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PP2A Antagonizes Phosphorylation of Bazooka by PAR-1 to Control Apical-Basal Polarity in Dividing Embryonic Neuroblasts

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SUMMARY

Bazooka/Par-3 (Baz) is a key regulator of cell polarity in epithelial cells and neuroblasts (NBs). Phosphorylation of Baz by PAR-1 and aPKC is required for its function in epithelia, but little is known about the dephosphorylation mechanisms that antagonize the activities of these kinases or about the relevance of Baz phosphorylation for NB polarity. We found that protein phosphatase 2A (PP2A) binds to Baz via its structural A subunit. By using phospho-specific antibodies, we show that PP2A dephosphorylates Baz at the conserved serine residue 1085 and thereby antagonizes the kinase activity of PAR-1. Loss of PP2A function leads to complete reversal of polarity in NBs, giving rise to an "upside-down" polarity phenotype. Overexpression of PAR-1 or Baz, or mutation of 14-3-3 proteins that bind phosphorylated Baz, causes essentially the same phenotype, indicating that the balance of PAR-1 and PP2A effects on Baz phosphorylation determines NB polarity.

INTRODUCTION

The generation of cell fate diversity in developing and adult organisms depends on the asymmetric division of stem cells. One of the best model systems to study this process is the embryonic neuroblast (NB) in the fruit fly *Drosophila*. Upon unequal cytokinesis, the NB gives rise to a ganglion mother cell (GMC), which divides only once more and produces two neurons or glia cells, and another NB that continues to divide asymmetrically (Wodarz, 2005; Knoblich, 2008; Zhong and Chia, 2008). A crucial prerequisite for asymmetric cell division is the polarization of the stem cell. NB polarity is controlled by a group of genes encoding cortical proteins which function in a hierarchy, leading to the asymmetric localization of the cell fate determinants Prospero, Brain Tumor (Brat), and Numb (Wodarz, 2005; Knoblich, 2008; Zhong and Chia, 2008).

The PDZ domain protein Baz, the *Drosophila* homolog of *C. elegans* and vertebrate PAR-3, is among the first proteins that localize to the apical NB cortex, where it recruits PAR-6 and aPKC to form the Baz/PAR-6/aPKC complex (Kuchinke et al., 1998; Schober et al., 1999; Wodarz et al., 1999, 2000; Petronczki and Knoblich, 2001). Together with the proteins Discs Large (Dlg), Lethal giant larvae (Lgl), and Scribble (Scrib), the Baz/PAR-6/aPKC complex is essential for the localization of cell fate determinants and their adaptor proteins Miranda (Mira) and Partner of Numb (Pon) to the basal cortex of mitotic NBs (Rhyu et al., 1994; Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Ohshiro et al., 2000; Peng et al., 2000; Albertson and Doe, 2003). The assembly and activity of the Baz/PAR-6/aPKC complex is controlled by a phosphorylation cascade triggered by activation of the mitotic kinase Aurora A, which leads to phosphorylation of PAR-6 and activation of aPKC (Wirtz-Peitz et al., 2008). aPKC in turn phosphorylates and inactivates Lgl at the apical cortex (Betschinger et al., 2003). Phosphorylation of Lgl leads to dissociation of Lgl from PAR-6 and aPKC and to its exchange for Baz. The Baz/PAR-6/aPKC complex finally phosphorylates Numb and prevents its localization to the apical NB cortex (Wirtz-Peitz et al., 2008). These findings clearly show that modification of polarity proteins by phosphorylation is an important mechanism to regulate their function in asymmetric cell division.

Baz itself is also a phosphoprotein, and some phosphorylation sites relevant for its function have been identified. Phosphorylation of S151 and S1085 by PAR-1 is crucial for Baz function and localization in cells of the follicle epithelium and in the oocyte (Benton and St Johnston, 2003). In rat PAR-3, phosphorylation of S827, corresponding to S980 in Baz, by aPKC results in decreased stability of the aPKC-PAR-3 interaction (Nagai-Tamai et al., 2002). Overexpression of a mutant version of PAR-3 that cannot be phosphorylated at this site (PAR-3 S827A) in MDCK cells leads to polarity defects similar to those observed upon overexpression of a dominant-negative form of aPKC (Suzuki et al., 2001; Nagai-Tamai et al., 2002).

In contrast, up to now little has been known about the mechanisms of Baz or PAR-3 dephosphorylation counteracting the activities of PAR-1 and aPKC kinases. Protein phosphatase 1 (PP1) has recently been shown to bind mouse PAR-3 and to dephosphorylate its serine residues S144 and S824, corresponding to S151 and S980 of Baz (Traweger et al., 2008). A function for protein phosphatase 2A (PP2A) as an antagonist of PAR-1 in photoreceptor cells of the *Drosophila* pupal retina was recently described (Nam et al., 2007). In this system, overexpression of PAR-1 or reduction of PP2A activity led to mislocalization of Baz and disturbed cell polarity.

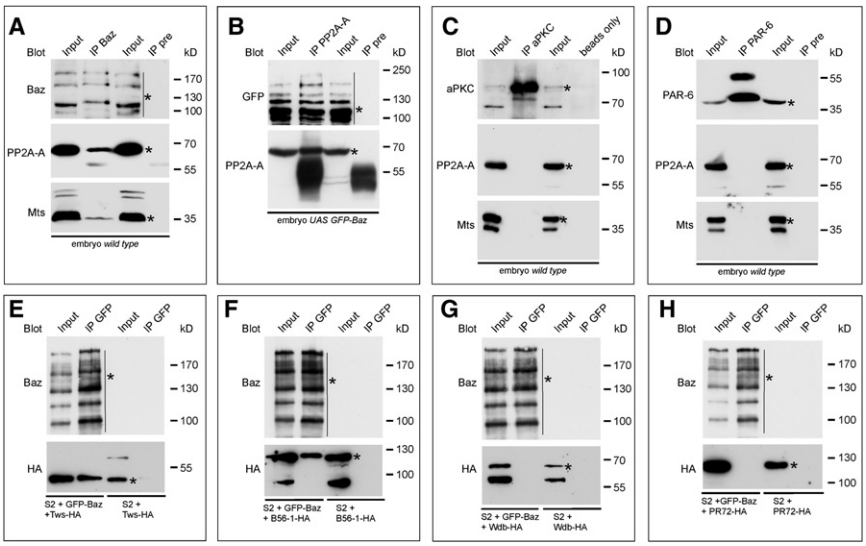

Figure 1. Baz Binds to PP2A In Vivo

(A) Wild-type embryonic extracts were immunoprecipitated with anti-Baz antiserum (IP Baz) or the corresponding preimmune serum (IP pre). Blots were probed for Baz, PP2A-A, and Mts.

(B) PP2A-A antibody was used for immunoprecipitation from extracts of embryos expressing GFP-Baz. Blots were probed for PP2A-A and GFP to detect GFP-Baz.

(C and D) Immunoprecipitates of wild-type embryonic extracts pulled down with anti aPKC (C) or anti PAR-6 antibody (D).

(E–H) The Tws and B56-1 regulatory B subunits of PP2A coimmunoprecipitate with GFP-Baz. S2 cells were cotransfected with GFP-Baz and HA-tagged Tws (E), B56-1 (F), Wdb (G), and PR72 (H). Lysates were precipitated with anti-GFP and probed for Baz and the HA-tag. Bands of interest are indicated by asterisks. Note that Baz always runs as a series of bands (marked by a bar next to the asterisks) in SDS-PAGE that are generated by proteolytic processing or degradation of the protein. In (E)–(H), S2 cells transfected only with the HA-tagged B subunits of PP2A were used as negative controls.

PP2A is a ubiquitously expressed serine-threonine-specific phosphatase which has crucial functions in various cellular processes such as cell cycle control, cytoskeleton reorganization, and cell polarity (Janssens and Goris, 2001). PP2A is a heterotrimeric enzyme complex consisting of a catalytic (PP2A-C) subunit, which is only stable if tightly bound to a structural subunit of 65 kD (PP2A-A). This core complex recruits one out of several regulatory subunits (PP2A-B) which provide substrate specificity (Janssens and Goris, 2001). Whereas in vertebrates two different A subunits and various regulatory B subunits are expressed, in *Drosophila* the A subunit is encoded by a single gene and only four genes for regulatory B subunits are annotated: Twins (Tws), Widerborst (Wdb), B56-1, and PR72.

In this study, we show that a heterotrimeric complex of PP2A-A, PP2A-C (Mts), and Tws binds to Baz and dephosphorylates Baz at the conserved serine residue 1085. Furthermore, impaired dephosphorylation of this site caused by lack of PP2A activity induces a dramatic phenotype in late-stage embryonic NBs characterized by a total reversal of apical-basal cell polarity. This finding can be phenocopied by overexpression of either PAR-1 or Baz. We furthermore show that the *Drosophila* homologs of PAR-5, 14-3-3ε and 14-3-3ζ (Leonardo), function in the same pathway to control apical-basal NB polarity.

RESULTS

PP2A Is Found in a Complex with Baz but Not with aPKC or PAR-6

In a yeast two-hybrid screen for interaction partners of the N-terminal region of Baz (aa 1–318), we isolated a clone corresponding to aa 399–590 of the structural A subunit of PP2A (PP2A-A) that specifically interacted with the Baz bait (see Figure S1 available online). In an independent experimental approach, we performed coimmunoprecipitation experiments from embryonic extracts using an antibody directed against Baz. A 39 kD band coimmunoprecipitating with Baz was analyzed by mass spectrometry, and three peptides matched Mts, the only catalytic subunit of PP2A annotated in the *Drosophila* genome (Figure S1).

In order to test whether PP2A is able to bind Baz in vivo, we performed coimmunoprecipitation experiments from embryonic lysates. To that aim, we generated polyclonal peptide antisera directed against PP2A-A. These antisera recognize a single band of 65 kD in western blots, which corresponds to the predicted size of PP2A-A (Figure 1A). A small proportion of PP2A-A and Mts coimmunoprecipitated with Baz (Figure 1A). Similarly, GFP-Baz was present in immunoprecipitates pulled down with the anti PP2A-A antibody (Figure 1B). In contrast, neither aPKC nor PAR-6 was found in a complex with PP2A (Figures 1C and 1D).

To clarify which of the four regulatory B subunits encoded in the *Drosophila* genome were associated with the PP2A-Baz complex, HA-tagged versions of all four B subunits were coexpressed with GFP-Baz in S2 cells and tested for coimmunoprecipitation with GFP-Baz. Both the Tws (Figure 1E) and the B56-1 (Figure 1F) regulatory subunits were found in a complex with GFP-Baz, whereas no interaction was found with Wdb (Figure 1G) or PR72 (Figure 1H).

Inhibition of PP2A Activity in S2 Cells Results in Increased Phosphorylation of Baz at Serine 1085

To test whether the association of Baz with PP2A controls the phosphorylation of one of the three previously described phosphorylation sites of Baz, we raised phospho-specific peptide antibodies against each of these phosphorylated serine residues (pS151, pS980, pS1085). S2 cells overexpressing GFP-Baz were treated for 4 hr with either DMSO as negative control, with okadaic acid (5 nM), or with cantharidin (0.5 μM). At these inhibitor concentrations, the inhibition of PP2A is more than 10-fold

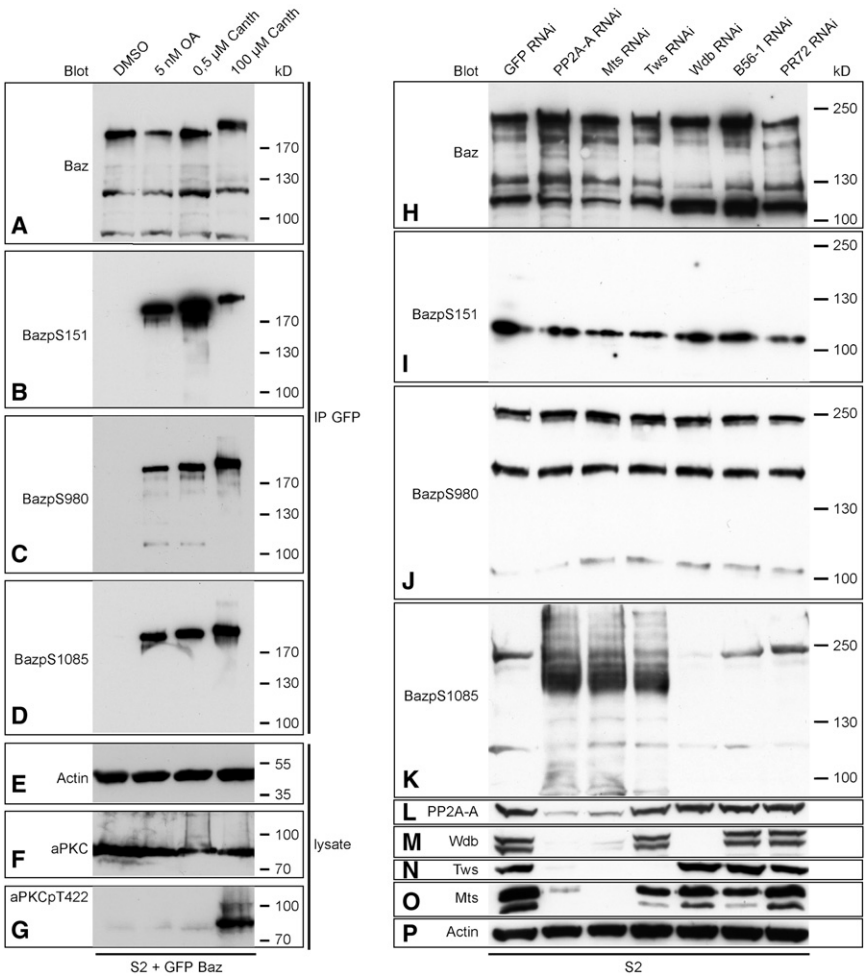

Figure 2. The Phosphorylation State of Three Conserved Serine Residues of Baz Can Be Monitored by Phospho-Specific Antibodies

(A–G) S2 cells expressing GFP-Baz were treated either with DMSO as negative control or with the phosphatase inhibitors okadaic acid (OA) and cantharidin (Canth) at the indicated concentrations. GFP-Baz was immunoprecipitated with anti-GFP antibody, and the precipitates were subsequently probed with anti-Baz (A), anti-BazpS151 (B), anti-BazpS980 (C), and anti-BazpS1085 (D). Lysates were also probed for actin (E), aPKC (F), and aPKCpT422 (G).

(H–P) Serine 1085 of Baz is specifically dephosphorylated by a heterotrimeric complex of PP2A-A, Mts, and Tws. S2 cells were treated with double-stranded RNA corresponding to different subunits of PP2A indicated on top (X RNAi). Double-stranded RNA corresponding to GFP was used as negative control. Lysates were subjected to western blots with the antibodies indicated on the left.

higher than the inhibition of PP1, another major cellular protein phosphatase that can be inhibited with these substances (Barford, 1996). Subsequently, cells were lysed, GFP-Baz was immunoprecipitated, and the precipitates were subjected to western blot with the phospho-specific antibodies (Figures 2B–2D). Whereas GFP-Baz from DMSO-treated cells was only weakly phosphorylated at any of the three investigated sites, treatment with the phosphatase inhibitors resulted in a strong increase of the band intensity with all three phospho-specific antibodies (Figures 2B–2D). In contrast, the total amount of Baz protein was not affected (Figure 2A). Treatment of the cells with 100 μM cantharidin, a 10-fold excess over the EC_{50} of PP1, did not lead to further increase of phosphorylation at any of the three serines (Figures 2B–2D). However, a distinct shift in the mobility of the Baz band was detectable, indicating that PP1 or other phosphatases affected by this high concentration of the inhibitor may dephosphorylate other, yet undescribed phosphorylation sites of Baz.

In contrast to the three phosphorylation sites in Baz, phosphorylation of aPKC at T422 was not affected by the low concentrations of cantharidin or okadaic acid that predominantly inhibit PP2A (Figure 2G). Increased phosphorylation of T422 was only observed after inhibition of PP1 by higher concentrations of cantharidin (Figure 2G).

While these inhibitor experiments pointed to PP2A as the phosphatase that specifically dephosphorylated the three serine residues of Baz, the specificity of the inhibitors is not high enough to exclude the involvement of other phosphatases, such as PP4 or PP6. We therefore knocked down PP2A by application of double-stranded RNA in S2 cells, which has been shown to work efficiently for PP2A subunits (Silverstein et al., 2002; Sathyanarayanan et al., 2004). This approach also allowed us to clarify which one of the different regulatory B subunits is involved in the dephosphorylation of Baz.

Knockdown of PP2A-A, Mts, and Tws resulted in strongly elevated phosphorylation of S1085 (Figure 2K), whereas phosphorylation of S151 and S980 remained unaffected (Figures 2I and 2J). In the absence of phosphatase inhibitors, only a smaller form of Baz (110 kD) was detectable with the anti-BazpS151 antibody (Figure 2I, cf. Figure 2B). Knockdown of Wdb, B56-1, and PR72 did not lead to elevated phosphorylation of S1085 (Figure 2K), indicating that Tws is the only regulatory B subunit of PP2A that functions in a complex with the core dimer of PP2A-A and Mts to dephosphorylate Baz at S1085. PP2A-A and Mts were mutually required to stabilize each other and the B subunits Tws and Wdb, whereas the knockdown of Wdb or Tws did not affect the stability of PP2A-A or Mts (Figures 2L–2O) (Silverstein et al., 2002).

Apical-Basal Polarity Is Totally Reversed in a Fraction of PP2A-Deficient Metaphase-NBs

In order to investigate the consequences of PP2A depletion in embryonic NBs, we examined embryos homozygous mutant for *PP2A-29B*^{GE16781}, a predicted null allele for the PP2A-A subunit which carries a P element insertion 38 bp 3′ of the

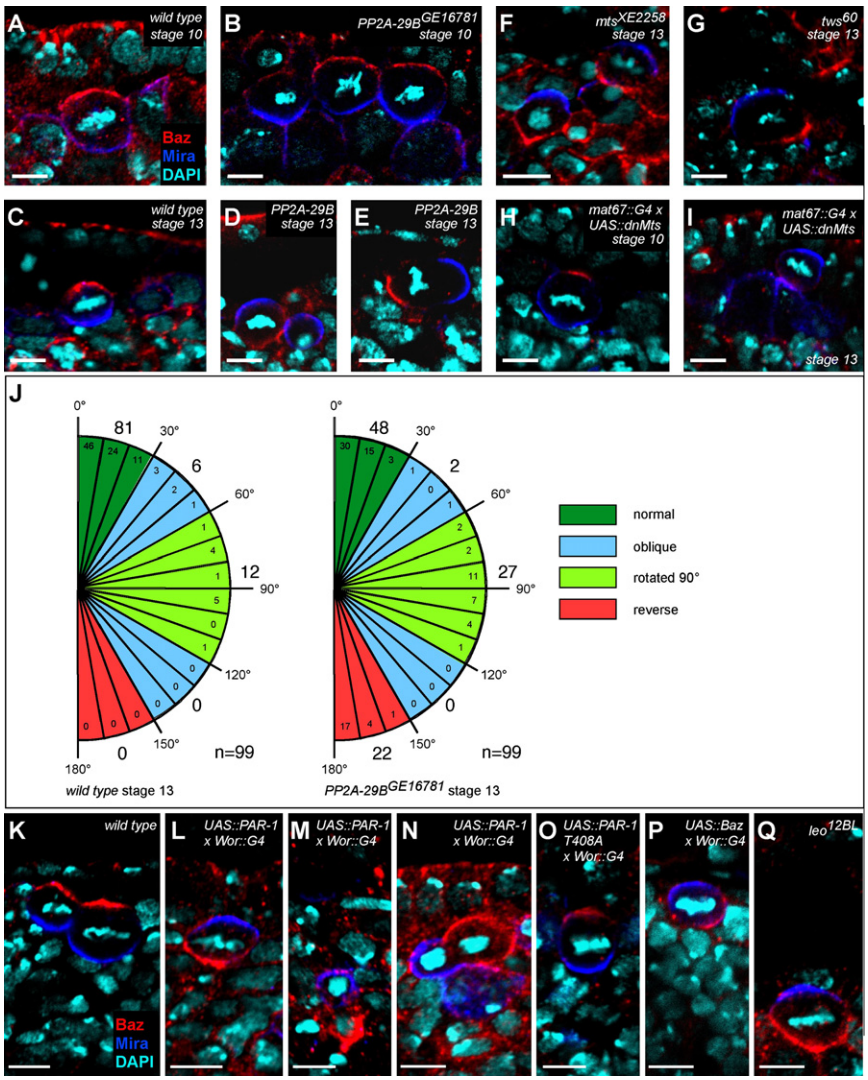

Figure 3. Loss of PP2A Function Leads to Complete Apical-Basal Polarity Reversal in a Fraction of Embryonic NBs

(A–I) Embryos of the indicated genotypes and developmental stages were stained for Baz (red), Mira (blue), and DAPI (turquoise).

(J) Quantification of spindle orientation in *wild-type* and *PP2A-29B*^{GE16781} mutant embryos. Spindle orientation was determined by drawing a line through the center of the NB that dissected the Baz crescent in the middle. The angle of that line to a line perpendicular to the plane of the overlying epithelium was measured in increments of 10°.

(K–Q) Stage 13 NB polarity is reversed upon overexpression of PAR-1 and Baz and in *leo* mutants. Scale bars = 5 μm. Apical is up.

start-codon and is embryonic lethal. The lethality of *PP2A-29B*^{GE16781} was fully rescued by ubiquitous expression of a UAS::PP2A-29B transgene (data not shown). In NBs at early stages of neurogenesis (stage 9–11), we did not observe any abnormalities regarding spindle orientation or localization of polarity proteins (Figure 3B). However, in late neurogenesis (stage 13–15), a significant percentage of *PP2A-29B*^{GE16781} mutant NBs showed either a spindle orientation parallel to the plane of the neuroectodermal epithelium (27%, n = 99; Figures 3E and 3J) or a total reversal of apical-basal cell polarity (22%; Figures 3D and 3J). Very few NBs with oblique spindle orientation were found in *PP2A-29B*^{GE16781} mutant embryos (Figure 3J). In the NBs with reversed polarity, Baz, which localizes to the apical cortex in wild-type (Figures 3A and 3C), formed basal crescents (Figure 3D), while Mira, which localizes to the basal cortex in wild-type (Figures 3A and 3C), formed apical crescents (Figure 3D). Analysis of other apically (Figures S2A–S2H) and basally (Figures S2I– S2N) localized proteins confirmed that cortical polarity was completely reversed in *PP2A* mutant embryos. To verify that this phenotype was due to decreased activity of PP2A, we analyzed embryos homozygous mutant for *mts*^{XE2258} (Figure 3F) (Wassarman et al., 1996) and *tws*^{60} (Figure 3G) (Uemura et al., 1993) and found essentially the same abnormalities. To analyze the functional requirement for PP2A in NB polarity by an additional approach, we expressed a dominant-negative version of Mts (UAS::dnMts) (Hannus et al., 2002) in a NB-specific pattern using wor::GAL4 or in a ubiquitous pattern using tubulin::GAL4 or mat67::GAL4. In all of these cases, we identified upside-down NBs at late stages of neurogenesis at a frequency comparable to embryos homozygous for mutations in *PP2A-A*, *mts*, or *tws* (Figure 3I). No polarity defects were observed in NBs at earlier stages of neurogenesis (Figure 3H), although the UAS::dnMts was expressed from the beginning of neurogenesis when the maternal tubulin::GAL4 or mat67::GAL4 driver lines were used. We did not detect any *PP2A-29B*^{GE16781} mutant ana- or telophase NBs showing reversed polarity, which is probably due to an arrest in mitosis upon loss of PP2A activity (Chen et al., 2007).

The NB Polarity Phenotype of *PP2A* Mutants Can Be Phenocopied by Overexpression of PAR-1

PAR-1 can phosphorylate Baz at relevant serine residues S151 and S1085 (Benton and St Johnston, 2003) and acts as an antagonist of PP2A with respect to cell polarity and Baz localization in pupal photoreceptor cells (Nam et al., 2007). In NBs, GFP-tagged PAR-1 localized predominantly to the basal cortex (Figure S3A). To test whether PAR-1 antagonizes PP2A also in the control of NB polarity, we overexpressed PAR-1 in NBs using the UAS-GAL4 system. The consequences on NB polarity at metaphase were essentially the same as in *PP2A* mutants and upon overexpression of dominant-negative Mts (Figure 3L; Table 1). Again, polarity defects were only observed in older NBs from stage 13

Table 1. Loss of PP2A and 14-3-3 Function and Gain of PAR-1 and Baz Function Causes NB Polarity Defects in Embryos at Stage 13–15

Genotype	NB Polarity (%)			
Metaphase	**Reversed**	**Rotated 90°**	**Normal**	**n**
wild-type	0.6	10.6	88.8	170
PP2A-29B^{GE16781}/ PP2A-29B^{GE16781}	26.5	24.9	49.6	163
UAS::PAR-1 x Wor::GAL4	19.0	22.1	58.9	168
UAS::PAR-1T408A x Wor::GAL4	2.6	12.9	84.5	162
UAS::Baz x Wor::GAL4	23.3	20.8	55.9	168
UAS::BazS1085E x Wor::GAL4	20.4	25.7	53.9	161
UAS::BazS1085A x Wor::GAL4	4.1	14.6	81.3	167
UAS::BazS151AS1085A x Wor::G4	0.6	8.1	91.3	160
14-3-3leo^{12BL}/ 14-3-3leo^{12BL}	25.8	20.5	53.7	160
14-3-3leo^{P1188}/ 14-3-3leo^{P1188}	18.4	17.9	63.7	163
14-3-3ε^{j2B10} / 14-3-3ε^{j2B10}	6.5	11.3	82.2	165
UAS::Baz, UAS::14-3-3leo x Wor::GAL4	7.9	12.7	79.4	161
UAS::Baz, UAS::14-3-3ε x Wor::GAL4	5.4	13.0	71.6	168
Telophase				
wild-type	0	4.9	95.1	61
UAS::Baz x Wor::GAL4	21.1	31.6	47.4	57

For measurements of NB polarity at metaphase, the angle between a line perpendicular to the plane of the ectodermal epithelium and a line from the center of the NB through the center of the Baz crescent was measured. For telophase NBs, the angle of a line connecting the centers of the two separating daughter cells to the line perpendicular to the plane of the epithelium was measured. Angles between 0° and 30° were scored as normal polarity, angles between 60° and 120° were scored as polarity rotated 90°, and angles between 150° and 180° were scored as reversed polarity. Oblique spindle orientations between 30° and 60° or between 120° and 150° were so rare (see Figure 3J) that they were not counted here. The numbers for wild-type and homozygous mutant *PP2A-29B^{GE16781}* NBs shown here differ from those shown in Figure 3J because they were obtained in independent experiments.

onward. The reversal of NB polarity was dependent on the kinase activity of PAR-1, as overexpression of a kinase-dead version of PAR-1 (PAR-1T408A) did not result in NB polarity defects (Figure 3O; Table 1). In contrast to the situation in *PP2A* mutants, overexpression of PAR-1 did not result in mitotic arrest at metaphase. Consequently, we did observe a significant number of ana- and telophase NBs that budded off the GMC to the apical side (Figure 3M), confirming our hypothesis that these NBs are entirely upside down. Besides NBs dividing with an upside-down polarity, we also observed an increased number of telophase NBs dividing in an orientation parallel to the plane of the neuroectodermal epithelium (Figure 3N).

Moderately Elevated Level of Baz Results in Reversed Apical-Basal NB Polarity

We next addressed the question of whether changes in Baz phosphorylation are responsible for the reversal of NB polarity upon loss of PP2A function or overexpression of PAR-1. Using different driver lines (Wor::GAL4, Pros::GAL4, asense::GAL4) and different temperatures, we determined that the effects of Baz overexpression were dosage dependent. Weak overexpression of wild-type Baz, either untagged or N-terminally tagged with GFP, led to complete reversal of NB polarity (Figure 3P) in a significant fraction of NBs from stage 13 onward (23.3%, Table 1). Similar to PP2A knockdown or PAR-1 overexpression, the number of dividing NBs showing a spindle orientation rotated by 90° was also increased (20.8%, Table 1). Furthermore, we frequently observed NBs with reversed polarity in ana- and telophase, budding off the GMC to the apical side, similar to the phenotype upon PAR-1 overexpression (Table 1). In order to visualize this event and to further elucidate if the reversed NB polarity was immediately established or was caused by relocalization of initially correctly targeted apical and basal proteins, we performed live imaging of embryos co-overexpressing wild-type Baz and Pon-GFP (Lu et al., 1999) (Movies S2 and S3). In wild-type embryos, Pon-GFP accumulated at the basal cortex and exclusively segregated into the basally localized GMC (Movie S1). By contrast, upon overexpression of Baz we frequently observed asymmetric divisions in parallel to the plane of the overlying epithelium (Movie S2) and also asymmetric divisions in which the GMC budded off to the apical side (Movie S3). In the latter case, Pon-GFP was directly targeted to the apical cortex without prior accumulation at the basal cortex (Movie S3).

Our observations bring up the question of whether the reversal of cortical apical-basal NB polarity was due to a mistargeting of apical and basal protein complexes without affecting the intrinsic asymmetry of the mitotic spindle, or whether the whole NB, including the mitotic spindle, was upside down. To investigate this, we stained centrosomes with γ-tubulin (Figure S4) and Cnn (data not shown). In wild-type, the apical centrosome of metaphase and anaphase NBs was significantly larger than the basal one (Figures S4A and S4B) (Kaltschmidt et al., 2000). In NBs showing reversed apical-basal polarity upon overexpression of Baz, the basal centrosome was larger than the apical centrosome (Figure S4C). In general, the centrosome close to the Baz crescent was larger than the centrosome opposite to the Baz crescent, irrespective of the orientation of the NB relative to the overlying epithelium (Figures S4C and S4D). From these observations, we conclude that the affected NBs do not only exhibit reversed apical-basal polarity of the cytocortex but are entirely upside down.

Next, we investigated if the phosphorylation of serine 1085 is crucial for the polarity reversal in NBs. Overexpression of Baz in which serine 1085 was exchanged for glutamate (BazS1085E), mimicking a constitutively phosphorylated protein, showed similar effects as overexpression of wild-type Baz (20.4% reversed polarity and 25.7% spindle rotated 90°, Table 1) without further enhancement of the phenotype, consistent with our observation that wild-type Baz gets phosphorylated on S1085 upon overexpression (Figure S5). In contrast, overexpression at the same level (Figure S6) of nonphosphorylatable Baz in which serine 1085 was exchanged for alanine (BazS1085A)

caused polarity reversal in only 4.1% of NBs and spindle misorientation in 14.6% of NBs (Table 1). Overexpression of a version of Baz in which both S151 and S1085 were mutated to alanine (BazS151AS1085A) did not cause any significant increase of NBs with abnormal polarity compared to wild-type (Table 1), suggesting that S151 contributes to a minor extent to the polarity defects observed upon overexpression of Baz.

14-3-3 Proteins Interact with Baz in the Control of Neuroblast Polarity

In *Drosophila*, two homologs of PAR-5 exist: 14-3-3ε and 14-3-3ζ (Leonardo, Leo). Both have been shown to interact with Baz posphorylated by PAR-1 and thereby exclude Baz from the basolateral membrane in the follicle cell epithelium (Benton and St Johnston, 2003). We have confirmed the phosphorylation-dependent interaction of 14-3-3ε with S1085 of Baz by coimmunoprecipitation experiments in transfected S2 cells (Figure S7). Interestingly, the binding of 14-3-3ε to Baz was inversely correlated with binding of aPKC to Baz (Figure S7), suggesting that phosphorylation of S1085 regulates the binding of Baz to aPKC.

In embryos mutant for a loss-of-function and a hypomorphic allele of *14-3-3ζ* (*14-3-3leo*^{12BL} and *14-3-3leo*^{P1188}), we detected the same phenotype of reversed apical-basal NB polarity as in *PP2A* mutants and upon PAR-1 overexpression (Figure 3Q, Table 1, and data not shown). In zygotic mutants of a null allele of *14-3-3ε*, *14-3-3ε*^{j2B10}, only a few (6.5%, Table 1) embryonic NBs showed reversed polarity, which could be due to the fact that the maternal component is stronger and longer lasting than that of *14-3-3ζ*. Indeed, zygotic mutant *14-3-3ε*^{j2B10} animals survive until late larval stages.

Based on these findings, we speculated that like in the follicle epithelium, 14-3-3 proteins may be required to exclude Baz from the basal cortex in NBs and thus to ensure proper NB polarity. To test our hypothesis, we co-overexpressed wild-type Baz and 14-3-3ζ or 14-3-3ε in NBs. Indeed, the percentage of NBs showing a reversed polarity was strongly decreased compared to overexpression of Baz alone (Table 1).

DISCUSSION

Apical-basal polarity of NBs is controlled by a relatively small number of proteins which assemble into protein complexes localized to the NB cortex in an asymmetric fashion (Wodarz, 2005; Knoblich, 2008). These cortical proteins interact with each other in a functional hierarchy. At the top of the hierarchy is Baz, because it can localize to the apical NB cortex in loss-of-function mutants for any of the other factors, including PAR-6, aPKC, Insc, Pins, and others (Schober et al., 1999; Wodarz et al., 1999; Yu et al., 2000; Rolls et al., 2003; Kim et al., submitted).

Here, we have shown that Baz gets frequently mislocalized to the basal NB cortex when it is moderately overexpressed or when it is excessively phosphorylated at S1085, either by overexpression of PAR-1 or by loss-of-function of PP2A. We expect that similar antagonistic activities of kinases and phosphatases regulate the phosphorylation state of additional sites of Baz/PAR-3 that are relevant in different cellular contexts. Loss of function of *14-3-3ζ* and to a lesser extent of *14-3-3ε* causes mislocalization of endogenous Baz in NBs, whereas overexpression of 14-3-3ζ and 14-3-3ε suppresses the mislocalization of overexpressed Baz. We therefore suggest that the ratio of Baz phosphorylated at S1085 to the amount of available 14-3-3 determines whether Baz gets mislocalized to the basal cortex. In this model, the 14-3-3 proteins function as a buffer to inactivate mislocalized, phosphorylated Baz. This inactivation could be explained by the inhibition of aPKC binding to Baz upon association of 14-3-3 with Baz. If the amount of overexpressed Baz exceeds the buffering capacity of 14-3-3, this would lead to the formation of active Baz/aPKC complexes at the basal cortex. These basally localized, active Baz/aPKC complexes may in turn affect the localization of PAR-1. The mammalian aPKC homolog PKCζ can phosphorylate PAR-1 at a conserved serine residue, and this phosphorylation causes a strong reduction of PAR-1 kinase activity and the release of PAR-1 from the plasma membrane (Hurov et al., 2004). If the same was true in *Drosophila*, it would explain the total reversal of NB polarity, because the now basally localized aPKC would phosphorylate PAR-1, which would cause its release from the membrane and the establishment of a new apical cortical domain at the previously basal cortex.

PAR-1, 14-3-3 proteins, and PP2A are strongly expressed during oogenesis, and maternal contributions may account for difficulties identifying requirements during early embryogenesis. On the other hand, eliminating maternal expression of these genes results in phenotypes too severe to allow the study of neurogenesis (Wassarman et al., 1996; Shulman et al., 2000; Benton et al., 2002). However, overexpression of a dominant-negative form of Mts from early neurogenesis onward also caused polarity reversal only in late-stage NBs. While this experiment does not exclude the possibility that the late onset of polarity reversal in NBs is due to the perdurance of the maternal gene products, it points to a fundamental difference in the mechanism of how NB polarity is controlled immediately after delamination as opposed to subsequent asymmetric divisions. The majority of late-stage NBs showing polarity reversal were not in direct contact with the overlying epithelium and thus may rely exclusively on intrinsic polarity cues, in contrast to NBs that have just delaminated and maintain contact to the overlying epithelium. Late-stage NBs lacking contact to the overlying epithelium show a higher variability of spindle orientation as compared to early-stage NBs in close contact to the epithelium (Siegrist and Doe, 2006). Thus, late-stage NBs may be particularly sensitive to changes in the phosphorylation state and general activity level of Baz, because they rely on Baz as the main cue for orienting their polarity axis.

It is interesting to note that mutations uncoupling spindle orientation from the localization of cell fate determinants commonly show fully random spindle orientation, including a variety of oblique orientations (Izumi et al., 2006; Siller and Doe, 2009). In contrast, hyperphosphorylation of Baz at S1085 resulted very rarely in oblique orientations, and spindles were always aligned with the asymmetric crescents of cell fate determinants. Although we currently do not have a good explanation for why there is a strong bias for either total reversal of polarity or misorientation of the spindle by 90°, our findings point to the existence of a spatial cue functioning upstream of Baz that defines a polarity axis perpendicular to the plane of the epithelium.

EXPERIMENTAL PROCEDURES

Antibodies

Phospho-specific antibodies against Baz phosphorylated at serine 151, 980, and 1085 were raised by injection of the phosphorylated peptides CLMVRRSpSDPNLL (pS151), CDALGRRpSISEKH (pS980), and CGMKKSSpSLESLQ (pS1085) into rabbits and subsequent affinity purification against the phosphorylated peptide.

Peptide antibodies directed against PP2A-A (PP2A-29B) were raised by injection of the peptides AASDKSVDDSLYPIAC (aa 2–16) and PYVRDLVSDPNPHVKC (aa 330–344) into rabbits (Eurogentec, Seraing, Belgium).

Immunoprecipitation and Western Blotting

For immunoprecipitations, wild-type embryos from an overnight collection were dechorionated and lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM HEPES [pH 7.44]) supplemented with protease inhibitors. S2 cells were lysed in the same buffer. After centrifugation, 2 μl rat anti-Baz (Wodarz et al., 1999), 2 μl rabbit anti-PP2A-A, 2 μl rabbit anti-PKCζ C20 (Santa Cruz Biotechnology, Inc.), 2 μl guinea-pig anti-PAR-6, 2 μl rabbit anti-GFP (Molecular Probes #A11122), or 2 μl of the corresponding preimmune serum was added to cell lysate corresponding to 500 μg total protein. Immune complexes were harvested using protein A/G-conjugated agarose (Roche), washed five times in lysis buffer, and boiled in 2× SDS sample buffer before SDS-PAGE and western blot. For mass spectrometry, immune complexes precipitated with rabbit anti-Baz were separated by SDS-PAGE, gels were silver stained, and selected bands were cut out. Bands were digested with trypsin and analyzed by MALDI-TOF at the ZMMK of the University of Cologne.

Western blotting was done according to standard procedures. Primary antibodies used for western blotting are listed in the Supplemental Data.

Immunohistochemistry

Embryos were fixed in 4% formaldehyde, phosphate buffer pH 7.4, according to standard procedures. Primary antibodies used for indirect immunofluorescence are listed in the Supplemental Data. Images were taken on a Zeiss LSM 510 Meta confocal microscope and processed using Adobe Photoshop.

Phosphatase Inhibition and RNA Interference in S2 Cells

Inhibition of phosphatases was achieved by incubation of S2 cells with okadaic acid (5 nM) or cantharidin (5 μM or 100 μM) for 4 hr, followed by lysis in lysis buffer supplemented with the same concentration of phosphatase inhibitors.

Knockdown of the different PP2A subunits by RNA interference in S2 cells was done as described (Silverstein et al., 2002; Sathyanarayanan et al., 2004).

SUPPLEMENTAL DATA

Supplemental Data include seven figures, three movies, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00173-7/.

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