgl that is phosphorylated by aPKC dissociates from it and is released from the cell cortex (Betschinger et al., 2003). In the absence of aPKC, the entire cortex becomes basal, and Miranda, an adaptor protein for Prospero and Brat, distributes uniformly throughout the cortex (Rolls et al., 2003). However, loss of Lgl results in uniform activation of aPKC in the cortex just as if the entire cortex were apical. Consequently, Miranda misdistributes into the cytoplasm and concentrates on mitotic spindles (Ohshiro et al., 2000; Peng et al., 2000).

The apical complex and the basal determinants dynamically change their localization as the cell cycle progresses. The apical complex accumulates at the apical cortex during late interphase, retains its apical localization during metaphase, and then initiates expansion through the cortex in anaphase (Petronczki and Knoblach,
2001; Schober et al., 1999; Wodarz et al., 1999). Miranda and its cargos are temporally found in the apical cortex in late interphase and, after spreading into the cytoplasm at the onset of mitosis, form the basal crescent that is complementary to the localization of the apical complex during metaphase (Hirata et al., 1995; Ikeshima-Kataoka et al., 1997). At late anaphase onwards, they are restricted to the GMC compartment, which is separated by the contractile ring from the neuroblast compartment.

It was recently shown that the mitotic kinase Aurora-A (AurA) has an important role in linking the cell cycle to the asymmetric cell division of neuroblasts and sensory organ precursors (SOPs) by phosphorylating Par-6 (Wirtz-Peitz et al., 2008). When AurA is inactive, aPKC binds to unphosphorylated Par-6 and Lgl and remains inactive. Phosphorylation of Par-6 by AurA blocks the interaction of Par-6 with aPKC, which in turn leads to activation of aPKC. Activated aPKC then phosphorylates Lgl to replace it with Baz. The Par complex that has recruited Baz is able to phosphorylate Numb, leading to an exclusion of Numb from the apical complex during metaphase (Hirata et al., 1995; Ikeshima-Kataoka et al., 1997). At late anaphase, the Par complex is able to synchronize aPKC activation with the entry into mitosis. Given the role of AurA as a positive regulator of aPKC signaling, it is also likely that dephosphorylation negatively regulates this signaling pathway.

The serine/threonine phosphatases are grouped into four major classes based on their sensitivity to inhibitors and requirement for divalent cations: protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B) and protein phosphatase 2C (PP2C) (Sontag, 2001). PP2A holoenzyme functions as a heterotrimeric complex comprising a catalytic C subunit (Microtubule star [Mts] in Drosophila (Snaith et al., 1996), a scaffolding A subunit (PP2A-A2 in Drosophila (Mayer-Jäckel et al., 1992)) and a regulatory B-subunit (Twins (Tws) (Shiomi et al., 1994; Uemura et al., 1993), Widerborst (Wdb) (Hannus et al., 2002) and PP2A-B' in Drosophila). The A-subunit can serve as a linker between the C- and B-subunits, and the B-subunit can influence the enzymatic activity and substrate specificity of the holoenzyme. In our genetic screen using the Drosophila compound eye, we identified mts as an enhancer of the aPKC-induced eye phenotype. The genetic and biochemical evidence indicating that Mts suppresses aPKC activity by enhancing dephosphorylation of Par-6 in neuroblasts uncovered an antagonistic role of PP2A in the aPKC signaling pathway.

### Results

Identification of the mts gene as a regulator of aPKC pathway

The Drosophila compound eye is composed of repetitive ommatidia that contain epithelial retinal cells (Fig. 1A). Because of the crystalline arrangement of ommatidia in the compound eye, it is sensitive to defects in epithelial polarity and therefore ideal for use in mutational screens for components involved in epithelial polarity. We undertook a modifier screen under a sensitized background to look for mutations that affected epithelial polarity. When the membrane-tethered aPKC [aPKC<sup>CAAX</sup> (Sotillos et al., 2004)] is expressed by GMR-GAL4 (Hay et al., 1997), it becomes expressed in all differentiated retinal cells, the apico-basal polarity of the retinal cells is severely impaired (data not shown), and the compound eye becomes small and rough (Fig. 1B). Kinase activity of aPKC<sup>CAAX</sup> is essential for inducing this eye phenotype, because the kinase-dead version, in which Lys293 is mutated to Trp (K293W), does not alter the eye morphology (data not shown). Using this system, we screened mutants that modify the aPKC<sup>CAAX</sup>-induced eye phenotype (data not shown) among lethal mutants available from stock centers, and identified mts as a phenotypic enhancer. When the GMR-GAL4/UAS-aPKC<sup>CAAX</sup> fly was crossed to the mts<sup>02496</sup> or mts<sup>XE-2258</sup> mutant fly, a smaller and rougher eye was observed (Fig. 1C,D), suggesting that Mts acts as an antagonist for the aPKC signaling pathway.

Mts is required for Miranda localization in embryonic neuroblasts

The mts gene is expressed ubiquitously during embryogenesis (Fig. 2A) and its protein product localizes to the cytoplasm in neuroblasts as well as in epithelial cells (Fig. 2B). Because a large amount of mts mRNA is maternally supplied (Fig. 2A), zygotic mts mutant embryos do not show significant defects with regard to cell polarity, and germline clones do not produce an egg. We therefore examined loss-of-function phenotypes in neuroblasts by overexpressing a dominant-negative mutant of Mts (dnMts) (Hannus et al., 2002), which lacks the N-terminal region of the phosphatase domain. In wild-type neuroblasts, the protein complex containing aPKC, Par-6 and Baz localizes to the apical cortex (Fig. 2C, arrowhead; and data not shown) and directs Miranda to the basal cortex at metaphase (Fig. 2C', arrowhead). In the dnMts-expressing embryos, the apical complex localizes to the apical cortex, and its distribution is broader than normal (Fig. 2D, arrowhead; and data not shown). By contrast,
localization of Miranda is severely affected, and it is distributed less asymmetrically along the cell cortex and into the cytoplasm, where it is concentrated on the mitotic spindles (Fig. 2D’, arrowhead). This phenotype resembles that of lgl (Ohshiro et al., 2000; Peng et al., 2000), raising the possibility that Mts functions in the same pathway as Lgl.

mts genetically interacts with lgl in a positive way

As there was no obvious defect in neuroblast division at stage 10 of embryogenesis in zygotic mtsXE-2258/mts02496 mutant embryos (Fig. 3A–A”, D; n=40), we introduced one copy of the lgl-null mutation into the mts background to examine whether mts genetically interacts with lgl. The lgl+/-, mtsXE-2258/mts02496 neuroblasts showed abnormal localization of Miranda (Fig. 3B, D; 61% cytoplasmic, 26% weakly basal, 13% normal; n=62), which was essentially indistinguishable from that seen in zygotic lgl mutant embryos (Fig. 3C). Since Lgl is directly phosphorylated and inactivated by αPKC, lowering αPKC activity was expected to rescue the phenotype caused by depriving a copy of lgl under the mts mutant background. Indeed, Miranda localization was considerably recovered when one copy of αPKC was inactivated (Fig. 3D; 20% cytoplasmic, 35% weakly basal, 45% normal; n=71). Genetic interaction between mts and lgl was also observed in other systems. When lgl+/lglR16 flies were cultivated at 27.5°C, the temperature-sensitive lglR16 partially lost its activity, and 86% of individual flies successfully developed into adulthood (Fig. 4A). Under this condition, when one copy of the mts02496 or mtsXE-2258 mutation was introduced, the eclosion rate was reduced to 72% and 42%, respectively. Furthermore, mts enhanced the lgl phenotype in the formation of extrasensory bristles in the adult notum, which are generated through successive asymmetric cell divisions of SOPs. The SOP cells segregate Numb into the pIIb daughter cell, which subsequently produces three inner cells: the glia, neuron and sheath cell. The sibling pIIa cell differentiates into two outer cells: a hair and a socket (Rhyu et al., 1994). In this process, Lgl regulates the asymmetric inheritance of Numb into pIIb and its loss of function leads to the transformation of inner cells into outer cells (Ohshiro et al., 2000). Although 28% of lgl+/lglR16 flies carried at least one defective bristle, such as a duplicated bristle or a socket without a bristle, as a result of the partial loss of lgl activity at the semi-restrictive temperature (27.5°C), the mts02496 and mtsXE-2258 mutations increased the frequency of defective bristle formation to 33% and 64%, respectively (Fig. 4B). These data suggest that Lgl is excessively inactivated in mts mutants.

Mts dephosphorylates Par-6

We next asked how Mts regulates Lgl activity. A simple possibility is that Mts directly dephosphorylates and inactivates Lgl. We tested this hypothesis using an in vitro dephosphorylation assay. Bacterially produced Lgl was phosphorylated by PKCζ, a mammalian homolog of αPKC, to test whether the phosphorylated Lgl was dephosphorylated by Mts. However, Mts was unable to dephosphorylate Lgl at all, which suggested that Mts is unlikely to be a phosphatase for Lgl (supplementary material Fig. S1). The same was true for αPKC. Autophosphorylation of PKCζ is necessary for its activation (Hirai and Chida, 2003), but it was not affected by Mts in the in vitro assay (supplementary material Fig. S1), nor by the overexpression of mts in embryos (data not shown). We then searched for other targets for Mts among the members of the αPKC pathway. Recent studies demonstrated that Par-6 directly associates with αPKC to suppress its kinase activity (Yamanaka et al., 2001). Phosphorylation of Par-6 on Ser34 by AurA abolishes this inhibitory effect of Par-6 on αPKC in neuroblasts (Wirtz-Peitz et al., 2008). Therefore, it is possible that Mts is a phosphatase for Par-6. Indeed, Mts efficiently dephosphorylates AurA-phosphorylated Par-6 in vitro, and this dephosphorylation was inhibited by lower concentrations of okadaic acid (OA), which is a PP2A-specific inhibitor (Fig. 5A). As previously described, AurA phosphorylates Par-6 not only on Ser34 but also on other Ser residue(s) in vitro (Wirtz-Peitz et al., 2008). Mts decreased phosphorylation of wild-type Par-6 and the Par-6 mutant form in which Ser34 is replaced by Ala (data not shown). Mts, therefore, appears to affect both Ser34 and other phosphorylation sites of Par-6.

To investigate whether Mts dephosphorylates Par-6 in vivo, we examined the effect of OA on Par-6 phosphorylation in Drosophila Schneider (S2) cells. As shown in the left panel of Fig. 5B, OA treatment induced changes in the electrophoretic mobility of Par-6.
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on SDS-PAGE. This mobility shift is a result of Par-6 phosphorylation because the upper bands were converted into the lower band by calf intestine alkaline phosphatase (CIAP) treatment (Fig. 5B, right panel), implying that Par-6 phosphorylation is normally repressed by Mts in S2 cells. From these in vitro and in vivo experiments, we concluded that Mts acts as the Par-6 phosphatase.

We next investigated whether Mts can physically interact with Par-6, aPKC and Lgl. S2 cells were transfected with a FLAG-Mts expression plasmid along with Myc-Par-6, Myc-aPKC and/or Lgl. After FLAG-Mts was immunoprecipitated, all these proteins were detected in the Mts complex (Fig. 5C). Par-6, especially, co-immunoprecipitated with Mts efficiently compared with aPKC and Lgl, suggesting that Par-6 is a major binding partner of Mts among the components of the aPKC pathway.

mts genetically promotes Par-6-mediated suppression of aPKC

If Mts is the Par-6 phosphatase, Mts would enhance the ability of Par-6 to attenuate aPKC, because Par-6 phosphorylation represses its inhibitory activity in neuroblasts. This implies that mts interacts positively with par-6 and negatively with aPKC in genetic tests. To investigate this as a possibility, we examined the genetic interactions of mts with par-6 and aPKC with regard to neuroblast phenotypes. At stage 16 of embryogenesis, Miranda is properly localized to the basal cortex in 100% of mitotic mtsXE-2258/mts02496 neuroblasts (Fig. 6A,D; n=34) and in 98% of mtsXE-2258/mts02496, aPKCk06403/+ neuroblasts (Fig. 6B,D; n=46). By contrast, 44% of mitotic neuroblasts in par-6 Δ226/+, mtsXE-2258/mts02496 zygotic mutants abnormally localized Miranda along the cortex or in the cytoplasm (Fig. 6C,D; n=82), which is similar to the phenotype Fig. 3. mts genetically interacts with lgl and aPKC in neuroblast asymmetric cell division. Localization of Miranda (Mira, A-C), Par-6 (A/H11032-C/H11032) and DAPI (A/H11033-C/H11033) in the mitotic neuroblasts of mtsXE-2258/mts02496 (A), lglf/+; mtsXE-2258/mts02496 (B) and lglf/lglf zygotic mutant embryos (C) at stage 10 of embryogenesis. Zygotic mtsXE-2258/mts02496 mutant neuroblasts do not show any defect in asymmetric cell division (arrowheads in A-A’), but introduction of one copy of lglf into the mts background causes delocalization of Miranda (arrowhead in B-B’), which is essentially indistinguishable from the phenotype that is observed in the lglf/lglf mutant (arrowhead in C-C’). (D) Quantification of cells containing no (=cytoplasm) and weak Miranda crescents in the dividing neuroblasts of mtsXE-2258/mts02496, lglf/+; mtsXE-2258/mts02496 and lglf/lglf; mtsXE-2258/mts02496, aPKCk06403/+ embryos. Lowering aPKC activity considerably reverted the defective localization of Miranda seen in the lglf/+, mtsXE-2258/mts02496 neuroblasts.

Fig. 4. Genetic interaction between mts and lgl in eclosion rate and bristle formation. (A) Effect of genetic interaction between mts and lgl on eclosion rate. When lglf/lglf flies were cultivated at 27.5°C, 86% of the individuals successfully develop into adulthood because of a partial loss of lglf function at this semi-restrictive temperature. mts02496 and mtsXE-2258 reduced the eclosion rate to 72% and 42%, respectively. Results are mean ± s.e.m. (B) Effect of genetic interaction between mts and lgl on the bristle formation in the adult notum, which is generated through the Lgl-regulated successive asymmetric cell division of SOP. 28% of lglf/lglf flies carry at least one defective bristle at 27.5°C. Introduction of one copy of the mts02496 or mtsXE-2258 mutation increases the frequency to 33% and 64%, respectively. The inset is a representative view of defective bristle formation. The white and black arrowheads indicate a duplication of macrochaeta and microchaeta, respectively. The white arrows indicate sockets without a bristle.
As shown in Fig. 6D, these mutants reduced the percentage of aPKC k06403/H11032 neuroblasts (Fig. 2D lgl observed when the aPKC signaling pathway is hyperactivated, such as in lgl mutant neuroblasts (Fig. 3C) or dnMts-expressing neuroblasts (Fig. 2D'). To test whether this defect depends on aPKC, we introduced one copy of aPKCk06403 into par-6Δ226/+; mtsXE-2258/mts02496 mutants and examined localization of Miranda. As shown in Fig. 6D, these mutants reduced the percentage of neuroblasts that delocalized Miranda (26%; n=34), indicating that the defective Miranda localization is caused by hyperactivation of aPKC. Thus mts has positive genetic interactions with par-6 in repressing aPKC signaling.

We also examined the genetic interaction between mts and aurA. Since AurA phosphorylates Par-6, AurA is expected to antagonize Mts function and hence a reduction of AurA activity in par-6Δ226/+; mtsXE-2258/mts02496 mutants should ameliorate their Miranda phenotype. Indeed, an introduction of one copy of the null aurA allele, aurAΔ770, significantly restored Miranda localization (Fig. 6D; 19%; n=37). These genetic interactions, together with the dephosphorylation assays in Fig. 5A,B, strongly suggest that Mts regulates neuroblast asymmetric cell divisions by modulating the phosphorylation levels of Par-6.

Mts is required for the formation or maintenance of epithelial polarity

We finally considered whether Mts contributes to construction of epithelial cell polarity. In wild-type ovaries, follicle cells, which surround a future oocyte, form an epithelial monolayer. aPKC is clearly concentrated at the apical side in these cells (Fig. 7A-A'). However, mosaic clones that were homozygous for mts failed to accumulate either aPKC or Dlg (Fig. 7B-B'). This phenotype did not resemble that of par-6 mutant cells (Fig. 7C-C'), which lose aPKC but not Dlg, and it was also distinct from that of the lgf/mutant mosaic clones, in which both aPKC and Dlg spread into the entire cell cortex (Fig. 7D-D'). These observations indicate that the epithelial cell polarity is impaired in mts mutant follicle cells, but its mechanism might be different from those that occur in the absence of Par-6 or Lgl.

Discussion

In this study, we showed that PP2A functions as a negative regulator of the aPKC signaling pathway in Drosophila neuroblasts. Although several studies have suggested that PP2A negatively regulates aPKC signaling in mammalian culture cells (Nunbhakdi-Craig et al., 2002; Zhang et al., 2008), the critical target(s) of PP2A is unknown in these studies. The substrates of aPKC, which include Lgl and aPKC (Zhang et al., 2008), the critical target(s) of PP2A is unknown. Nevertheless, our data suggest that Mts negatively regulates aPKC signaling in neuroblasts.}

**Fig. 5.** Mts associates with and dephosphorylates Par-6. (A) In vitro dephosphorylation assay of Par-6. Bacterially produced MBP-Par-6 was phosphorylated by AurA kinase in the presence of [γ-32P]ATP and then was co-incubated with a Drosophila Mts complex or human PP2A complex in the absence or presence of 50 μM okadaic acid (OA). In the upper panel, MBP-Par-6 protein (arrowhead) was stained with Coomassie brilliant blue R250. Asterisk indicates an unknown protein contained in the human PP2A reagent. The middle panel shows autoradiography of MBP-Par-6. In the lower panel, the PP2A level included in the reaction was examined by western blotting using an anti-PP2A antibody that recognizes both Drosophila Mts and human PP2A C subunit. Mobility of Mts on SDS-PAGE is slightly slower than that of human PP2A, suggesting that some modification may be present on Mts. (B) Inhibition of Mts activity by OA induces Par-6 phosphorylation in S2 cells. S2 cells were treated with 250 nM okadaic acid for 1 hour at 25°C and then the cell lysate was probed with the anti-Par-6 antibody on western blotting. OA induces electrophoretic mobility shift of Par-6 on SDS-PAGE (left panel). This mobility shift is caused by phosphorylation of Par-6 because calf intestine alkaline phosphatase (CIAP) treatment reverts the mobility (right panel). (C) Mts tightly associates with Par-6. S2 cells transfected with the indicated plasmids were subjected to immunoprecipitation using an anti-FLAG antibody. The immunoprecipitates were probed with anti-FLAG, anti-Myc and anti-Lgl antibodies to detect in vivo association of Mts with each protein. Expression of Myc-Par-6 increases the amount of Lgl that is contained in the Mts complex. This co-immunoprecipitation of Lgl is presumably mediated by direct association of Lgl with Myc-Par-6 rather than association of Lgl with FLAG-Mts, because Lgl tightly binds to Myc-Par-6 in S2 cells (data not shown).
Par-6 is a direct target of PP2A in the aPKC pathway

Our co-immunoprecipitation assays of overexpressed Par-6, aPKC or Lgl with Mts in S2 cells indicated that all these molecules can form a complex either directly or indirectly. Among these, the association of Mts with aPKC and Lgl is relatively weaker than the association with Par-6, although a previous study suggested that PP2A associates with aPKC to suppress its kinase activity in mammalian cultured cells (Nunbhakdi-Craig et al., 2002). Our results indicate that, in S2 cells, Par-6 most efficiently forms a complex with Mts. Consistently, the in vitro dephosphorylation assay showed that PP2A effectively dephosphorylates AurA-phosphorylated Par-6 but not the auto-phosphorylated PKCζ or the PKCζ-phosphorylated Lgl. We infer from these results that Par-6 is a direct target of Mts. Substrate specificity of PP2A is greatly influenced by the B-subunit incorporated into the holoenzyme (Sonntag, 2001). Thus, differences in the affinity with Mts among the three tested molecules in our co-immunoprecipitation assays might, therefore, partly reflect the B-subunit(s) that is expressed in the normal range. Thus, a probable normal function of Mts is to promote the inhibitory function of Par-6 on aPKC without affecting its function as an essential subunit of the aPKC complex.

PP2A suppresses aPKC function through Par-6

Par-6 is an essential cofactor for aPKC activity, and it is known to keep aPKC inactive in the absence of AurA-dependent phosphorylation of Par-6 in neuroblasts. The complete deprivation of Par-6 results in the uniform distribution of Miranda into the cell cortex (Petronczki and Knoblich, 2001), which is reminiscent of the asymmetric cell division of SOPs as well as of neuroblasts. Targeting Par-6. Among them, tws mutants often show bristle duplications that are due to defective cell fate decisions of the SOP (Shiomi et al., 1994), as lglts3 flies show. Since this lglts3 phenotype is enhanced by mts, mts is also likely to be involved in the same pathway. Furthermore, a recent study demonstrated that Tws, together with Mts, is included in the aPKC complex to regulate the asymmetric cell division of larval neuroblasts (Chabu and Doe, 2009). These results suggest that Mts uses Tws to target Par-6 in the asymmetric cell divisions of SOPs as well as of neuroblasts.

Fig. 7. Ovarian follicle cell clone analyses of mts, par-6 and lgl. Wild-type ovarian follicle cells (monolayered cells indicated by the white bracket) show the typical apico-basal epithelial polarity (A–A′′′′), in which aPKC accumulates apically (A′) and Dlg localizes at the basolateral membrane (A″). In mtsXE-2258 follicle cell clones (GFP-negative cells outlined with dots in B–B″), both aPKC and Dlg signals decrease to an undetectable level (B′, B″), and the monolayered epithelial structure was disrupted. Both par-6Δ226 (C–C″) and lglts3 (D–D″) mutant cells are apparently different from the mtsXE-2258 with regard to aPKC or Dlg localization. In the par-6Δ226 cells, aPKC disappears whereas Dlg is present, and in the lglts3 cells, aPKC spreads into the entire cell cortex. In addition, both the par-6Δ226 and lglts3 mutant cells often overproliferate.

neuroblasts and SOPs enter mitosis. Our biochemical and genetic evidence reveals that Mts dephosphorylates Par-6 to suppress the aPKC pathway, suggesting an antagonistic role for Mts against AurA in the regulation of cell polarity that is governed by aPKC signaling.

Par-6 is a direct target of PP2A in the aPKC pathway

Our co-immunoprecipitation assays of overexpressed Par-6, aPKC or Lgl with Mts in S2 cells indicate that all these molecules can form a complex either directly or indirectly. Among these, the association of Mts with aPKC and Lgl is relatively weaker than the association with Par-6, although a previous study suggested that PP2A associates with aPKC to suppress its kinase activity in mammalian cultured cells (Nunbhakdi-Craig et al., 2002). Our results indicate that, in S2 cells, Par-6 most efficiently forms a complex with Mts. Consistently, the in vitro dephosphorylation assay showed that PP2A effectively dephosphorylates AurA-phosphorylated Par-6 but not the auto-phosphorylated PKCζ or the PKCζ-phosphorylated Lgl. We infer from these results that Par-6 is a direct target of Mts. Substrate specificity of PP2A is greatly influenced by the B-subunit incorporated into the holoenzyme (Sonntag, 2001). Thus, differences in the affinity with Mts among the three tested molecules in our co-immunoprecipitation assays might, therefore, partly reflect the B-subunit(s) that is expressed in the normal range. Thus, a probable normal function of Mts is to promote the inhibitory function of Par-6 on aPKC without affecting its function as an essential subunit of the aPKC complex (Fig. 8).

The difference between epithelial cells and neuroblasts

Whereas AurA seems to be active only during the mitotic phase in cell-cycling cells, mitotically inactive or interphase epithelial cells...
exhibit concrete apicobasal polarity. How do those cells activate aPKC signaling even though AurA is inactive? This apparent paradox raises several possibilities. Par-6 phosphorylation is required for aPKC activation in epithelial cells but might be mediated by kinase(s) other than AurA. Alternatively, aPKC might be activated by mechanisms other than the phosphorylation of Par-6. Indeed, it has been reported that the active form of Cdc42 binds to the CRIB domain of Par-6 to relieve its inhibitory effect on aPKC, leading to the activation of aPKC (Yamanaka et al., 2001).

Although obvious defects are not detected in the epithelial cells of zygotic mts mutant embryos, ovarian follicle cell clones of mts show dramatic defects in their epithelial polarity. This follicle cell phenotype is different from that caused by hyperactivation of aPKC, as observed in the lgl mutant (Fig. 7), suggesting that the action of Mts is mechanistically different in the maintenance of follicle cell polarity from that observed in neuroblasts. In photoreceptor cells, Mts operates antagonistically against Par-1 kinase, which restricts polarity from that observed in neuroblasts. In photoreceptor cells, Mts operates antagonistically against Par-1 kinase, which restricts polarity from that observed in neuroblasts.

The role of negative regulation in the aPKC pathway
AurA-mediated Par-6 phosphorylation is a key step in initiating the asymmetric segregation of the cell fate determinants in the neuroblast cell cycle. Once Par-6 is phosphorylated, aPKC will be continuously activated during the mitotic phase. The apical domain would overwhelm the entire cortex unless an antagonistic reaction occurred. PP2A will be able to balance AurA in Par-6 phosphorylation during mitosis. Thus, PP2A, together with the antagonistic ligand Lgl, might have a role in maintaining aPKC activity at an appropriate level to create both apical and basal domains in the cortex during mitosis. Although both Mts and Lgl negatively regulate aPKC signaling, Mts operates on aPKC activity by directly regulating the cell-signaling cascade, whereas Lgl does so through the direct physical association as a substrate. Therefore, they are different in their mechanisms of action.

When neuroblasts complete cell cleavage, the basal membrane is largely segregated into the GMC, and the entire cell cortex of neuroblasts appears to become apical. It is therefore necessary to repolarize in order to make the apical and basal domains in the cell cortex for the onset of the next cell cycle. To do so, Par-6 phosphorylation must be removed before entering the next cell cycle, to reset the configuration of the apical complex. We propose a model in which Mts actively dephosphorylates Par-6 to reset the membrane polarity after the completion of each division cycle. In this context, it will be important to examine whether Mts function depends on the cell-cycle stage in neuroblasts.

Global control of cell polarity by multiple phosphatases
In eukaryotes, serine/threonine phosphatases are categorized into four major groups: PP1, PP2A, PP2B and PP2C. Recent studies have shown that PP1c1a affects Par-3 activity through the regulation of a phosphorylation-dependent interaction of Par-3 with 14-3-3 or PKCζ (Traweger et al., 2008). We also identified a Pp1-87B mutation as an enhancer of the aPKCζ-induced eye phenotype in our genetic screen and found defects in localization of Miranda as well as in epithelial cell polarity in the Pp1-87B mutant. Furthermore, Sousa-Nunes and colleagues reported recently that protein phosphatase 4 (PP4), which is a PP2A family member, regulates Miranda localization in Drosophila neuroblasts, although the direct substrate of PP4 is not yet clear (Sousa-Nunes et al., 2009). Thus, other classes of phosphatases in addition to PP2A are involved in the regulation of cell polarity in various cellular contexts. Further delineation of phosphatase functions and the crosstalk between phosphatases should help us to understand the global control of cellular processes regulated by cell polarity.

Materials and Methods
Flies strains and genotypes
In the eye screen, GMR-GAL4; UAS-aPKCζ A5/SM1 female flies were crossed to males with a known lethal mutation, and the eye phenotypes of their progeny were analyzed. Both mts mutants, mtsQ6294 and mtsW2218, were from the Bloomington Drosophila Stock Center. mtsQ6294 contains a P-element that is located 320 bp upstream of the translation start site, and mtsW2218 is probably a null allele in which there is a 16 bp (−7 to 9) deletion that spans the translation start site. Other mutants used in this study were lgl2, lgl3 (Obshuro et al., 2000), aPKCζA5460 (Rols et al., 2000), par-6F67 (Petronczki and Knoblich, 2001) and aruk4770 (W.M. and F.M., unpublished results). aruk4770 was generated by an imprecise excision of P[EPgy2]aure[A03490] and is a null allele that removes the most part of the aruk coding sequence. Overexpression of dominant-negative Mts (dnMts) was induced by crossing UAS-dnMts to maternal-GAL4 V32. To investigate the genetic interaction between mts and lgl, an Igl2 female was mated with an Igl2 male carrying an additional mts mutation, and the offspring were cultivated at 27.5°C. The eclosion rate was calculated by counting the number of flies of interest and comparing it with the number of balanced-inheriting flies. For follicle cell clone analysis, progeny from the cross of mts FRT40A to hsFLP; ubiquitin-GFP FRT40A were heat-shocked at 37°C for 2 hours twice over 2 days at the L3 and prepupal stages and they were dissected to isolate ovaries from females of the appropriate genotype at day 2 to 3 after eclosion.

In situ hybridization
RT-PCR-amplified mts cDNA (310-1836 of RA isoform) was cloned into the pSP718 vector (Roche). The antisense and sense single-stranded RNA were produced by in vitro transcription with SP6 or T7 RNA polymerase and labeled with digoxigenin (DIG)-UTP using the DIG RNA Labeling Kit (Roche). The DIG-labeled RNA probe was incubated with the parafomaldehyde (PFA)-fixed white embryos in hybridization buffer (50% formamide, 5× SSC, 0.1% Tween 20) at 55°C overnight to allow hybridization. The hybridized RNA was detected using an alkaline phosphatase-conjugated anti-DIG antibody (Roche) and was visualized with an NBT-BCIP coloring reaction.

Immunohistochemistry
For staining of the embryos, dechorionated embryos were fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature with vigorous shaking. The fixed embryos were blocked with 5% skimmed milk in PBS with 0.2% Tween 20 for 20 minutes at room temperature with vigorous shaking. The fixed embryos were blocked with 5% skimmed milk in PBS with 0.2% Tween 20 for 2 hours at room temperature and then incubated with the primary antibody in the blocking solution at 4°C overnight, followed by detection using fluorescent secondary antibodies. For follicle cell staining, ovaries dissected from 2- to 3-day-old females were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature and were stained as described above. The antibodies used in this study were mouse anti-PP2A (1:200, P47720, BD Biosciences), mouse anti-Mira-3 (1:100, F381-0), rabbit anti-PKCζ (1:400, sc-216, Santa Cruz Biotechnology), rabbit anti-Par-3 (1:200, A3700), mouse anti-Dig (1:80, 4F3, Developmental Studies Hybridoma Bank), mouse anti-Sld (1:100, M18, Developmental Studies Hybridoma Bank) and chicken anti-GFP (1:2000, GFP-1010, Aves). A confocal microscope (BioRad Radiance 2000, BioRad) was used to acquire images.

S2 cell experiments
S2 cells were maintained at 25°C in Schneider’s Drosophila Medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics. Transfection was performed using the Effectene reagent (Qiagen) according to the manufacturer’s protocol. One day after transfection, cells were suspended in lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Triton X-100, 50 mM NaF, 50 mM p-glycerophosphate, 1 mM DTT, protease inhibitor cocktail). The cleared cell lysate was subjected to immunoprecipitation with mouse anti-FLAG M2 agarose (A-2220, Sigma), and the immunoprecipitate was probed with rabbit anti-FLAG (1:1000, Bethyl), or rabbit anti-Lgl (50% formamide, 5× SSC, 0.1% Tween 20) at 55°C overnight to allow hybridization. The hybridized RNA was detected using an alkaline phosphatase-conjugated anti-DIG antibody (Roche) and was visualized with an NBT-BCIP coloring reaction.
antibody (1:1000, N2). Par-6 RNAs was performed as described previously (Worby et al., 2001). 10% of the cells were used in each experiment. Par-6 double-stranded RNA corresponding to the entire coding region in 400 µl serum-free Schneider’s Drosophila medium for 1 hour at 25°C and then 800 µl normal medium culture was added. After 4 days, the cells were stimulated with 250 nM okadaic acid (Sigma) for 1 hour at 25°C and then lysed in 20 mM Tris-HCl pH 7.5, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT and the protease inhibitor cocktail for western blotting analysis with anti-Par-6 antibody. For CIAP treatment, the cell lysate was reacted with 5 U CIAP (Takara) for 1 hour at 37°C.

In vitro dephosphorylation assay
Recombinant Par-6 protein (full-length PA isoform) was prepared as a fusion with maltose binding protein (MBP). One microgram of MBP-Par-6 protein was phosphorylated with 12.5 µg recombinant human Aurora A (Cell Signaling) at 30°C for 1 hour in a 5 µl reaction (20 mM HEPES-Na, pH 7.4, 10 mM MgCl2, 1 mM DTT, 25 µM ATP) that contained 0.375 µCi [γ-32P]ATP. This mixture was then passed through a Zeba Desalt Spin Column (Thermo Scientific) to remove unincorporated ATP. In vitro phosphorylated MBP-Par-6 was incubated at 30°C for 2 hours with the Mts complex immunoprecipitated from white embryos using rabbit anti-PBP2A antibody (ab33537, Abcam) or 10 ng human PP2A (Millipore). The reaction was followed by SDS-PAGE followed by autoradiography. Okadaic acid was added to the reaction at a final concentration of 50 µM.

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