

The Rap1–Rgl–Ral signaling network regulates neuroblast cortical polarity and spindle orientation

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A crucial first step in asymmetric cell division is to establish an axis of cell polarity along which the mitotic spindle aligns. *Drosophila melanogaster* neural stem cells, called neuroblasts (NBs), divide asymmetrically through intrinsic polarity cues, which regulate spindle orientation and cortical polarity. In this paper, we show that the Ras-like small guanosine triphosphatase Rap1 signals through the Ral guanine nucleotide exchange factor Rgl and the PDZ protein Canoe (Cno; AF-6/Afadin in vertebrates) to modulate the NB division

axis and its apicobasal cortical polarity. Rap1 is slightly enriched at the apical pole of metaphase/anaphase NBs and was found in a complex with atypical protein kinase C and Par6 in vivo. Loss of function and gain of function of Rap1, Rgl, and Ral proteins disrupt the mitotic axis orientation, the localization of Cno and Mushroom body defect, and the localization of cell fate determinants. We propose that the Rap1–Rgl–Ral signaling network is a novel mechanism that cooperates with other intrinsic polarity cues to modulate asymmetric NB division.

Introduction

Asymmetric cell division is a key process in development and stem cell biology. In an asymmetric cell division, one daughter cell retains the self-renewal capacity of the mother stem cell and keeps on dividing, whereas the other daughter cell is committed to initiating a differentiation program. A crucial first step in an asymmetric cell division is to establish an axis of cell polarity along which the mitotic spindle aligns. Extrinsic and intrinsic mechanisms regulate the spindle orientation and the final asymmetry of the division. *Drosophila melanogaster* stem cells have been extensively studied during the last few decades, providing a deep insight into both types of mechanisms (Doe, 2008; Knoblich, 2008; Morrison and Spradling, 2008). *Drosophila* neural stem cells, called neuroblasts (NBs), divide asymmetrically, mainly through intrinsic polarity cues. In the embryonic central nervous system (CNS), NBs delaminate from the neuroectoderm (NE) inheriting the apicobasal polarity of the neuroepithelial cells. Intrinsic signals, mostly polarized at the apical NB cortex, tightly couple the spindle orientation along the apicobasal axis with the asymmetric location of cell fate determinants at the basal pole of the NB. In this way, these determinants are secreted to the basal and smaller daughter cell, called the

ganglion mother cell (GMC). The apical and bigger daughter cell continues dividing as an NB, always budding off smaller GMCs into the embryo in the same, highly stereotyped, basal orientation (Wodarz and Huttner, 2003; Chia et al., 2008; Knoblich, 2008; Siller and Doe, 2009). Extrinsic signals emanating from the NE also participate in regulating spindle orientation and cortical polarity in the NB, though the nature of these signals remains elusive (Siegrist and Doe, 2006).

Here, we show that the Ras-like small GTPase Rap1 contributes to regulate asymmetric NB division through the Ral guanine nucleotide exchange factor Rgl, Ral, and the PDZ domain-containing protein Canoe (Cno; AF-6/Afadin in vertebrates; Miyamoto et al., 1995; Asha et al., 1999; Mirey et al., 2003). Rap1 has a key and evolutionary conserved role in regulating morphogenesis, integrin- as well as cadherin-mediated cell–cell adhesion, and junction formation. In addition, Rap1 has adhesion-independent functions that suggest a central function of Rap1 in signal transduction (Asha et al., 1999; Knox and Brown, 2002; Caron, 2003; Mirey et al., 2003; Price et al., 2004; Wang et al., 2006; Kooistra et al., 2007; O’Keefe et al., 2009). Ral proteins are Ras-like GTPases that can be activated through a Ras-dependent mechanism in mammalian cell lines (Yaffe et al., 2001) and downstream of Rap1–Rgl in *Drosophila*

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Abbreviations used in this paper: aPKC, atypical PKC; Baz, Bazooka; Cnn, centrosomine; Cno, Canoe; CNS, central nervous system; DE-cad, *Drosophila* E-cadherin; GMC, ganglion mother cell; Insc, Inscuteable; L’sc, Lethal of Scute; Mira, Miranda; mNB, metaphase NB; Mud, Mushroom body defect; NB, neuroblast; NE, neuroectoderm; Pins, Partner of Insc; Pros, Prospero; Scrib, Scribbled; WT, wild type.

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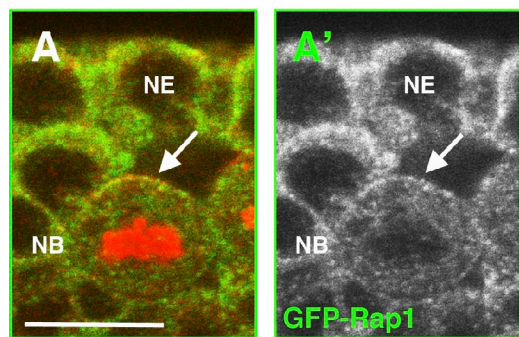
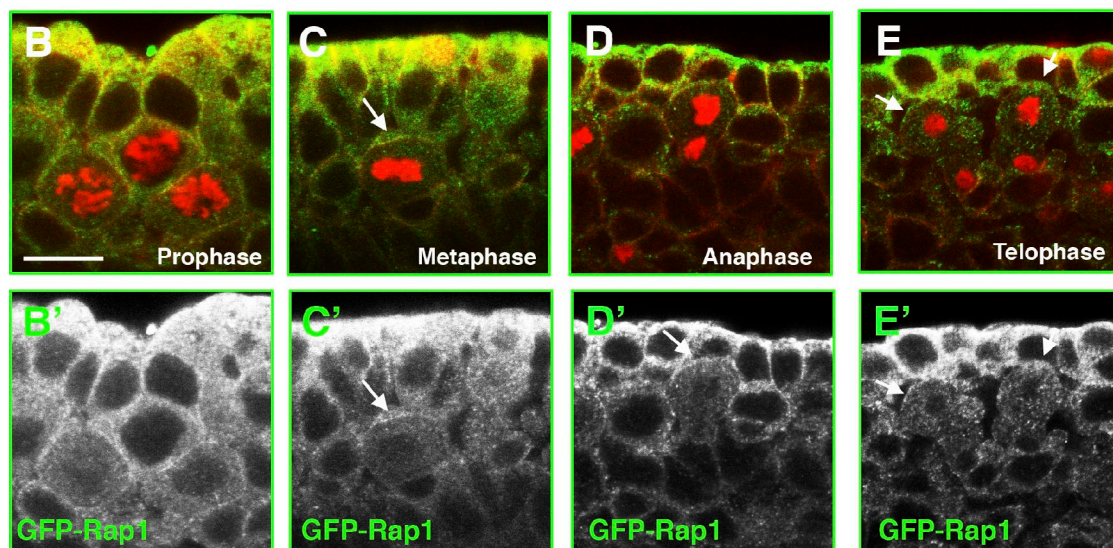
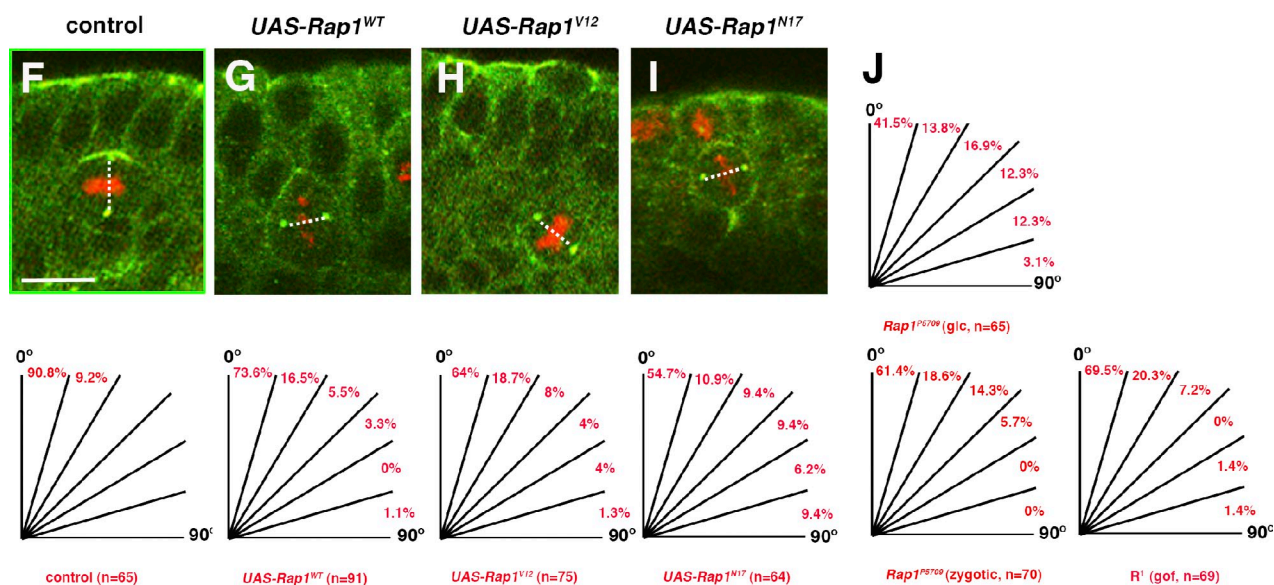
GFP-Rap1/Baz/PH3**GFP-Rap1/Scrib/PH3****Baz/Cnn/PH3**

Figure 1. Rap1 is present in mNBs, and it is required for spindle orientation. (A and A') GFP-Rap1 appears in a punctate pattern, which is uniformly distributed in the NE and enriched at the apical pole of NBs (arrows), where Rap1 colocalizes with Baz. The DNA (PH3) is in red. (B–E') GFP-Rap1 location throughout mitosis. (B and B') At prophase, GFP-Rap1 is mainly cytoplasmic. (C–D') At metaphase, it accumulates at the NB apical pole (C and C'),

(Mirey et al., 2003). The Rap/Ras–Rgl–Ral GTPase signaling network is highly conserved between *Drosophila* and mammals (Moskalenko et al., 2001; Mirey et al., 2003). Intriguingly, Rap1 interacts physically with Cno/AF-6, and the Ral guanine nucleotide exchange factor Rgl has been predicted as a potential partner of Cno (Drosophila Interactions Database; Boettner et al., 2000, 2003), a novel regulator of asymmetric NB division (Speicher et al., 2008). Our results now show that loss and gain of function of Rap1, Rgl, and Ral proteins affect the NB spindle orientation, the generation of unequal-sized progeny, and the localization of apical proteins, such as Cno and the microtubule-associated protein Mushroom body defect (Mud; Numa in vertebrates; Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006). Bazooka (Baz; Par3 in vertebrates) and the atypical PKC (aPKC; Schober et al., 1999; Wodarz et al., 1999, 2000) were affected to a lesser degree. Failures in the basal localization of the cell fate determinants Numb, Prospero (Pros), and its adaptor protein Miranda (Mira; Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Ikeshima-Kataoka et al., 1997; Shen et al., 1997; Schuldt et al., 1998) were also detected in *Rap1* and *Ral* mutants. Moreover, co-immunoprecipitation experiments from embryo extracts showed that Rap1 is in a complex with aPKC and Par6. Additionally, Rgl synergistically cooperated with other apical proteins, such as Partner of Inscuteable (Insc; Pins), Insc, and Mud to regulate spindle orientation. Taking all data into account, we propose that the Rap1–Rgl–Ral signaling network is a novel intrinsic mechanism that cooperates with other apical proteins to regulate cortical polarity and spindle orientation in NBs.

Results and discussion

Rap1 is present in the embryonic neuroepithelium and in NBs

In an attempt to further characterize the protein network that along with Cno modulates asymmetric NB division (Speicher et al., 2008), we wanted to analyze in detail the expression and function of the Cno-interacting partner Rap1 (Boettner et al., 2000). GFP-Rap1, a fusion protein that is expressed under the control of the endogenous *Rap1* promoter (Knox and Brown, 2002), was detected throughout the NE, evenly distributed in the cytoplasm, and in the delaminated metaphase NBs (mNBs), with a slight enrichment at the apical pole along with the Baz apical crescent (Fig. 1, A and A'). Looking in more detail throughout the NB mitotic cycle, GFP-Rap1 was found uniformly distributed at prophase and started to accumulate apically at metaphase (Fig. 1, B–C'). During anaphase, this apical enrichment was still detected, and by telophase, GFP-Rap1 began to delocalize (Fig. 1, D–E'). Hence, Rap1 was a potential candidate for regulating the process of asymmetric NB division.

Rap1 is required for the mitotic axis orientation and apical proteins localization in mNBs

To determine a possible function of Rap1 in the cell division axis orientation, we analyzed the effect of expressing wild-type (WT), constitutively active (V12), and dominant-negative (N17) forms of Rap1 (hereafter referred to as *Rap1* mutants). A maternal Gal-4 line (V32) was used to drive expression of these transgenes. The spindle orientation was altered when the Rap1 signal was impaired, in clear contrast with control embryos, in which most NBs showed a normal spindle orientation along the apicobasal axis of cell polarity (Fig. 1, F–I). To further support these results, we decided to look at additional *Rap1* mutant conditions: *Rap1*^{P5709} germline clones, a complete loss of maternal and zygotic product, *Rap1*^{P5709}-only zygotic loss, and *R*¹ (*Roughened*¹), a gain-of-function mutation in the *Rap1* locus (Hariharan et al., 1991). Clear defects in the spindle orientation were detected in all these mutants (Fig. 1 J). Apical cortical polarity was also affected in *Rap1* mutants. The Par complex proteins Baz and aPKC were mislocalized in 10.1% of mNBs ($n = 178$) and 1.4% ($n = 143$) in *UAS-Rap1*^{WT} embryos, respectively, in 11.2% ($n = 161$) and 11.3% ($n = 115$) in *UAS-Rap1*^{V12} embryos, and in 28.8% ($n = 145$) and 19.8% ($n = 101$) in *UAS-Rap1*^{N17} embryos (Fig. 1, G–I). No defects in Baz ($n = 65$ mNBs) or aPKC ($n = 66$) were observed in control embryos. Much more penetrant phenotypes were observed for the apical proteins Cno and Mud in those mutant backgrounds (Fig. 2, A–I). The localization of the Gai subunit and Pins, other apical proteins key for regulating spindle alignment (Parmentier et al., 2000; Schaefer et al., 2000; Yu et al., 2000), were, however, not affected (Fig. 2, J–M; and not depicted). Hence, Rap1 is required for the correct establishment of cortical polarity and spindle orientation through a pathway that includes Cno and Mud but is Gai and Pins independent.

Rap1 mutants show cell fate determinant mislocalization and equal-sized daughter cells

Given the defective localization of apical proteins we observed in *Rap1* mutants, we predicted that cell fate determinants would be misplaced in mNBs. In fact, whereas in control embryos Numb was found in basal crescents in most mNBs, clear defects in Numb localization were detected in *Rap1* mutants, including *Rap1*^{P5709} germline clones and *R*¹ mutant conditions (Fig. 3, A–Q). Another cell fate determinant, the transcription factor Pros and its adaptor protein Mira also showed altered location in a significant number of the mNBs analyzed compared with control embryos (Fig. 3, R–Y). These defects were partially or completely rescued at telophase (compensatory mechanism known as telophase rescue; Fig. 3 Y). Therefore, Rap1 is required for the correct establishment of apical polarity in the NB and for the proper location

where it still can be detected at anaphase (D and D'). (E and E') At telophase, GFP-Rap1 starts to delocalize. (F–I, top) Cnn at the centrosomes reveals spindle orientation. Arrows point to GFP-Rap1 at the apical pole of the dividing NB. (F) In control embryos, the mitotic spindle (depicted by dotted lines) orientates along the apicobasal axis. (G–I) In *Rap1* mutants, the spindle was misorientated. (F–I, bottom) Percentages of spindles aligned in each 15° bin for the genotypes indicated. (J) The same analysis is shown in additional *Rap1* mutant conditions (see also Rap1 is required for the mitotic axis orientation and apical protein localization in mNBs in the Results). glc, germline clone; gof, gain of function. Bars, 10 μ m.

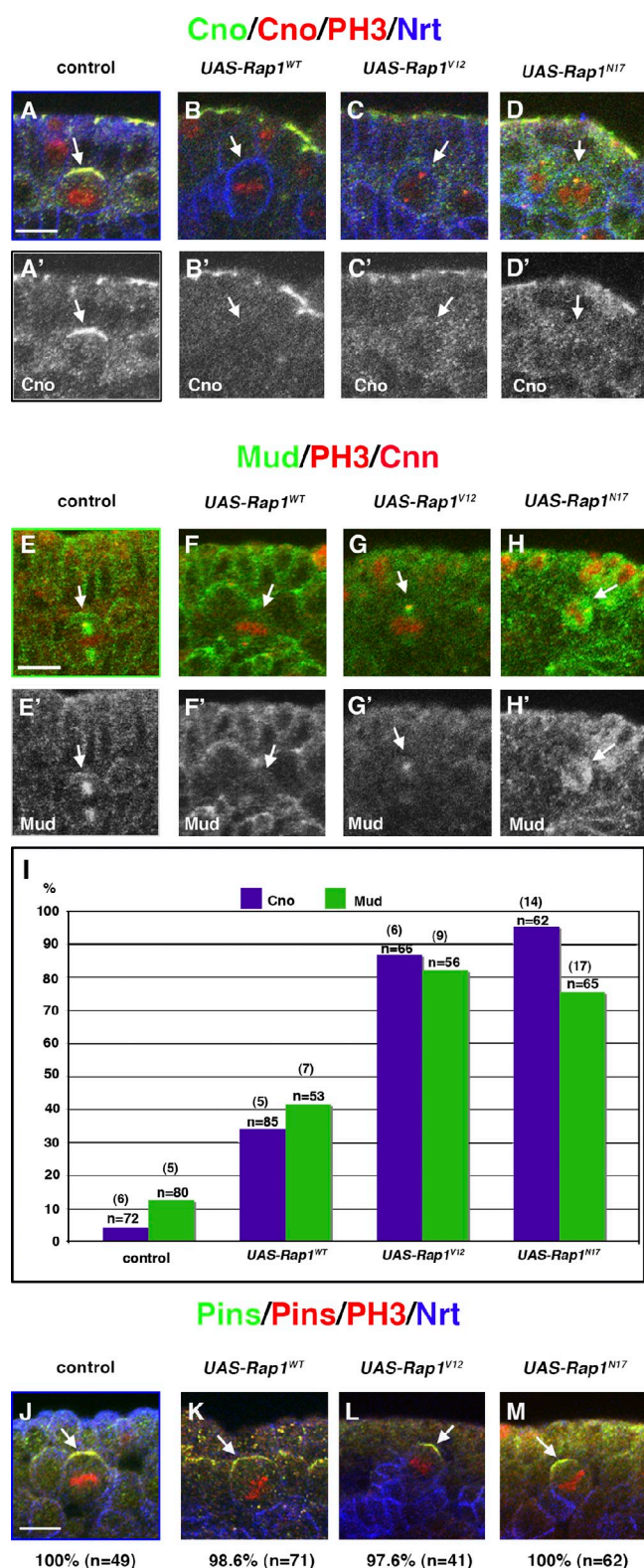


Figure 2. Rap1 is required for the proper localization of apical proteins in mNBs. (A and A') In control embryos, Cno (in yellow: green plus red channels) appears in an apical crescent in mNBs (arrows). (B–D') In *Rap1* mutants, Cno was absent (B–C') or mislocalized (D and D'). Note that Cno location is not affected in the NE. Cnn is only shown in C and D. Neurotactin (Nrt) labels membranes. (E and E') In control embryos, Mud appears apically in mNBs (arrows) as well as associated with centrosomes and microtubules. (F–H') In *Rap1* mutants, the apical location of Mud frequently fails (arrows). (I) Quantitations of Cno- and Mud-defective localization in

of asymmetrically segregating factors. Mud, whose distribution was altered in *Rap1* mutants, is not required for apicobasal cortical polarity (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006; Cabernard and Doe, 2009). Hence, the failure in cell fate determinant location in *Rap1* mutants was caused, at least in part, by the mislocalization in these mutants of Cno and aPKC/Baz, all key factors for the proper formation of determinant basal crescents in mNBs (Speicher et al., 2008; Wirtz-Peitz et al., 2008). Intriguingly, another distinctive feature of asymmetric NB division, the generation of unequal-sized daughter cells, was also altered in *Rap1* mutants (Fig. S1, A–D). In addition, the asymmetric division within the well-characterized RP2 neuron lineage (Broadus et al., 1995) was affected in these mutants (Fig. S1, E–H). Hence, Rap1 regulates multiple aspects of the asymmetric NB division.

Given that Rap1 is required for a proper cell–cell adhesion in epithelial tissues and that this might influence the analysis of the underlying NBs in *Rap1* mutants, we looked in detail to epithelial cell polarity in *Rap1*^{V12} and *Rap1*^{N17} mutant embryos (Fig. S2). Different polarity proteins, such as *Drosophila* E-cadherin (DE-cad), aPKC, and Scribble (Scrib), were analyzed. In *Rap1*^{V12} mutants, aPKC was reduced, but DE-cad and Scrib were not affected. Similar results were found in *Rap1*^{N17}, though in this case, clear defects in the morphology/integrity of the epithelia were observed. Remarkably, however, no correlation was found between epithelial morphology and NB polarity/orientation defects in these mutants (for an example see Fig. S2, C, C', F, and F'). From this analysis, we conclude that Rap1 is required for both processes, epithelial integrity and NB polarity, and that these functions are independent. Additionally, to discard that the defects observed in *Rap1* mutant NBs were caused by earlier defects in NB specification, we analyzed in detail the process of NB delamination at stage 9 in these mutants. In *Rap1*^{V12}, NBs were organized in the windowlike arrangement typical of this stage in WT embryos (Fig. S3, A and D). About 31% of *Rap1*^{N17} mutant embryos showed NB disorganization, probably caused by the early role of Rap1 in morphogenetic events, such as gastrulation (Fig. S3, G and H; see also Fig. 5 in Asha et al. [1999]). In a percentage of *Rap1*^{V12} and *Rap1*^{N17} mutant embryos, the number of NBs seemed also to be affected, but importantly, NB delamination was properly achieved in both cases (Fig. S3, compare E, F, and I–K with B and C). Thus, NB alterations in polarity and spindle orientation are not merely a consequence of an impaired NB specification process.

Rgl and Ral mutants display abnormal NB spindle orientation and apicobasal cortical polarity

Given the phenotypes we found in *Rap1* mutants in the CNS NBs, we wondered whether the Rap1 effectors Rgl and Ral also had a function in this system. We found that ΔRgl mutant embryos

Rap1 mutants. The experiment was completed once (n = total number of mNBs analyzed; number of embryos analyzed in each case is shown between brackets). (J–M) Pins location is not altered in *Rap1* mutants. Arrows point to Pins at the apical pole of the mNB. Percentages indicate the frequency of Pins correct localization. Bars, 10 μ m.

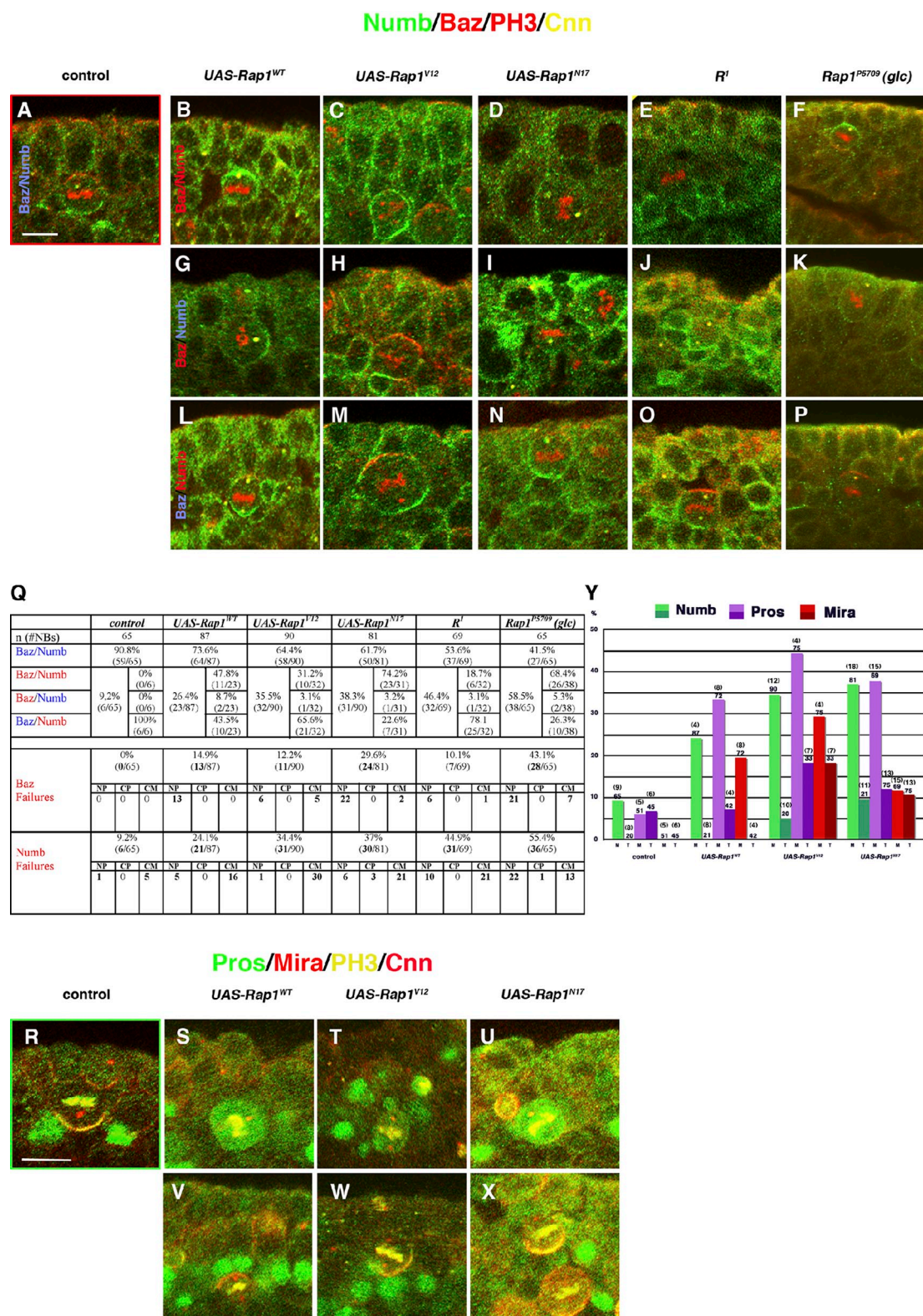
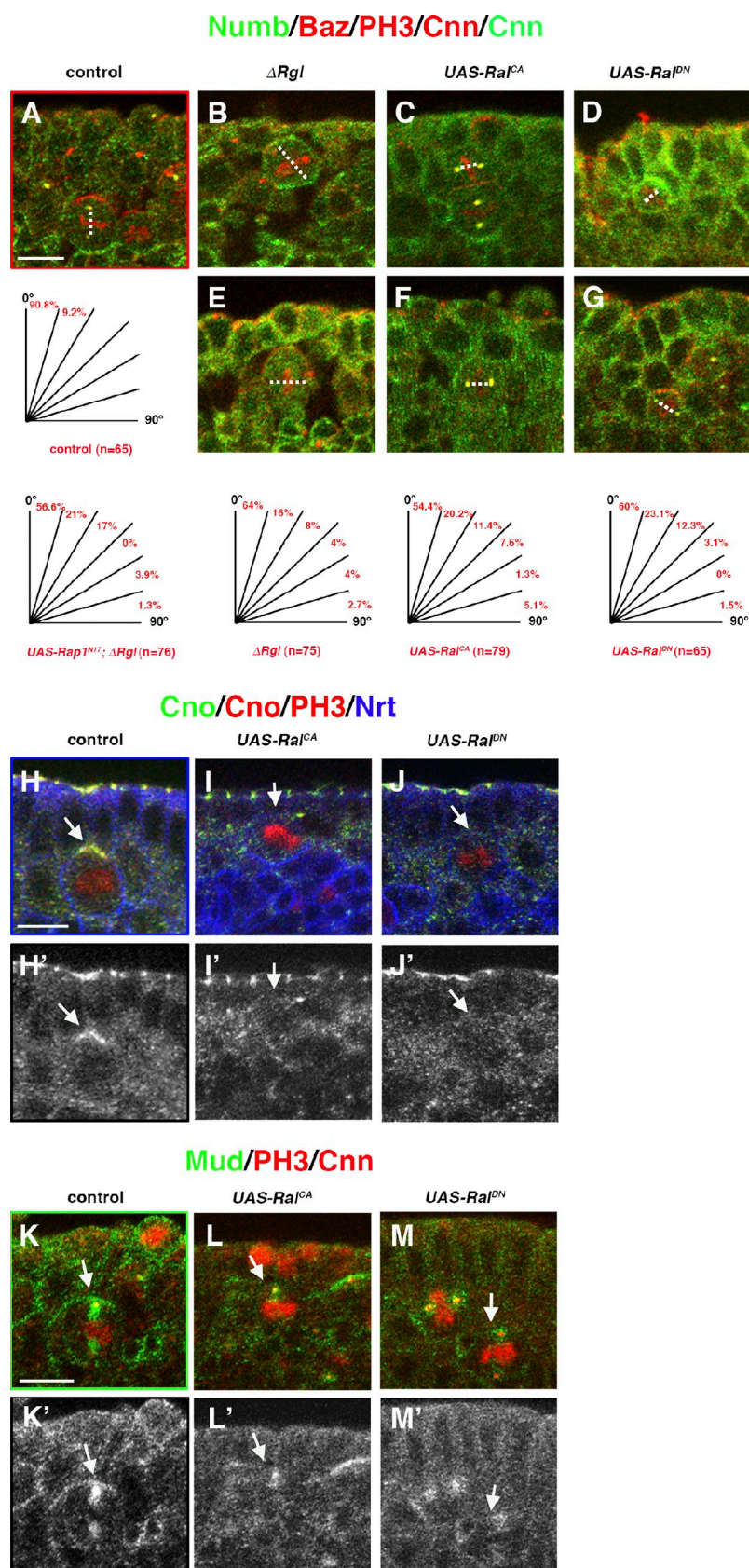


Figure 3. *Rap1* mutants show cell fate determinant mislocalization. (A) In control embryos, Numb appears at basal crescents in mNBs, just opposite to the Baz apical crescent. (B–P) In *Rap1* mutants, Numb location failed. Three examples of each mutant phenotype are shown: (1) Baz and Numb have wrong locations (Baz/Numb, first row); (2) only Baz is mislocalized (Baz/Numb, second row); and (3) only Numb is mislocalized (Baz/Numb, third row). (Q) Quantitations of these three phenotypes. The particular defects observed in the *Rap1* mutants specified are described: NP, not present; CP, cytoplasmic; CM, cortical mislocalization. (R) In control embryos, Pros and Mira colocalize in a basal crescent in mNBs. PH3 is in red/green channels. (S–X) In *Rap1* mutants (two examples are shown of each mutant genotype), Pros and Mira localization was altered. They were found at the cytoplasm (S–U and X), not present (S–V), or mislocalized (V and W). (Y) Quantitations of Pros, Mira, and Numb location failures in *Rap1* mutants at metaphase and at telophase (telophase rescue is shown by darker color bars). The experiment was completed once (n = total number of mNBs analyzed; number of embryos analyzed in each case is shown between brackets). M, metaphase; T, telophase; glc, germline clone. Bars, 10 μ m.

Figure 4. *Rgl* and *Ral* mutants display abnormal NB spindle orientation and apicobasal cortical polarity. (A) In control embryos, Baz and Numb form crescents at the apical and basal poles of mNBs, respectively, and the spindle aligns along the apicobasal axis (dotted lines). (B–G) In *Rgl* and *Ral* mutants, the localization of Baz and Numb was altered (two different examples of each genotype are shown). The spindle orientation also failed in *Rgl* and *Ral* mutants. Bottom diagrams show the percentages of spindles aligned in each 15° bin for each genotype analyzed. This analysis was also performed in *UAS-Rap1^{N17}; ΔRgl* double mutant embryos (see also *Rgl* and *Ral* mutants display abnormal NB spindle orientation and apicobasal cortical polarity in the Results). (H–M') The location of Cno (H–J') and Mud (K–M') failed in *Ral* mutants. Arrows point to the apical pole of the mNB. Nrt, Neurotactin. Bars, 10 μm.



(Mirey et al., 2003) displayed mitotic spindle misorientation (Fig. 4, B and E), the same phenotype that showed embryos expressing activated (*Ral^{CA}*) and dominant-negative forms (*Ral^{DN}*)

of *Ral* (Fig. 4, C, D, F, and G). Moreover, double mutants *Rap1^{N17}; ΔRgl* showed a spindle phenotype more similar to that shown by ΔRgl single mutants (Fig. 4), suggesting that *Rgl* is

acting in the same pathway of Rap1, downstream of it (Fig. 1 I). Additionally, in both ΔRgl and Ral mutants, the apicobasal cortical polarity was altered. Baz was misplaced in 9.3% of the ΔRgl NBs analyzed ($n = 75$; 0% in control embryos, $n = 65$) and in 16.4% ($n = 79$) and 15.4% ($n = 65$) of NBs in Ral^{CA} or Ral^{DN} embryos (Fig. 4, B–G). The localization of Cno and Mud also showed (similar to that observed in $Rap1$ mutants) more penetrant phenotypes. Although in control embryos, 4.2 and 12.5% of mNBs displayed failures in Cno and Mud localization, respectively, in Ral^{CA} mutants, the percentages were 71% of NBs ($n = 62$) for Cno localization and 51.2% for Mud ($n = 80$; Fig. 4, H–I' and K–L'). In Ral^{DN} mutant embryos, the failures observed corresponded to 76.8% ($n = 56$) for Cno and 64.3% ($n = 56$) for Mud (Fig. 4, J, J', M, and M'). Finally, the basal localization of Numb was also affected in ΔRgl , in 48% of mNBs ($n = 75$; Fig. 4, B and E), and in Ral mutants. Specifically, in Ral^{CA} mutants, Numb failures were 27.8% ($n = 79$) and 29.2% ($n = 65$) in Ral^{DN} mutant embryos (Fig. 4, C, D, F, and G). Thus, Rgl–Ral functions in the embryonic NBs of the CNS to regulate cortical polarity and spindle orientation downstream of Rap1.

The Rap1–Rgl–Ral signaling network functions in a complex with aPKC and Par6 cooperating with other apical proteins to regulate asymmetric NB division

Here, we have shown that Rap1 functions in asymmetric NB division in the embryonic CNS to modulate NB apicobasal cortical polarity and mitotic axis orientation. Rap1 would act through the Rgl–Ral–Cno signaling network to regulate Mud localization and, hence, spindle alignment. The localization of the apical protein Pins, which is attached to the cortex through the heterotrimeric G α i subunit (Fig. 5 F; Schaefer et al., 2000; Nipper et al., 2007), was not dependent on Rap1 signaling and neither was the location on the G α i subunit. Pins, through Discs Large and Khc-73, is key for spindle location (Siegrist and Doe, 2005; Johnston et al., 2009). Hence, both pathways, Rap1–Rgl–Ral and G α i–Pins could cooperate to properly orientate the mitotic axis. To test this possibility, we analyzed the spindle orientation in double mutants ΔRgl , $pins^{\Delta 50}$. In this genetic background, 46.4% of the mNBs ($n = 69$) showed a correct spindle alignment (0–15° window) compared with the 64.0% ($n = 75$; $P = 0.0439$) and the 66.1% ($n = 56$; $P = 0.0314$) of mNB defects found in ΔRgl or $pins^{\Delta 50}$ single mutants, respectively (Fig. 5 A). In addition, $insc^{P49}$; ΔRgl and mud^{Δ} ; ΔRgl double mutants also showed a strong cooperation in regulating spindle orientation (Fig. 5, B and C). In $insc^{P49}$; ΔRgl double mutants, 36.1% of the mNBs ($n = 72$) displayed a WT spindle orientation (0–15° window) versus the 64.0% ($n = 75$; $P = 0.0009$) and the 57.9% ($n = 57$; $P = 0.0204$) of mNB failures observed in the single mutants ΔRgl and $insc^{P49}$, respectively. The interaction between Rgl and mud was statistically significant when comparing the expressivity, not the penetrance, of the phenotype. In other words, mud^{Δ} ; ΔRgl double mutants showed many more cases of spindle alignment defects in the 75–90° window (11.8%, $n = 59$) compared with the single mutants ΔRgl (2.7%, $n = 75$; $P = 0.0429$) and mud^{Δ} (1.6%, $n = 63$; $P = 0.286$). This last interaction between mud and Rgl suggests

that the Rap1–Rgl–Ral pathway is modulating something else, independent of Mud, which is important for spindle alignment. It has previously been reported that there are two independent and redundant apical pathways in NBs. One of these pathways is formed by Baz, aPKC, Par6, and Insc, and the other pathway is formed by G α i–Pins (Cai et al., 2003). Even though there are some interdependence between both pathways at prophase for their apical location (Yu et al., 2000), they become much more independent from metaphase onwards. For example, Pins localizes asymmetrically in 81% of the $insc$ mutant mNBs analyzed (Cai et al., 2003). This would explain why the Baz and aPKC failures found in $Rap1$ mutants, only at metaphase, are not affecting Pins location. Hence, the Rap1–Rgl–Ral pathway synergistically cooperates with other apical polarity cues to correctly position the mitotic spindle.

The Rap1 signaling network also contributes to the establishment of the cortical polarity and, consequently, to the proper segregation of determinants at the basal NB pole. This effect seems to be mainly mediated through its interacting partner Cno, which has been shown to be required for this process (Speicher et al., 2008). Trying to understand how Rap1 is initially polarized at the apical NB pole, we performed in vivo coimmunoprecipitation assays with GFP–Rap1 and different apical proteins. Although Pins, G α i, or Mud did not show any positive result, we found that both aPKC and Par6 were able to coimmunoprecipitate with Rap1 (Fig. 5 D). Thus, these Par complex proteins can help to locate Rap1 at the apical NB pole. The localization of these proteins, Baz and aPKC, also key for cell fate determinant localization, were altered to a lesser degree in $Rap1$ mutant mNBs. This effect of Rap1–Rgl–Ral on Baz and aPKC may respond to the establishment in normal conditions of a positive feedback loop of the Rap1 pathway on the Par protein complex (Par6–Baz/Par3–aPKC) to facilitate their stabilization at metaphase (Fig. 5 E). Intriguingly, Rap1B has been shown to act upstream of this complex and of Cdc42 to regulate neuronal polarity in mammalian cell cultures (Schwamborn and Püschel, 2004). The low penetrant phenotypes observed for Baz and aPKC location in $Rap1$ mutants suggests though that Rap1 signal is not the major driving force initially positioning the Par proteins. This might be driven, at least in part, by extrinsic signals coming from the NE (Siegrist and Doe, 2006). The nature of those extrinsic cues remains, however, elusive.

Materials and methods

Drosophila strains and genetics

The following mutant stocks and fly lines were used: *GFP–Rap1* (Knox and Brown, 2002), *UAS–Rap1^{WT}*, *UAS–Rap1^{V12}*, and *UAS–Rap1^{N17}* (a gift from R. Reuter, Universität Tübingen, Tübingen, Germany), *Rap1^{P5709}* (a gift from N. Brown, University of Cambridge, Cambridge, England, UK), *R¹* (Bloomington Stock Center), *pins^{Δ50}* (Schaefer et al., 2000), *insc^{P49}* (Bloomington Stock Center), *mud^Δ* (Bloomington Stock Center), *UAS–Ral^{CA}*, *UAS–Ral^{DN}*, ΔRgl (a gift from J. Camonis, Institut Curie, Institut National de la Santé et de la Recherche Médicale, Paris, France; Mirey et al., 2003), and *maternal–GAL4 V32* (a gift from J.A. Knoblich, Institute of Molecular Biotechnology, Vienna, Austria). All the crosses of *GAL4–UAS* were performed at 29°C. *yw* was used as the reference control WT strain. Balancer chromosomes containing different *lacZ* or *GFP* transgenes were used for identifying homozygous mutant embryos.

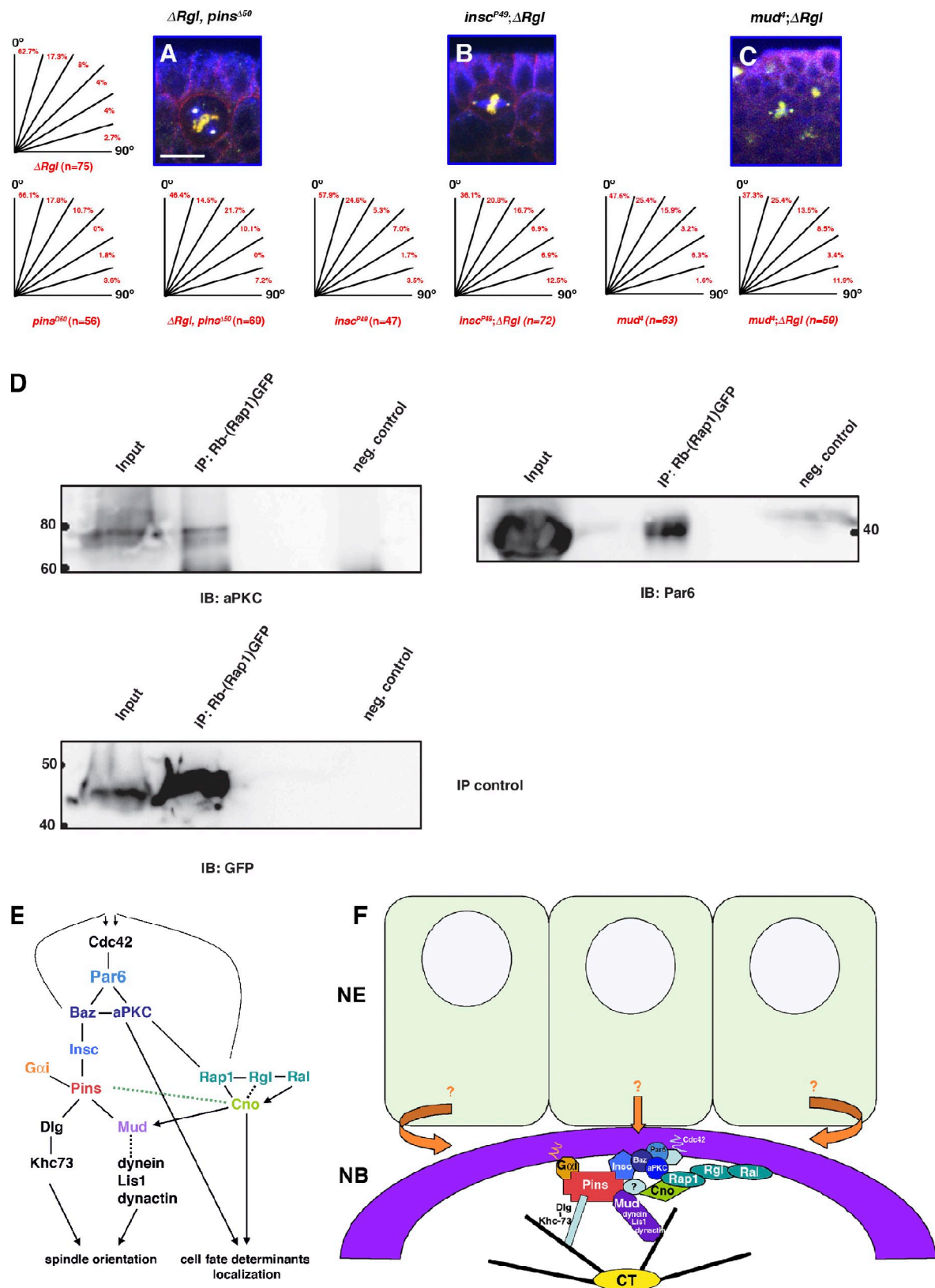


Figure 5. **Rap1-Rgl-Ral network functions in a complex with aPKC and Par6 cooperating with other apical proteins to regulate asymmetric NB division.** (A–C) Spindle orientation fails in a statistically significant higher degree in *ΔRgl, pins^{Δ50}*, *insc^{P49}, ΔRgl*, and *mud^Δ, ΔRgl* double mutant mNBs compared with *pins^{Δ50}*, *insc^{P49}*, *mud^Δ*, and *ΔRgl* single mutants, respectively. Percentages of spindles aligned in each 15° bin for the genotypes indicated are shown.

Generation of germline clones

w; pr pwn P{ry^{47.2}=hsFLP}38/Cyo; Rap1^{P5709} P{FRT(w^{hs})}2A/TM2 females were crossed with w; P{ovo^{D1-18}}3L P{FRT(w^{hs})}2A/TM3, Sb males. Mitotic recombination was induced in 24–48-h larvae for 2 h at 37°C. Virgins from this cross were mated with Rap1^{P5709}/TM6lacZ males, and the embryos (without maternal and zygotic Rap1 products) were used for the phenotypic analysis.

Immunofluorescence

Embryo fixation and antibody staining were performed by standard protocols (4% formaldehyde for 20 min) with the exceptions mentioned at the end of this paragraph. The following primary antibodies were used: sheep anti-GFP at 1:400 (Osenses); rabbit anti-β-galactosidase at 1:1,000–1:10,000 (Cappel); mouse anti-β-galactosidase at 1:8,000 (Promega); rabbit anti-PKC-ζ at 1:1,000 (C-20; Santa Cruz Biotechnology, Inc.); rabbit anti-Cno at 1:400 (Speicher et al., 2008); guinea pig anti-Numb at 1:250 (a gift from Y.-N. Jan, University of California, San Francisco, San Francisco, CA; Rhyu et al., 1994); rabbit anti-PH3 at 1:400 (Millipore); rabbit anticentrosomine (Cnn) at 1:400 (a gift from T.C. Kaufman, Indiana University, Bloomington, IN); mouse anti-Neurotactin at 1:200 (Speicher et al., 1998); rabbit anti-Baz at 1:200 (a gift from A. Wodarz, Georg-August-Universität Göttingen, Göttingen, Germany; Wodarz et al., 1999); rabbit anti-Mira at 1:2,000 (a gift from F. Matsuzaki, RIKEN Center for Developmental Biology, Kobe, Japan; Ikeshima-Kataoka et al., 1997); mouse anti-Pros at 1:100 (Developmental Studies Hybridoma Bank); mouse anti-Mud at 1:100 (a gift from F. Matsuzaki; Izumi et al., 2006); rabbit anti-Scrib at 1:4,000 (a gift from C. Doe, University of Oregon, Eugene, OR); rabbit anti-Even-skipped at 1:3,000 (Frasch et al., 1987); rat anti-DE-cad at 1:20 (Developmental Studies Hybridoma Bank); rabbit anti-Pins at 1:200 and rabbit anti-Gai at 1:1,000 (both gifts from J.A. Knoblich); mouse anti-α-tubulin at 1:1,000 (Sigma-Aldrich); rat anti-Lethal of Scute (L'sc) at 1:2,000 (Martín-Bermudo et al., 1991); and mouse anti-Wingless at 1:50 (Developmental Studies Hybridoma Bank). Secondary antibodies coupled to biotin (Vector Laboratories) plus streptavidin 488 (Invitrogen), Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 633 (Invitrogen) were used. For immunostaining with the anti-Cno antibody, embryos were fixed by using the heat-methanol method (Tepass, 1996). For α-tubulin staining, embryos were fixed with 37% formaldehyde for 1 min. L'sc signal was enhanced by use of reagents (Tyramide Signal Amplification; DuPont).

Spindle orientation analysis

Taking as a reference the overlying epithelia, angles formed between the axis delineated by the NB spindle and the apicobasal polarity axis of epithelial cells were measured using Photoshop (Adobe).

Microscope image acquisition

Fluorescent images were recorded by using an upright microscope (DM-SL with Spectral Confocal acquisition software; Leica). All images were taken with an HCX Plan APOchromat 63×/1.32-0.6 NA oil confocal scanning objective. Figs. 1 (A–E') and 5 (A–C) were acquired with an additional electronic zoom (z = 4). Fig. S1 (E–H) was recorded by using a microscope (Axio Imager.A1; EC Plan Neofluar 63×/1.25 NA oil objective; Carl Zeiss) and a camera (AxioCam HRC; Carl Zeiss). Images were assembled by using Photoshop CS3.

Coimmunoprecipitations

For immunoprecipitations, 0–7-h embryos were homogenized in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 1 mM EDTA, 1% Triton X-100, 1 mM NaF, 100 μM Na₂VO₄, 2 mM PMSF, and protease inhibitors [Complete; Roche]). Embryo extracts were centrifuged at 4°C for 15 min at 14,000 rpm (18,700 g) and then for 5 min in the same conditions. Precleared extracts were incubated with rabbit polyclonal antibody to GFP Sepharose beads (Abcam) for 2 h at 4°C. The beads then were washed three times with lysis buffer without inhibitors, resuspended in

protein set buffer (Fluka), and heated at 95°C for 5 min. Precipitates were resolved by SDS-PAGE and immunoblotted with mouse anti-GFP (Takara Bio, Inc.) at 1:2,000, rabbit anti-PKC-ζ at 1:500, or rabbit anti-Par6 (a gift from J.A. Knoblich) at 1:2,000. Each experiment was repeated at least three times.

Online supplemental material

Fig. S1 shows that Rap1 mutants show equal-sized daughter cells and defects in the RP2 lineage. Fig. S2 shows that epithelial polarity defects in Rap1 mutants are not correlated with NB polarity and spindle orientation failures. Fig. S3 shows that NB delamination is not affected in Rap1 mutant embryos. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201108112/DC1>.

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Bar, 10 μm. (D) Rap1 forms a complex in vivo with aPKC and Par6. GFP-Rap1 embryo lysates were subject to immunoprecipitation (IP) with rabbit anti-GFP antibody bound to beads and probed on immunoblots (IB) with anti-aPKC, anti-Par6, and with anti-GFP (as an immunoprecipitation control). In negative (neg.) controls, WT embryo lysates were immunoprecipitated with the same rabbit anti-GFP antibody bound to beads. Each coimmunoprecipitation was repeated at least three times. Molecular masses are given in kilodaltons. (E and F) Diagram (E) shows the genetic and physical relationships among the apical proteins represented. Continuous line indicates a physical interaction. Dotted lines indicate potential physical interactions. Cno and Pins are in a complex in vivo, but they do not physically interact (green dotted line). Arrows indicate genetic relationships. (F) Location of proteins at the apical mNB cortex and their link with the mitotic spindle. Orange arrows represent unknown extrinsic signals coming from the NE. CT, centrosome; Dlg, Discs Large; Tub, tubulin.

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