Apical–basal polarity in *Drosophila* neuroblasts is independent of vesicular trafficking

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ABSTRACT **The possession of apical–basal polarity is a common feature of epithelia and neural stem cells, so-called neuroblasts (NBs). In *Drosophila*, an evolutionarily conserved protein complex consisting of atypical protein kinase C and the scaffolding proteins Bazooka/PAR-3 and PAR-6 controls the polarity of both cell types. The components of this complex localize to the apical junctional region of epithelial cells and form an apical crescent in NBs. In epithelia, the PAR proteins interact with the cellular machinery for polarized exocytosis and endocytosis, both of which are essential for the establishment of plasma membrane polarity. In NBs, many cortical proteins show a strongly polarized subcellular localization, but there is little evidence for the existence of distinct apical and basolateral plasma membrane domains, raising the question of whether vesicular trafficking is required for polarization of NBs. We analyzed the polarity of NBs mutant for essential regulators of the main exocytic and endocytic pathways. Surprisingly, we found that none of these mutations affected NB polarity, demonstrating that NB cortical polarity is independent of plasma membrane polarity and that the PAR proteins function in a cell type–specific manner.**

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INTRODUCTION

The separation of the plasma membrane into distinct apical and basolateral membrane domains is crucial for the establishment and maintenance of cell polarity in epithelia. One essential mechanism to generate membrane asymmetry is the targeted exocytosis of apical and basolateral transport vesicles to the plasma membrane, which requires recognition between the vesicles and their target membrane (Schuck and Simons, 2004; Rodriguez-Boulan *et al.*, 2005). This process, termed vesicle tethering, is mediated by the exocyst, an evolutionarily conserved octameric protein complex consisting of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Hsu *et al.*, 1996; TerBush *et al.*, 1996; Kee *et al.*, 1997; Matern *et al.*, 2001; He and Guo, 2009). In *Drosophila*, several studies demonstrated the importance of the exocyst complex for the maintenance of epithelial apical–basal polarity. In embryos mutant for *exo84*, the transmembrane protein Crumbs (Crb) mislocalizes to enlarged recycling endosomes (Blankenship *et al.*, 2007). The resulting phenotype strongly resembles the *crb* loss-of-function phenotype (Tepass *et al.*, 1990), demonstrating that the proper delivery of Crb to the apical plasma membrane domain is essential for epithelial integrity. Trafficking of *Drosophila* E-Cadherin (DE-Cad), another transmembrane protein important for epithelial polarity, from the recycling endosome to the plasma membrane was shown to depend on the exocyst components Sec5, Sec6, and Sec15 (Langevin *et al.*, 2005).

In addition to targeted exocytosis, the regulation of endocytosis is also crucial for the control of epithelial apical–basal polarity. Already the earliest steps of endocytosis, including the α-adaptin–dependent sorting of receptors into clathrin-coated vesicles and the dynamin-dependent scission of vesicles at the plasma membrane, are required for epithelial cell polarity (Shivas *et al.*, 2010). In *Drosophila*, mutations in genes that regulate the fusion of vesicles with early endosomes, namely *avalanche*, encoding a syntaxin, the small GTPase Rab5, the Rab5 effector *rabenosyn-5*, and *Vps45* (*vesicular protein sorting 45*) cause the loss of epithelial polarity (Lu and Bilder, 2005; Menut *et al.*, 2007; Morrison *et al.*, 2008). Epithelial cells lacking the function of any of these genes show loss of the zonula adherens and mislocalization of apical proteins to the basolateral

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Abbreviations used: ada, α-adaptin; aPKC, atypical protein kinase C; Baz, Bazooka; Brat, Brain tumor; Cdc42, cell division control protein 42; Chc, clathrin heavy chain; Crb, Crumbs; DAPI, 4′,6-diamidino-2-phenylindole; DE-Cad, *Drosophila* Epithelial Cadherin; elav, embryonic lethal abnormal vision; Ept, Erupted; ESCRT, Endosomal sorting complex required for transport; Exo, Exocyst; FRT, flipase recombinase target; Hrs, hepatocyte growth factor regulated tyrosine kinase substrate; Lgd, lethal (2) giant discs; MARCM, mosaic analysis with a repressible cell marker; Mira, Miranda; NBs, neuroblasts; NIP, Numb-interacting protein; Onr, onion rings; Par, partitioning-defective; PDZ, postsynaptic density 95, Discs-large, Zonula occludens-1; Pon, Partner of Numb; Pros, Prospero; Rab, Ras related GTP binding protein; Sara, smad anchor for receptor activation; Sec, secretory; Shi, Shibire; TSG101, tumor susceptibility gene 101; Vps, vesicular protein sorting.

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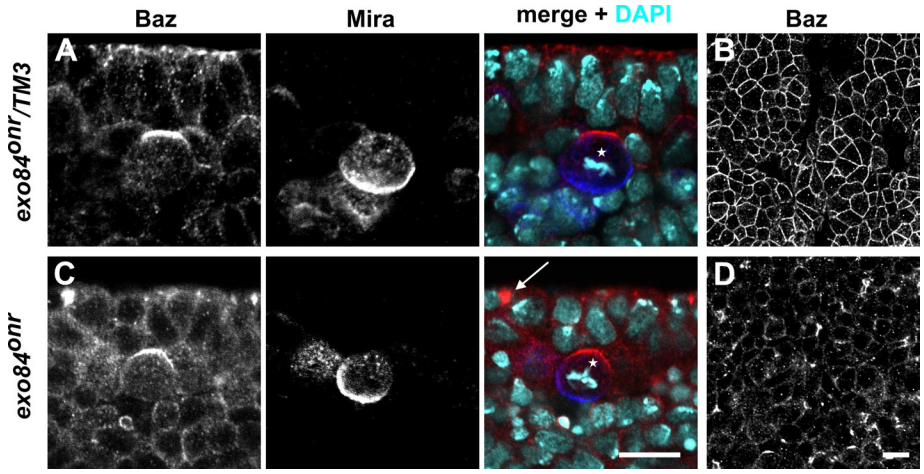

FIGURE 1: NB polarity is normal in *exo84*^{onr} mutant embryos. (A and B) Baz (red) and Mira (blue) localization in an embryo heterozygous for *exo84*^{onr}. Baz localizes to the apical margin of the lateral membrane in epithelial cells and to an apical crescent in NBs (A, star), whereas Mira is not expressed in the epithelium and forms a basal crescent in NBs. (B) An optical section of the same embryo as shown in A at the plane of the zonula adherens. (C and D) Baz and Mira localization in an embryo homozygous mutant for *exo84*^{onr}. Baz is mislocalized to scattered aggregates (arrow) in the neuroectoderm of *exo84*^{onr} mutant embryos, whereas Baz and Mira localization in NBs is indistinguishable from wild type (C, star). (D) An optical section of the same embryo as shown in C at the plane of the zonula adherens. In A and C apical is up. Scale bars, 10 μm. DNA is stained with 4′,6-diamidino-2-phenylindole (DAPI) (turquoise).

membrane. Proteins of the endosomal sorting complex required for transport (ESCRT) machinery, which are required for sorting of endocytosed cargo into multivesicular bodies, are also indispensable for epithelial polarity. Loss of function of tumor susceptibility gene 101 (TSG101, named Erupted [Ept] in *Drosophila*), a component of the ESCRT-I complex, and Vps25, a component of the ESCRT-II complex, cause the loss of apical–basal polarity and extensive overproliferation (Moberg *et al.*, 2005; Thompson *et al.*, 2005; Vaccari and Bilder, 2005).

Cell polarity in many different cell types is controlled by atypical protein kinase C (aPKC) and the evolutionarily conserved PDZ-domain proteins Bazooka/Par-3 (Baz) and Par-6, which form the so-called Par/aPKC complex (Suzuki and Ohno, 2006). Loss of any of the Par/aPKC proteins in epithelial cells leads to the complete loss of apical–basal polarity (Muller and Wieschaus, 1996; Petronczki and Knoblich, 2001; Harris and Peifer, 2007; Kim *et al.*, 2009). It was shown that vesicle trafficking and the Par/aPKC complex, together with the small Rho GTPase Cdc42, control epithelial apical–basal polarity in a mutually dependent manner (Harris and Tepass, 2008).

As in epithelial cells, the Par/aPKC complex has an essential function in the control of apical–basal polarity in neuronal stem cells of *Drosophila*, so-called neuroblasts (NBs; Wodarz, 2005; Knoblich, 2008). The Par/aPKC complex localizes to the apical cortex in mitotic NBs and is responsible for the basal localization of the cell fate determinants Prospero (Pros), Brain tumor (Brat), and Numb, as well as their adaptor proteins Miranda (Mira) and Partner of Numb (Pon) (Schober *et al.*, 1999; Wodarz *et al.*, 1999; Petronczki and Knoblich, 2001; Bello *et al.*, 2006; Betschinger *et al.*, 2006; Lee *et al.*, 2006; Kim *et al.*, 2009).

In contrast to the clear evidence for the function of targeted vesicle trafficking in apical–basal polarity in epithelial cells, data addressing the involvement of vesicle trafficking in the establishment and maintenance of polarity in *Drosophila* NBs are lacking. We therefore analyzed the polarity of NBs in several mutant conditions affecting different steps of vesicle trafficking. Surprisingly, we did not detect any defect of apical–basal NB polarity in any of the mutants analyzed, leading to the conclusion that targeted vesicle trafficking is dispensable for proper polarization of NBs. Moreover, our data indicate that the PAR/aPKC complex may control polarity of NBs in a manner fundamentally different from its function in epithelial cells.

RESULTS

Loss of exocyst function does not affect polarity of embryonic NBs

The function of the exocyst complex is crucial for apical–basal epithelial polarity (Blankenship *et al.*, 2007). We therefore checked whether NB polarity is affected in embryos mutant for the exocyst component Exo84. *exo84*^{onr} represents a hypomorphic allele of *exo84*, and *exo84*^{onr} mutant embryos display epithelial defects caused by impaired apical targeting of the transmembrane protein Crumbs. This in turn results in mislocalization of proteins normally localized to the apical junctional region, including Baz, aPKC, DE-Cad, and Armadillo, to aggregates along the basolateral membrane of epithelial cells (Blankenship *et al.*, 2007). Consistent with this report, we observed that Baz was lost from the apical junctional region in the embryonic neuroectoderm and localized to intracellular aggregates in *exo84*^{onr} mutant embryos (Figure 1, C and D; compare to control, Figure 1, A and B). Despite of these severe epithelial polarity defects, Baz and Mira localized as in wild-type embryos in mitotic NBs of *exo84*^{onr} mutant embryos (Figure 1C). Although we cannot exclude that the Exo84 protein encoded by the hypomorphic *exo84*^{onr} allele possesses residual Exo84 activity sufficient to control polarity in NBs, this result indicates that the polarization mechanisms in epithelial cells and NBs are different.

Dynamin function is not required for NB polarity

Epithelial apical–basal polarity depends on endocytosis to maintain the proper balance between apical and basolateral transmembrane proteins and lipids in the plasma membrane (Shivas *et al.*, 2010). To test whether dynamin dependent endocytosis is required for regulating cortical polarity of *Drosophila* NBs we used a temperature-sensitive allele of dynamin (*shibire*^{1}, *shi*^{1}) and blocked endocytosis during embryonic neurogenesis. We observed severe disruption of epithelial organization (Figure 2, C and D), probably as a result of disturbed Notch signaling (Poodry, 1990) in *shi*^{1} embryos reared at the restrictive temperature of 29°C but not at the permissive temperature of 22°C (Figure 2, A and B). Although epithelial organization was severely disturbed in *shi*^{1} embryos reared at 29°C and NBs frequently failed to ingress and remained positioned in the cell layer facing the outside of the embryo (Figure 2, C and D), the apical localization of Baz and the basal localization of Mira in NBs was unaffected (Figure 2C). The same observation was made for *shi*^{1} mutant NBs in third-instar larvae shifted to 34°C (Figure 2F; Chabu and Doe, 2008), indicating that NB polarity is not dependent on dynamin function.

Larval NBs mutant for different regulators of vesicle trafficking exhibit normal polarity

Recently it was shown that different components of the vesicle trafficking machinery are crucial for the maintenance of apical–basal cell

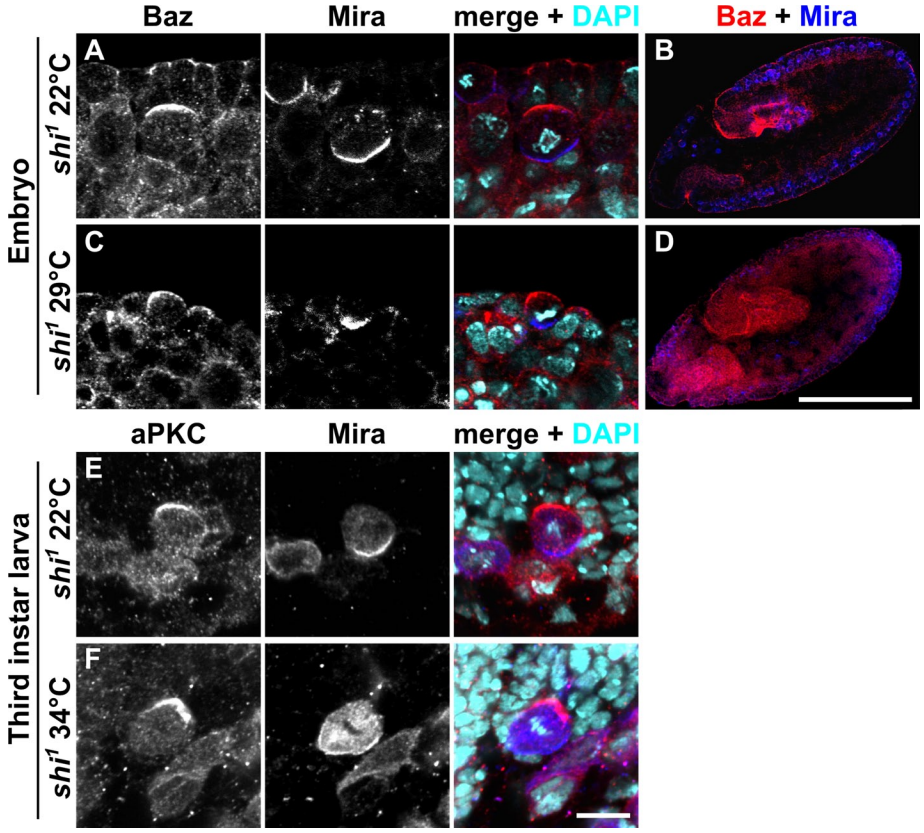

FIGURE 2: Blocking dynamin-dependent endocytosis does not affect NB polarity. (A–D) Baz (red) and Mira (blue) localization in *shi*^{1} embryos reared at the permissive temperature (22°C) (A and B) and at the restrictive temperature (29°C) (C and D). Embryos are at stage 10. (E and F) Baz and Mira localization in NBs of *shi*^{1} wandering third-instar larvae reared at 22°C (E) and at 34°C (F). Except for B and D, apical is up. In B and D, dorsal is up, anterior is to the left. Scale bar except for B and D, 10 µm. Scale bar for B and D, 200 µm. DNA is stained with DAPI (turquoise).

polarity in epithelial cells of *Drosophila* (Langevin *et al.*, 2005; Lu and Bilder, 2005; Moberg *et al.*, 2005; Thompson *et al.*, 2005; Vaccari and Bilder, 2005; Blankenship *et al.*, 2007). Null mutations in most of the genes coding for the components of the vesicle-trafficking machinery either cause arrest during oogenesis if the gene product is missing in germline cells or cause the death of the animal at the end of embryogenesis or during early larval development when the zygotic gene product is missing (Murthy *et al.*, 2003; Murthy and Schwarz, 2004; Wucherpfennig *et al.*, 2003; Beronja *et al.*, 2005). This complicates the analysis of NB polarity in these mutants because embryonic phenotypes are at least partially rescued by residual maternal gene product, and zygotic mutant animals do not develop far enough to analyze the phenotype of NBs at third larval instar.

To circumvent these problems, we generated positively labeled homozygous mutant clones containing NBs in the brains of heterozygous larvae using mosaic analysis with a repressible cell marker (MARCM; Lee and Luo, 1999). As a control, clones were generated with a wild-type chromosome harboring the same FRT site as the gene under investigation (Figure 3A). In dividing NBs of these control clones, Mira and aPKC formed crescents at opposing poles of the NB cortex (Figure 3A). To check whether the MARCM technique is suitable for causing polarity defects in larval NBs, we generated positively labeled clones of the amorphic *aPKC* allele *aPKC*^{k06403} (Wodarz *et al.*, 2000; Rolls *et al.*, 2003). Loss of *aPKC* in embryonic and larval NBs leads to mislocalization of Mira to the whole NB cortex (Rolls *et al.*, 2003; Kim *et al.*, 2009). NBs in *aPKC*^{k06403} clones lacked any detectable aPKC staining, consistent with the fact that *aPKC*^{k06403} is a null allele (Figure 3B). Concomitantly, Mira was distributed all around the cell cortex, demonstrating that the MARCM technique is a suitable method to study the effect of mutations on NB polarity.

To investigate exocyst function in third-instar larval NBs, we generated positively labeled clones homozygous mutant for the loss-of-function alleles *sec5*^{E10} (Figure 3C; Murthy *et al.*, 2003), *sec6*^{Ex15} (Figure 3D; Murthy *et al.*, 2005), and *sec15*^{1} (Figure 3E; Mehta *et al.*, 2005). NBs in these clones displayed normal localization of aPKC and Mira, indistinguishable from control clones (Figure 3A). The efficiency of the MARCM technique in the reduction of the protein levels for the protein under investigation could only be checked for Sec15. Here, Sec15 levels were strongly reduced in the clones as compared to adjacent cells expressing a wild-type allele of *sec15* (Supplemental Figure S1, A and B). For Sec5 and Sec6, no antibodies suitable for immunohistochemical stainings were available.

Rab5 has been implicated in the control of apical–basal polarity in epithelial cells of *Drosophila* (Lu and Bilder, 2005). Therefore we checked whether loss of *Rab5* in larval NBs affects cell polarity. NBs of MARCM clones homozygous mutant for *Rab5*^{2}, a *Rab5*-null mutation (Wucherpfennig *et al.*, 2003), had a normal distribution of aPKC and Mira (Figure 3F). In a complementary approach, we interfered with Rab5 function by overexpression of a constitutively active and a dominant-negative version of Rab5 in embryos (Zhang *et al.*, 2007). In both cases, NB polarity was unaffected (Figure 4, A and B), consistent with our loss-of-function data (Figure 3F). We also overexpressed a dominant-negative version of Rab11 (Zhang *et al.*, 2007), which has been demonstrated to affect the function of recycling endosomes. Again, NB polarity was normal under these conditions (Figure 4C), further corroborating our hypothesis that cortical NB polarity is established by a mechanism independent of endosomal trafficking.

Next we generated MARCM clones homozygous for the *ada*^{3}-null allele of the vesicle coat protein α-adaptin (Gonzalez-Gaitan and Jackle, 1997). NBs in *ada*^{3} clones displayed normal distribution of aPKC and Mira (Figure 3G). The same result was obtained in MARCM clones for the *ada*^{ear4} allele (data not shown). *ada*^{ear4} is a hypomorphic mutation in α-adaptin that specifically affects asymmetric cell divisions in sensory organ precursor cells (Berdnik *et al.*, 2002). We also tested the function in NB polarization of another vesicle coat protein, clathrin heavy chain, by overexpression of a dominant-negative version in larval NBs. Again, apical–basal NB polarity was unaffected under these conditions (Figure 4D).

To analyze the role of ESCRT proteins for polarity of larval NBs, we generated MARCM clones homozygous for null alleles of *Hrs* (*Hrs*^{D28}), *Vps25* (*Vps25*^{A3}), and *erupted/TSG101* (*ept*^{2}) (Lloyd *et al.*, 2002; Moberg *et al.*, 2005; Vaccari and Bilder, 2005). MARCM clones for *Hrs*^{D28} and *Vps25*^{A3} showed normal NB polarity (Figure 3, H and I). We rarely observed small clones of cells homozygous for *ept*^{2}, but

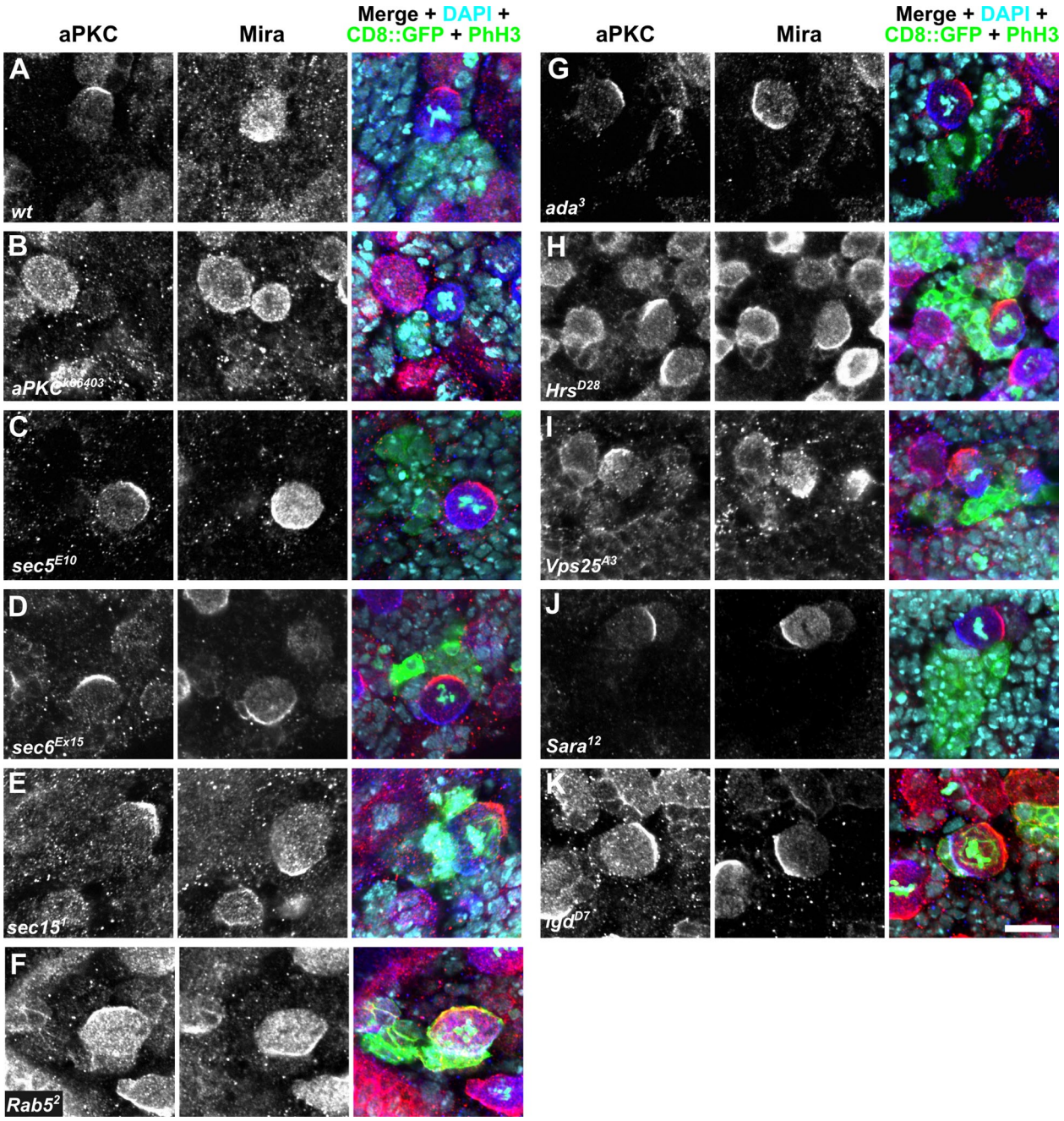

FIGURE 3: Dividing larval neuroblasts mutant for different vesicle-trafficking genes do not exhibit polarity defects. Larval neuroblasts in wild-type (A) and mutant (B–K) MARCM clones were stained for aPKC (red), Mira (blue), GFP, and phospho-histone H3 (both in green). The genotype of the mutant clones is indicated. CD8::GFP (green) marks the homozygous mutant cells. Scale bar, 10 μm. DNA is stained with DAPI (turquoise).

we never observed NBs in these clones (data not shown). It is possible that these clones result from inappropriate NB differentiation, but it is more likely that NB loss is caused by cell lethality of the mutation.

We also tested two additional mutations in genes involved in vesicular trafficking that had been shown to affect asymmetric division of larval sense organ precursor cells for defects in apical–basal NB polarity. Neither larval NBs mutant for the *Sara*^{12}-null allele of *Smad anchor for receptor activation* (*Sara*) (Bokel *et al.*, 2006) nor NBs mutant for the *lgd*^{D7}-null allele of *lethal (2) giant discs* (*lgd*) (Jaekel and Klein, 2006) showed polarity defects (Figure 3, J and K).

Table 1 summarizes the genes and alleles that were used to generate MARCM clones and the effects on Mira and aPKC localization in NBs homozygous for the indicated alleles.

DISCUSSION

Our data strongly indicate that vesicle trafficking is not involved in polarization of NBs, in contrast to epithelia, where it is essential for polarity. In epithelial cells vesicle trafficking controls cell polarity mainly by regulating the levels of the transmembrane proteins Crumbs and DE-Cad at the membrane (Langevin *et al.*, 2005; Lu and Bilder, 2005; Blankenship *et al.*, 2007). In NBs no asymmetrically

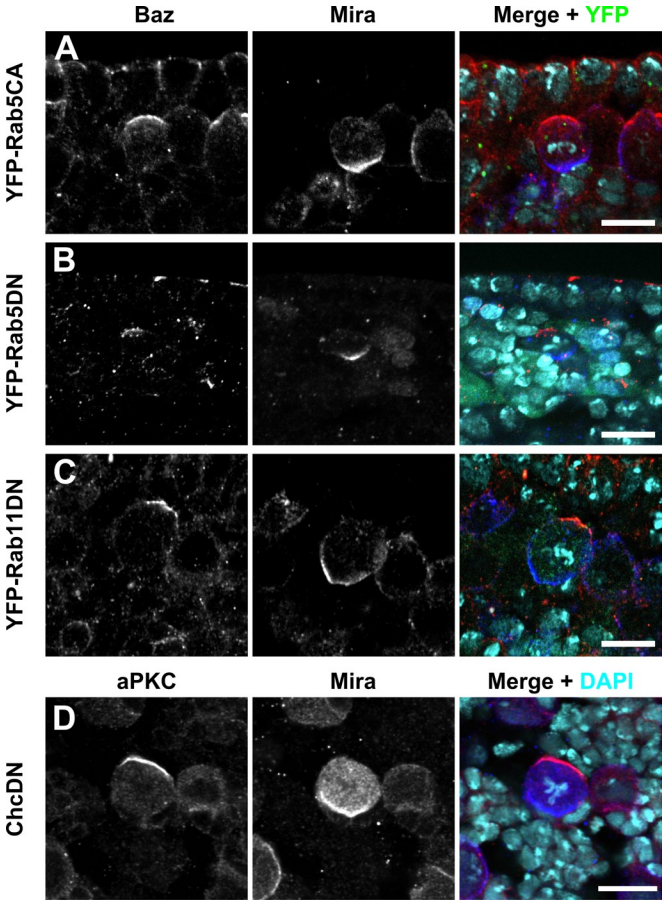

FIGURE 4: NB polarity is independent of Rab5, Rab11, and clathrin function. (A–C) Embryos overexpressing the yellow fluorescent protein–tagged, constitutively active (CA) or dominant-negative (DN) versions of the Rab proteins indicated on the left were stained for Baz (red) and Mira (blue). Overexpression was driven by tubulin::GAL4 (A) or worniu::GAL4 (B and C). (D) A larval brain overexpressing dominant-negative clathrin heavy chain under control of worniu::GAL4 was stained for aPKC (red) and Mira (blue). DNA was stained with DAPI (turquoise). Apical is up. Scale bar, 10 μm.

localized transmembrane protein has been described so far, except for one: Numb-interacting protein (NIP) is a multipass transmembrane protein that colocalizes with Numb at the basal cortex of dividing NBs. In *Drosophila* Schneider cells, Numb and NIP colocalize at the plasma membrane, and RNA interference–mediated knockdown of NIP results in a release of Numb from the plasma membrane (Qin *et al.*, 2004). Whether NIP is required for proper localization of Numb in dividing NBs is not known since no null mutation in *moladietz*, the gene encoding NIP, is available. It has also not been studied whether Numb may be required for the asymmetric localization of NIP in NBs.

So, how could cell polarity be established and maintained in NBs? Baz, PAR-6, and aPKC are all localized to the apical junctional region of the neuroectodermal epithelium at the time when NBs ingress from the epithelium. Thus the components of the PAR/aPKC complex are already apically enriched in NBs prior to their first division. We furthermore know that Baz can associate with the plasma membrane by direct binding to phosphoinositide lipids (Krahn *et al.*, 2010). However, there is no evidence for an asymmetric distribution of phosphoinositides in NBs, which might cause the asymmetric localization of Baz. In analogy to the mechanism that operates in the *Caenorhabditis elegans* zygote (Gonczy, 2008), we favor the hypothesis that the apical localization of Baz is stabilized by a mutual repression mechanism involving phosphorylation of Baz by the basally localized kinase PAR-1 (Benton and St Johnston, 2003; Krahn *et al.*, 2009) and phosphorylation of PAR-1 by aPKC (Hurov *et al.*, 2004; Kusakabe and Nishida, 2004). Although this mechanism may be sufficient to stably polarize an NB, extrinsic cues from adjacent neuroectodermal cells contribute to the positioning of the Baz crescent to the apical cortex (Siegrist and Doe, 2006).

In conclusion, our work shows for the first time that cortical polarity in NBs can be established even when intracellular vesicular trafficking is blocked, in striking contrast to the situation in epithelia. Although we cannot completely rule out the possibility that the lack of polarity phenotypes in NBs homozygous for the mutations that we analyzed may be due to the perdurance of the respective wild-type protein in the clones, we consider this possibility unlikely. It has been shown that the same mutations that we analyzed in NBs cause strong polarity phenotypes when clones are induced in epithelia.

		Miranda localization		aPKC localization	
Gene	Allele	Normal	Mislocalized	Normal	Mislocalized
sec5	$sec5^{E10}$	18	0	18	0
sec6	$sec6^{Ex15}$	23	0	23	0
sec15	$sec15^{1}$	14	0	14	0
Rab5	$Rab5^{2}$	18	0	18	0
alpha-adaptin	ada^{3}	20	0	20	0
Hrs	Hrs^{D28}	21	0	21	0
Vps25	$Vps25^{A3}$	14	0	14	0
Sara	$Sara^{12}$	15	0	15	0
erupted (TSG101)	ept^{2}	No clones obtained			
lgd	lgd^{d7}	15	0	15	0
aPKC	$aPKC^{k06403}$	0	10	Not detectable	Not detectable

Numbers indicate in how many dividing NBs in MARCM clones of the indicated genotype Mira and aPKC were normal or mislocalized. All analyzed NBs were in late prophase to anaphase. "No clones obtained" indicates that under the chosen experimental conditions no clones containing NBs homozygous for the indicated null alleles were recovered.

TABLE 1: Summary of MARCM experiments to study the function of vesicular trafficking during asymmetric division of larval NBs.

Furthermore, in some of our experiments we can rule out perdurance, for example in the experiments using the *shi*^{1} allele, and these also showed no polarity defects in NBs.

Our findings imply that the PAR/aPKC complex can function in different ways, to polarize only the cortex, as in NBs or the *C. elegans* zygote, or the cortex and the plasma membrane, as in epithelia and probably also in neurons. In the future it will be important to dissect these different mechanisms at the molecular level in order to understand the function of the PAR proteins in a specific cellular context.

MATERIAL AND METHODS

Fly stocks and genetics

The following stocks and alleles were used in this study: *w*^{1118} *shi*^{1} (#7068), *Df(3R)Espl3/TM6C cu*^{1} *Sb*^{1} *Tb*^{1} *ca*^{1} (#5601), *elav::Gal4 UAS-mCD8::GFP hsFLP* (#5146), *y*^{1} *w*; FRT42D GAL80/CyO y*$^{+}$ (#9917), *y*^{1} *w*; GAL80 FRT40A/CyO* (#5192), *y*^{1} *w*;; GAL80 FRT80B* (#5191), *y*^{1} *w*;; FRT82B GAL80* (#5135), *y*^{1} *w*; P{w[+mC] = tubP-GAL4}LL7/TM3 Sb*^{1} (#5138), *w*;; UAS-Chc.DN* (#26874) (Bloomington *Drosophila* Stock Center, Indiana University, Bloomington, IN; stock number given in parenthesis), and *onr*$^{142\text{-}5}$*/TM3 hb-lacZ* (Giansanti *et al.*, 2004; Blankenship *et al.*, 2007), *w; ada*^{ear4} *FRT40A* (Berdnik *et al.*, 2002), *w; ada*^{3} *FRT40A* (Gonzalez-Gaitan and Jackle, 1997), *w; Hrs*^{D28} *FRT40A* (Lloyd *et al.*, 2002), *w; Rab5*^{2} *FRT40A* (Wucherpfennig *et al.*, 2003), *w; lgd*^{d7} *FRT40A (Jaekel and Klein, 2006), w; FRT42D Sara*^{12} (Bokel *et al.*, 2006), *w; FRT42D aPKC*^{k06403} (Wodarz *et al.*, 2000; Rolls *et al.*, 2003), *w; FRT42D Vps25*^{A3} (Vaccari and Bilder, 2005), *w;; FRT80B ept*^{2} (Moberg *et al.*, 2005), *y w; sec5*^{E10} *FRT40/CyO y*$^{+}$ (Murthy *et al.*, 2003), *w; FRT42D sec6*^{Ex15} (Murthy *et al.*, 2005), *w;; FRT82B sec15*^{1}*/TM3* (Mehta *et al.*, 2005), *y*^{1} *w*; P{UASp-YFP.Rab5.Q88L}24*, *y*^{1} *w*; P{UASp-YFP.Rab5.S43N}01*, *y*^{1} *w*; P{UASp-YFP.Rab11.S25N}06* (Zhang *et al.*, 2007), and *w; worniu::GAL4* (Siegrist and Doe, 2005).

Embryos maternally and zygotically mutant for *onion rings*$^{142\text{-}5}$ *(Exo84*^{onr}*)* were obtained as described in Blankenship *et al.* (2007), with the exception that *Exo84*^{onr} was balanced over *TM3 ftz::lacZ* to facilitate genotyping by the absence of lacZ expression.

To generate embryos lacking *shibire* (*shi*) function, *w*^{1118} and *shi*^{1} flies were allowed to lay eggs for 1 h at 22°C; embryos were kept at 22°C for 5 h and were then shifted to 29°C for 2 h. As a control *w*^{1118} and *shi*^{1} embryos were kept at 22°C for 2 h instead.

w^{1118} *shi*^{1} wandering third-instar larvae were shifted to 34°C at 96 h after larval hatching for 6 h and directly dissected and fixed.

To generate positively labeled MARCM clones, the following crosses were conducted (females listed first):

1) *elav::Gal4 UAS-mCD8::GFP hsFLP; GAL80 FRT40A/CyO* × *w; ada*^{3} *FRT40A/CyO*
2) *elav::Gal4 UAS-mCD8::GFP hsFLP; GAL80 FRT40A/CyO* × *y w; sec5*^{E10} *FRT40/CyO y*$^{+}$
3) *elav::Gal4 UAS-mCD8::GFP hsFLP; GAL80 FRT40A/CyO* × *w; rab5*^{2} *FRT40A/CyO*
4) *elav::Gal4 UAS-mCD8::GFP hsFLP; GAL80 FRT40A/CyO* × *w; Hrs*^{D28} *FRT40A/CyO*
5) *elav::Gal4 UAS-mCD8::GFP, hsFLP; GAL80 FRT40A/CyO* × *w; lgd*^{d7} *FRT40A/CyO*
6) *elav::Gal4 UAS-mCD8::GFP hsFLP; GAL80 FRT40A/CyO* × *w; FRT40A*
7) *elav::Gal4 UAS-mCD8::GFP hsFLP; FRT42D GAL80/CyO* × *w; FRT42D vps25*^{A3}*/CyO*
8) *elav::Gal4 UAS-mCD8::GFP hsFLP; FRT42D GAL80/CyO* × *w; FRT42D Sara*^{12}*/CyO*
9) *elav::Gal4 UAS-mCD8::GFP hsFLP; FRT42D GAL80/CyO* × *w; FRT42D sec6*^{Ex15}*/CyO*
10) *elav::Gal4 UAS-mCD8::GFP hsFLP; FRT42D GAL80/CyO* × *w; FRT42D aPKC*^{k06403}*/CyO*
11) *elav::Gal4 UAS-mCD8::GFP hsFLP; FRT42D GAL80/CyO* × *w; FRT42D*
12) *elav::Gal4 UAS-mCD8::GFP hsFLP;; GAL80 FRT80B/CyO* × *w;; ept*^{2} *FRT80B/TM6B*
13) *elav::Gal4 UAS-mCD8::GFP hsFLP;; GAL80 FRT80B/CyO* × *w;; FRT80B*
14) *elav::Gal4 UAS-mCD8::GFP hsFLP; FRT82B GAL80* × *w;; FRT82B sec15*^{1}*/TM6B*
15) *elav::Gal4 UAS-mCD8::GFP hsFLP; FRT82B GAL80* × *w;; FRT82B*

For induction of MARCM clones, eggs were collected for a period of 24 h. After incubation at 25°C for another 24 h, larvae were heat shocked during L1 once for 2 h in a 37°C water bath to induce mitotic recombination. Brains of wandering third-instar larvae were dissected and stained.

Immunohistochemistry

Embryos and brains of wandering third-instar larvae were fixed in 4% formaldehyde, phosphate-buffered saline (PBS), pH 7.4. The primary antibodies used were rabbit anti-PKCζ C20 (Santa Cruz Biotechnology, Santa Cruz, CA) 1:1000, rabbit anti-Baz (Wodarz *et al.*, 1999) 1:1000, guinea pig anti-Mira (Kim *et al.*, 2009) 1:1000, rabbit anti-Sara (Bokel *et al.*, 2006) 1:200, guinea pig anti-Hrs (Lloyd *et al.*, 2002) 1:1000, mouse anti–phospho-histone H3 (6G3; Cell Signaling Technology, Danvers, MA) 1:1000, mouse anti–green fluorescent protein (GFP; Invitrogen, Carlsbad, CA) 1:1000, mouse anti–β-galactosidase JIE7 (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) 1:50, and guinea pig anti-Sec15 (Mehta *et al.*, 2005) 1:500. Secondary antibodies conjugated to Cy2, Cy3, Cy5 (Jackson Laboratories, West Grove, PA), Alexa Fluor 647, and Alexa Fluor 488 (Invitrogen) were used at 1:400. Images were taken on a Zeiss (Wetzlar, Germany) LSM 510 Meta confocal microscope and processed using Gimp and Inkscape.

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