Kinase-activity-independent functions of atypical protein kinase C in Drosophila

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Summary
Polarity of many cell types is controlled by a protein complex consisting of Bazooka/PAR-3 (Baz), PAR-6 and atypical protein kinase C (aPKC). In Drosophila, the Baz–PAR-6–aPKC complex is required for the control of cell polarity in the follicular epithelium, in ectodermal epithelia and neuroblasts. aPKC is the main signaling component of this complex that functions by phosphorylating downstream targets, while the PDZ domain proteins Baz and PAR-6 control the subcellular localization and kinase activity of aPKC. We compared the mutant phenotypes of an aPKC null allele with those of four novel aPKC alleles harboring point mutations that abolish the kinase activity or the binding of aPKC to PAR-6. We show that these point alleles retain full functionality in the control of follicle cell polarity, but produce strong loss-of-function phenotypes in embryonic epithelia and neuroblasts. Our data, combined with molecular dynamics simulations, show that the kinase activity of aPKC and its ability to bind PAR-6 are only required for a subset of its functions during development, revealing tissue-specific differences in the way that aPKC controls cell polarity.

Key words: PAR complex, Asymmetric cell division, Cell polarity, Epithelial development

Introduction
Polarity is a universal feature of many different cell types, including epithelial cells, migrating cells and cells that divide asymmetrically, for instance the zygote of the nematode C. elegans or neural stem cells in Drosophila and vertebrates (Knust and Bossinger, 2002; Nelson, 2003; Wodarz and Huttner, 2003; Cowan and Hyman, 2004). Although these cell types are quite different in their function, shape and subcellular organization, the mechanisms that are involved in the establishment and maintenance of cell polarity have been conserved in evolution. One of the key regulators of cell polarity is the so-called PAR-3–PAR-6–aPKC complex, consisting of the PDZ (Postsynaptic density 95, Discs large, Zonula occludens 1) domain-containing proteins PAR-3 [also known as Bazooka (Baz) in Drosophila], PAR-6 and atypical protein kinase C (aPKC) (Wodarz, 2002; Suzuki and Ohno, 2006). This protein complex operates at the top of a functional hierarchy that in epithelia is responsible for the subdivision of the plasma membrane into an apical and a basolateral membrane domain and for the positioning of the zonula adherens (ZA), an adhesion belt encircling the apex of epithelial cells (Bilder et al., 2003; Johnson and Wodarz, 2003; Tanentzapf and Teapass, 2003; Hutterer et al., 2004; Harris and Peifer, 2005).

In Drosophila, the function of the PAR-3–PAR-6–aPKC complex in epithelial development has been studied in the embryonic ectoderm and in the somatic follicle epithelium of the ovary. The components of the PAR-3–PAR-6–aPKC complex are localized in the cortex underlying the apical plasma membrane and in the most apical region of the lateral cortex, overlapping with the subapical region (SAR) and the ZA (Wodarz et al., 2000; Petronczki and Knoblich, 2001; Harris and Peifer, 2005). Loss-of-function mutants for components of the PAR-3–PAR-6–aPKC complex show a loss of the apical plasma membrane domain, fail to assemble a ZA and lose the columnar structure typical of the wild-type embryonic ectoderm and the follicle epithelium (Müller and Wieschaus, 1996; Wodarz et al., 2000; Cox et al., 2001; Huyhn et al., 2001; Petronczki and Knoblich, 2001; Rolls et al., 2003; Hutterer et al., 2004). The components of the complex are mutually dependent on each other for their correct subcellular localization, with the exception of Baz, for which the initial apical localization during cellularization is independent of the other complex members (Wodarz et al., 2000; Petronczki and Knoblich, 2001; Rolls et al., 2003; Hutterer et al., 2004; Harris and Peifer, 2005).

The PAR-3–PAR-6–aPKC complex is also required for setting up apical-basal polarity in neuroblasts (NBs), the stem cells of the central nervous system. Here, the complex localizes to the apical cortex and forms a crescent in pro- and metaphase (Kuchinke et al., 1998; Schober et al., 1999; Wodarz et al., 1999; Wodarz et al., 2000; Petronczki and Knoblich, 2001). The cell fate determinants Prospero, Brain Tumor (Brat) and Numb and their adapter proteins Miranda and Partner of Numb localize to the basal cortex of the NB and form crescents that do not overlap with the localization of the PAR-3–PAR-6–aPKC complex (Rhyu et al., 1994; Hirata et al.,...
null allele 

strong defects in some tissues and phenotypes indistinguishable from wild type in others. We propose that the functional requirement for aPKC is tissue specific and that both the binding to PAR-6 and the kinase activity of aPKC are only required for a subset of its functions.

Results

Isolation of four hypomorphic alleles of aPKC

In a genetic screen for EMS-induced mutations in maternally expressed genes that affect the patterning and integrity of the first instar larval epidermis, we isolated four mutations that showed cuticle phenotypes indicative of the loss of epithelial integrity reminiscent of mutations in the crumbs gene (Fig. 1) (Tepass et al., 1990; Luschnig et al., 2004). All four alleles were viable but female sterile in transheterozygous combinations with each other and with the aPKCgsu417 mutation (Table 1) (Luschnig et al., 2004). aPKCgsu417 has been classified as null allele because it is homozygous lethal and because no detectable protein is made in cells that are homozygous for this mutation (Wodarz et al., 2000; Rolls et al., 2003). Three of the four new aPKC alleles were homozygous viable, with the exception of aPKCgsu417, which is lethal at the second larval instar stage (Table 1) (Luschnig et al., 2004). In animals homozygous mutant for any of the four new aPKC alleles full-length mutant proteins were detectable by western blot (data not shown). These results together with the phenotypic and molecular analysis presented below show that all four mutations are hypomorphic alleles of aPKC that retain partial functionality.

Fig. 1. aPKC mutant embryos derived from germ-line clones show loss of epithelial integrity in the epidermis. (A) Cuticle preparation of a wild-type embryo showing a contiguous cuticle with ventral denticle bands (arrowheads) and the head skeleton (arrow). (B-F) Cuticles of aPKC mutant embryos derived from germ-line clones show only crumbs of cuticle (B,C,F) or rudimentary patches of contiguous cuticle (D,E) consisting of long white lines, indicative of a breakdown of epithelial tissue structure. The phenotypes of the aPKCgsu417 (B) and aPKCgsu417 (C) class I alleles are indistinguishable from the null allele aPKCgsu417 (F), whereas the phenotypes of the aPKCgsu265 (D) and aPKCgsu417 (E) class II alleles are much milder. Arrowheads in D point to rudimentary denticle bands. The cuticle phenotype of par-6a265 null mutant embryos derived from germ-line clones (G) resembles the aPKC null mutant phenotype. Anterior is to the left in all panels. Scale bar: 100 μm.
Embryos derived from germ-line clones of the hypomorphic aPKC alleles loose apical-basal polarity in the embryonic ectoderm. Embryos homozygous mutant for the null allele aPKC<sup>k06403</sup> develop normally because of maternally provided aPKC and die during larval stages when the maternal pool of aPKC is exhausted (Rolls et al., 2003). To analyze the phenotype of the new aPKC alleles during development of embryonic ectodermal epithelia in the absence of maternally provided wild-type aPKC, we generated germ-line clones of all four hypomorphic aPKC alleles, of the null allele aPKC<sup>k06403</sup>, and of the null allele par-6<sup>Δ226</sup>. Cuticle preparations of germ-line clone embryos from two hypomorphic aPKC alleles that we refer to as class I alleles (aPKC<sup>psu69</sup> and aPKC<sup>psu141</sup>) showed only scattered crumbs of cuticle (Fig. 1B,C), very similar to the

### Table 1. Complementation analysis of aPKC alleles

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<tr>
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<th>aPKC&lt;sup&gt;psu69&lt;/sup&gt;</th>
<th>aPKC&lt;sup&gt;psu141&lt;/sup&gt;</th>
<th>aPKC&lt;sup&gt;psu265&lt;/sup&gt;</th>
<th>aPKC&lt;sup&gt;psu417&lt;/sup&gt;</th>
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<tr>
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<td>+</td>
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<tr>
<td>aPKC&lt;sup&gt;psu265&lt;/sup&gt;</td>
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<td>aPKC&lt;sup&gt;psu417&lt;/sup&gt;</td>
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++, Adult animals hatch in the expected Mendelian ratio; +, adult animals hatch in each experiment but numbers were less than expected from Mendel’s laws; o, occasional adult escapers hatched; –, no adult animals were observed.

Fig. 1. Apical-basal polarity of the ectodermal epithelium is disrupted in aPKC and par-6 mutant embryos derived from germ-line clones. The ectodermal epithelium of wild-type and mutant embryos at stage 7 was stained for aPKC (A-E), PAR-6 (F-J), Baz (K-O), Nrt, Crb and Dlg (P-T). Scale bars: 10 μm (A-O); 10 μm (P-T). (U) Ultrastructure of the wild-type embryonic epidermis at stage 11. There is a contiguous electron dense ZA (U<sup>′</sup>, red arrows) in the apical portion of the lateral membrane. By contrast, aPKC<sup>psu141</sup> mutant embryos at the same stage do not possess a contiguous ZA (V<sup>′</sup>,V<sup>′</sup>). Instead, spot adherens junctions are found in ectopic positions along the lateral membrane (V<sup>′</sup>, arrow). Scale bars: 0.5 μm (U–V<sup>′</sup>). In all images apical is to the top.
cuticle phenotype of aPKC<sup>606403</sup> (Fig. 1F), par-6 (Fig. 1G), crb and sdt mutants, which show complete disintegration of the embryonic epidermis (Tepass et al., 1990; Wodarz et al., 1995; Grawe et al., 1996; Bachmann et al., 2001; Hong et al., 2001; Petronczki and Knoblich, 2001; Hutterer et al., 2004; Harris and Peifer, 2005). The hypomorphic class II alleles (aPKC<sup>mut126</sup> and aPKC<sup>mut147</sup>) showed a weaker phenotype with contiguous patches of cuticle, including remnants of denticle belts (Fig. 1D,E).

In order to understand the development of this terminal phenotype in the aPKC germ-line clone embryos, we analyzed, by confocal microscopy, the subcellular localization of the mutant aPKC proteins and of several other proteins with a polarized localization in epithelia. Wild-type aPKC was localized to spots at the most apical region of the lateral plasma membrane of the ectodermal epithelium from cellularization onwards (Fig. 2A) (Wodarz et al., 2000; Harris and Peifer, 2005). By contrast, in embryos derived from germ-line clones, both mutant aPKC proteins encoded by the class I alleles were not localized to the cortex at all and instead showed a diffuse, cytoplasmic localization (Fig. 2B). aPKC proteins encoded by the class II alleles were localized similarly to wild-type aPKC at cytoplasmic localization (Fig. 2B). aPKC proteins encoded by the null allele aPKC<sup>Δ226</sup> were localized to the apical cortex at gastrulation (Fig. 2H), as it was in wild type. In the case for the four hypomorphic aPKC alleles, whereas aPKC class II alleles produced weaker phenotypes in every aspect that we analyzed.

Ultrastructural examination of aPKC<sup>mut1141</sup> (class I) mutant embryos revealed that the neuroectodermal epithelium did not possess a contiguous ZA, consistent with the observed mislocalization of DE-cadherin (Fig. 2V). Instead, spot adherens junctions were found in ectopic positions along the lateral plasma membrane (Fig. 2V).

Mutations in crb, sdt and several other genes that result in loss of epithelial cell polarity and failure to form a ZA cause massive apoptosis in epithelial cells from mid-embryogenesis onwards (Tepass and Knust, 1993; Grawe et al., 1996). This was also the case for the four hypomorphic aPKC alleles, as detected by TUNEL and staining for the activated caspase Drice (supplementary material Fig. S2B). Apoptotic cells frequently were not internalized and engulfed by macrophages, as in wild type (supplementary material Fig. S2C) but remained on the surface of the embryo (supplementary material Fig. S2D).

aPKC is required for the correct localization of PAR-6 and Miranda in embryonic neuroblasts

In wild-type NBs at metaphase, aPKC, PAR-6 and Baz colocalize in an apical cortical crescent whereas Miranda forms a basal cortical crescent (Fig. 3A,H,O). In order to study the requirement for aPKC during the asymmetric division of embryonic neuroblasts, we analyzed germ-line clone embryos by confocal microscopy. The mutant aPKC proteins encoded by the class I alleles were localized diffusely in the cytoplasm and never formed apical crescents (Fig. 3BC; supplementary material Table S1). Complete mislocalization of aPKC to the cytoplasm was also observed in par-6Δ226 mutant embryos (Fig. 3G). By contrast, the mutant aPKC proteins encoded by the class II alleles localized to the apical cortex of neuroblasts in 78% and 53% of cases, respectively (Fig. 3DE; supplementary material Table S1).

PAR-6 never formed apical crescents in NBs of aPKC<sup>606403</sup> null mutant embryos (Fig. 3M) and in only 29% of germ-line clone aPKC<sup>mut89</sup> mutant embryos (Fig. 3I). In embryos mutant for the other three hypomorphic alleles the apical localization of PAR-6 in NBs was also reduced, but not to the same extent as in the null and aPKC<sup>mut89</sup> mutants (Fig. 3J-L; supplementary material Table S2).
The apical localization of Baz in NBs was only slightly affected in germ-line clone embryos of all four hypomorphic aPKC alleles (Fig. 3P-S; supplementary material Table S3). Only in \( \text{aPKC}^{\text{par-6}-\text{null}} \) null mutant embryos was apical localization of Baz reduced to 30% (Fig. 3T; supplementary material Table S3). In \( \text{par-6}^{\text{D2256}} \) mutant embryos, Baz was still localized to the apical cortex of NBs in 67% of the embryos (Fig. 3U).

The PAR-3–PAR-6–aPKC complex is required for the basal localization of cell fate determinants in metaphase NBs (Schöber et al., 1999; Wodarz et al., 1999; Petronczki and Knoblich, 2001; Rolls et al., 2003). In NBs of \( \text{aPKC}^{\text{par-6}-\text{null}} \) null mutant embryos the adapter protein Miranda never localized as a basal crescent as in wild type but instead was found all around the cortex, at the apical cortex or diffuse in the cytoplasm (Fig. 3T; supplementary material Table S4). The four hypomorphic aPKC alleles resulted in Mira being either localized to the basal cortex or to the whole cortex to different degrees, with the weakest defects seen in the \( \text{aPKC}^{\text{par-6}-\text{null}} \) class II mutants and the strongest defects seen in the \( \text{aPKC}^{\text{par-6}-\text{null}} \) class II mutants (Fig. 3P-S; supplementary material Table S4).

We noted that the orientation of the metaphase plate and of the asymmetric crescent of Baz was abnormal in a fraction of aPKC mutant NBs in embryos derived from germ-line clones (Fig. 3S,T). The quantification of these defects is shown in supplementary material Fig. S3.

Female sterility in animals mutant for the hypomorphic aPKC alleles

Females homozygous for any of the three viable hypomorphic alleles were sterile and their ovaries lost the monolayered organization of the follicle epithelium (Fig. 4B). In follicle cells that were in contact with germ-line cells PAR-6 was apically localized (Fig. 4B), similar to wild-type follicle cells (Fig. 4A). By contrast, follicle cells that did not touch germ-line cells because of the multilayering of the epithelium showed diffuse cortical staining for PAR-6 (Fig. 4B). In many mutant egg chambers, the follicle epithelium was discontinuous and failed to ensheathe the germ-line cells (Fig. 4B). In all transheterozygous combinations of the hypomorphic aPKC alleles the females were sterile and showed the same defects as observed in homozygous mutant females.

Follicle cell polarity is normal in clones of cells homozygous for hypomorphic aPKC alleles

To analyze the effect of the mutations in the new aPKC alleles on the integrity and polarity of the follicle epithelium, we generated follicle cell clones. Although follicle cell clones of the null allele \( \text{aPKC}^{\text{par-6}-\text{null}} \) showed a complete loss of apical-basal polarity and extensive multilayering of the mutant cells (Fig. 4E) (Cox et al., 2001; Abdelilah-Seyfried et al., 2003), clones of each of the new aPKC alleles showed only subtle defects, if any (Fig. 4C,D). In general, follicle cells mutant for any of the hypomorphic aPKC alleles showed normal apical-basal polarity with respect to the subcellular localization of the mutant aPKC itself and of the apical markers Baz, PAR-6 and Crb (Fig. 4C,D; and data not shown). In rare cases we observed egg chambers with follicle cell clones that had gaps in the follicle epithelium (Fig. 4D).

Oocyte determination and polarity are unaffected in germ-line clones of the hypomorphic aPKC alleles

To investigate the functionality of the proteins encoded by the four new aPKC alleles in germ-line cells of the ovary without compromising aPKC function in the follicle epithelium, we generated clones of homozygous mutant germ-line cells by FLP-FRT-mediated mitotic recombination. Oocyte determination occurred normally in the vast majority of egg chambers with germ-line clones (93.7%, \( n=111 \)), as demonstrated by expression of the oocyte-specific Orb protein in only one of the 16 germ-line cells after stage 4 of oogenesis (Fig. 4F,G). By contrast, in only 28% (\( n=64 \)) of germ-line clones of the null allele \( \text{aPKC}^{\text{par-6}-\text{null}} \), was an oocyte specified (Fig. 4H), consistent with published data (Cox et al., 2001). The germ-line clone oocytes mutant for any of the four new alleles did not show any obvious defects in the localization of Staufen, Gurken (Fig. 4I,K; 100% normal localization of both proteins, \( n=49 \)) and Vasa (data not shown) at stage 9-10, indicating that the anterior-posterior polarization of the oocyte was unaffected. The localization of Staufen and Gurken in \( \text{aPKC}^{\text{par-6}-\text{null}} \) mutant
The four hypomorphic aPKC alleles encode mutant proteins with changes in single, highly conserved amino acid residues. Sequencing of genomic DNA from the aPKC locus of the mutant chromosomes revealed the presence of point mutations leading to single amino acid changes in all four alleles (Fig. 5A). All four mutations alter amino acid residues that are identical in the aPKCs of all species analyzed so far, indicating that these residues are functionally important (supplementary material Fig. S4).

The position of the altered amino acids resulting from the four novel alleles allowed predictions regarding the functions that may be affected by the mutations. The mutation in aPKCpsu69 changes cysteine 122 to tyrosine (Fig. 5A; supplementary material Fig. S4). Since this residue is positioned close to the PB1 domain that is required for binding of aPKC to PAR-6 (Noda et al., 2003; Wilson et al., 2003; Hirano et al., 2005), we tested whether the binding to PAR-6 was affected in this mutant protein. To that aim, we expressed N-terminally GFP-tagged versions of full-length wild-type aPKC and the four mutant versions of aPKC in Drosophila S2 cells and checked for binding to the endogenous PAR-6 by coimmunoprecipitation experiments. Whereas GFP-wt aPKC, GFP-aPKCpsu141, GFP-aPKCpsu265 and GFP-aPKCpsu417 all bound endogenous PAR-6, the binding of GFP-aPKCpsu69 to PAR-6 was completely abolished, irrespective of whether the anti-PAR-6 antibody or the anti-GFP antibody was used for coimmunoprecipitation (Fig. 5B). By contrast, both GFP-wt aPKC and all four mutant versions of GFP-aPKC bound equally well to Lgl and to Baz (data not shown), two additional known binding partners of aPKC (Wodarz et al., 2000; Betschinger et al., 2003).

The mutation in aPKCpsu417 changes phenylalanine 423 to isoleucine (Fig. 5C). This residue is located within the activation loop of the kinase and lies right next to threonine 422, which is the predicted phosphorylation target of phosphatidylinositol dependent kinase 1 (PDK1; supplementary material Fig. S4) (Chou et al., 1998; Le Good et al., 1998). Since phosphorylation of T422 is sequence specific, we predicted that mutation of F423 prevents phosphorylation of T422 by PDK1 and thus blocks activation of aPKC. A phosphospecific antibody is available that distinguishes aPKC phosphorylated at T422 from the unphosphorylated form. In S2 cells transfected with wild-type GFP-aPKC, the GFP-tagged protein was recognized by the phosphospecific antibody, in contrast to the mutant GFP-aPKCpsu141 (Fig. 5C). In extracts of heads of flies homozygous for aPKCpsu265 and aPKCpsu417, the GFP-tagged aPKC protein was recognized by the phosphospecific antibody specific for aPKC phosphorylated at T422 (Fig. 5C).

The mutation in aPKCpsu265 changes the conserved alanine residue A291 to valine (Fig. 5A). A291 lies in the immediate vicinity of K293 (supplementary material Fig. S4), a lysine residue in the ATP binding pocket of the kinase domain that is essential for the kinase activity of all aPKC family members (Newton, 1995). The mutation in aPKCpsu141 changes the conserved glycine residue G347 in the kinase domain to an asparagine residue (Fig. 5A). To test whether any of the four mutations described here affect the kinase activity of the mutant proteins, we performed in vitro kinase assays, and for comparison included the mutant protein GFP-aPKCpsu265 which carries a point mutation leading to the exchange of the active site lysine residue for alanine which renders it kinase-dead (Sotillos et al., 2006).
et al., 2004). The full-length wild-type GFP-aPKC fusion protein and the GFP-aPKC<sup>psu69</sup> fusion protein showed comparable levels of kinase activity when tested for autophosphorylation or phosphorylation of a GST-Baz fusion protein containing the aPKC target site S980 in radioactive in vitro kinase assays (Fig. 5D). By contrast, the mutant fusion proteins GFP-aPKC<sup>psu141</sup>, GFP-aPKC<sup>psu265</sup> and GFP-aPKC<sup>psu417</sup> showed strongly reduced kinase activity in both assays with GFP-aPKC<sup>psu141</sup> being indistinguishable in its activity from the kinase-dead mutant GFP-aPKC<sup>K293A</sup> (Fig. 5D). To further consolidate this finding, we performed in vitro kinase
assays using a phosphospecific antibody directed against pS980 of Baz (Krahn et al., 2009), which is homologous to pS827 of mammalian PAR-3 (Nagai-Tamai et al., 2002). This highly substrate-specific assay confirmed the findings from the radioactive kinase assays and showed that GFP-aPKC<sup>psu141</sup> and GFP-aPKC<sup>psu265</sup> also have little if any kinase activity in vitro (Fig. 5E).

Effects of the mutations on ATP binding and protein folding

To test if the observed loss of kinase function for the point mutations A291V (aPKC<sup>psu265</sup>), G347N (aPKC<sup>psu141</sup>) and F423I (aPKC<sup>psu417</sup>) is either due to changed substrate affinity or, alternatively, to a possible destabilization of the folded state, free energy calculations based on extended molecular dynamics simulations were carried out. Since the crystal structure of <i>Drosophila</i> aPKC has not been solved, we used the published structure of human PKC<sup>α</sup> (Messerschmidt et al., 2005) (Fig. 6) as the basis for our calculations, which is 67% identical to <i>Drosophila</i> aPKC. All three residues affected by the mutations in <i>Drosophila</i> aPKC are identical to the homologous residues in human PKC<sup>α</sup> (supplementary material Fig. S4). Moreover, the regions harboring the three mutations are highly conserved in both proteins, such that the relevant free energy double differences are expected to be unaffected by distant sequence differences. A similar argument holds for the structural elements (Fig. 6A, blue), which were not resolved in the crystal structure and, therefore, had to be modeled. Table 2 shows the relevant differences as well as the double differences $\Delta G_{\text{fold}(3/5)}$ and $\Delta G_{\text{bind}}$. The latter express the change in folding free energy and ATP binding free energy, respectively, caused by the mutation. Our free energy calculations predict a 14 times lower ATP binding affinity for the A272V mutation (homologous to <i>Drosophila</i> A291V) as compared with the wild type, which provides a plausible explanation for the observed loss of function. The ATP binding affinity of the G328N mutation (homologous to <i>Drosophila</i> G347N), in contrast, is higher by a factor of 50 and, therefore, loss of function of that mutant probably has other causes. Because in this case the folding equilibrium constant is larger by four orders of magnitude, we suggest misfolding as the most likely alternative explanation. For the F404I mutant (homologous to <i>Drosophila</i> F423I), both the ATP binding affinity as well as the folding free energy are nearly unchanged, thus ruling out both of these possible explanations. Also, this residue is unlikely to affect the ATP binding affinity of aPKC because of the large distance between F404 and the ATP-binding pocket (Fig. 6A).

**Table 2. Calculated free energy differences between wild-type protein and the three mutant proteins**

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<tr>
<th>Mutant</th>
<th>$\Delta G_f$</th>
<th>$\Delta G_{\text{ATP}}$</th>
<th>$\Delta G_{\text{G3}}$</th>
<th>$\Delta G_{\text{G5}}$</th>
<th>$\Delta G_{\text{G3/5}}$</th>
<th>$\Delta G_{\text{G3/5(u)}}$</th>
<th>$\Delta G_{\text{G3/5(f)}}$</th>
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<td>A272V</td>
<td>$-101.4\pm1.0$</td>
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<tr>
<td>F404I</td>
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<td>$9.0\pm0.6$</td>
<td>$-2.9\pm1.2$</td>
<td>$-3.1\pm0.9$</td>
<td>$0.8\pm1.0$</td>
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$\Delta G_f$ denotes the free energy difference for the folded and ligand-free states, $\Delta G_{\text{ATP}}$ for the Mg$^{2+}$-ATP bound complex, $\Delta G_{\text{G3}}$ for the tripeptide, and $\Delta G_{\text{G5}}$ for the pentapeptide. $\Delta G_{\text{G3/5}}=\Delta G_f-\Delta G_{\text{ATP}}$ denotes the ATP binding free energy difference between wild type and the respective mutant, $\Delta G_{\text{G3/5(u)}}$ between $\Delta G_f$ and $\Delta G_{\text{G3}}$, and $\Delta G_{\text{G3/5(f)}}$ between $\Delta G_f$ and $\Delta G_{\text{G5}}$. All energies are given in kJ/mol.
The four hypomorphic alleles of aPKC result in strong mutant phenotypes in embryonic epithelia

Embryos derived from germ-line clones for any of the four hypomorphic aPKC alleles showed severe defects in ectodermal epithelia. Their cuticle phenotypes, in particular those of the class I alleles, were very similar to those of crb, sdt, baz and par-6 mutants, which have almost complete loss of apical-basal polarity and integrity of ectodermal epithelia (Tepass et al., 1990; Grabe et al., 1996; Müller and Wieschaus, 1996; Tepass, 1996; Bachmann et al., 2001; Hong et al., 2001; Petronczki and Knoblich, 2001). This was confirmed at the ultrastructural level, since the defects in ZA formation in aPKC mutant embryos were very similar to those of crb, sdt and baz mutants (Grabe et al., 1996; Müller and Wieschaus, 1996; Tepass, 1996; Bachmann et al., 2001; Hong et al., 2001). Consistent with these observations, Crb was not localized to the plasma membrane at all and instead was in cytoplasmic vesicles in class I aPKC mutant embryos. It is conceivable that the trafficking of Crb to the apical plasma membrane is dependent on direct phosphorylation by aPKC (Sotillos et al., 2004). Alternatively, because Crb and its binding partner Sdt/PALS1 can directly bind to PAR-6 (Hurd et al., 2003; Lemmers et al., 2004; Wang et al., 2004; Kemppens et al., 2006) and aPKC and PAR-6 are mutually dependent on each other for their correct localization in the epidermis, the mislocalization of Crb in aPKC mutant embryos could also be caused by the mislocalization of PAR-6. It has recently been reported that Cde42 and the PAR-3–PAR-6–aPKC complex are required to suppress apical endocytosis in tissues undergoing strong morphogenetic movements, such as the neurogenic ectoderm (Harris and Tepass, 2008). Our finding that in aPKC mutant embryos Crb is in vesicles instead of being localized to the apical plasma membrane is fully consistent with this model.

The severity of the mutant phenotypes in the epidermis differed considerably between class I and class II alleles. This difference cannot be accounted for by a difference in the kinase activity of the mutants, since both proteins encoded by the class II alleles and the protein encoded by the class I allele aPKCpsu141 showed the same reduction in kinase activity, which was indistinguishable from the kinase-dead aPKCk293A protein. We therefore speculate that the difference may be caused by the effects of the mutations on the scaffolding function of aPKC. The protein encoded by the class I allele aPKCpsu141 is unable to bind PAR-6 and thus cannot assemble a functional PAR-3–PAR-6–aPKC complex. Similarly, the protein encoded by the class I allele aPKCpsu141 carries a mutation in the recognition sequence for PDK1 and thus may fail to bind PDK1. Moreover, the failure of the aPKCpsu141 protein to be phosphorylated by PDK1 may prevent a conformational change that allows additional proteins to bind aPKC (Hirai and Chida, 2003).

Quite surprisingly, the class II allele aPKCpu417 is the only one of the four hypomorphic aPKC alleles that is lethal in homozygosity. Since it is viable in combination with all other alleles, including the null allele aPKCk06403, we believe that the lethality of this allele is caused by a second site hit and not by the loss of aPKC function.

Binding to PAR-6 and kinase activity of aPKC are dispensable for the function of aPKC in the follicle epithelium

In the follicle epithelium, even in large clones of follicle cells mutant for any of the four hypomorphic alleles the localization of the mutant aPKC itself, PAR-6 and Baz was normal. Also, the mutant follicle cells were always organized in a monolayer. By contrast, follicle cell clones with the null allele aPKCk06403 showed severe defects. aPKCk06403 mutant cells completely lost polarity, failed to localize PAR-6 and Baz to the apical cortex and piled up on top of each other. Thus, although the presence of aPKC is essential, neither the binding between aPKC and PAR-6 nor the kinase activity of aPKC is required for normal development of the follicle epithelium during oogenesis.

All females homozygous mutant for any of the three viable aPKC alleles showed multilayering of the follicle epithelium, whereas follicle cell clones of the same alleles never showed this phenotype. Nonetheless, even in areas with multilayered tissue organization, follicle cells that were in contact with germ-line cells showed normal apical-basal polarity. Thus, we favor the idea that in ovaries of animals homozygous mutant for one of the hypomorphic aPKC alleles the simultaneous loss of aPKC function in germ-line cells and follicle cells may disturb the interaction between these cell types early during egg chamber formation, which could cause the multilayering of the follicle epithelium.

Why are the mutant phenotypes of the four hypomorphic aPKC alleles much stronger in the embryonic ectodermal epithelium than in the adult follicle epithelium? This is not the case for the null allele of aPKC, which causes an almost complete loss of cell polarity and tissue integrity in both tissues. The reason may lie in the different ways these two epithelia are formed. The polarity of the embryonic ectodermal epithelium is established at cellularization, when no basement membrane is present, and thus relies predominantly on apical cues, in particular the PAR-3–PAR-6–aPKC complex and the Crb-Sdt complex (Tepass et al., 1990; Wodarz et al., 1995; Müller and Wieschaus, 1996; Bachmann et al., 2001; Hong et al., 2001; Harris and Peifer, 2004). By contrast, the establishment of polarity in the follicle epithelium relies predominantly on basal cues provided by the basement membrane and only later on apical and lateral cues provided by the contact with germ-line cells and adjacent epithelial cells (Tanentzapf et al., 2000; Schneider et al., 2006; Mirouse et al., 2009).

aPKC function in embryonic neuroblasts

Regarding their subcellular localization in embryonic NBs, the mutant proteins encoded by the four hypomorphic aPKC alleles behaved as they do in the epidermis: the mutant proteins encoded by the class I alleles never formed apical crescents in NBs, whereas the proteins encoded by the class II alleles were localized to the apical cortex in the majority of NBs. Similarly, apical localization of PAR-6 was abolished in the majority of NBs mutant for class I aPKC alleles but was largely normal in class II mutant NBs. Vice versa, aPKC was completely mislocalized in NBs mutant for par-6. Thus, we conclude that aPKC and PAR-6 are codependent for
their proper localization in NBs, but that the kinase activity of aPKC is not essential in this context.

In contrast to the mislocalization of aPKC and PAR-6 in the hypomorphic aPKC mutant NBs, the localization of Baz was normal in most cases. However, only 30% of NBs mutant for the null allele aPKC<sup>650401</sup> showed apical localization of Baz. This finding suggests that neither the binding between aPKC and PAR-6 nor the kinase activity of aPKC is required for the apical localization of Baz in NBs, as long as aPKC is available as a binding partner of Baz.

Support for this interpretation comes from our finding that the mutant proteins encoded by all four hypomorphic aPKC alleles retain their ability to bind Baz. An earlier study reported that apical localization of Baz in larval NBs was independent of aPKC (Rolls et al., 2003). This result could reflect a different requirement for aPKC function in embryonic and larval NBs. Alternatively, residual maternal aPKC protein still present in larval NBs could explain this apparent discrepancy.

The PAR-3–PAR-6–aPKC complex is required for the localization of cell fate determinants, including Prospero and Miranda, which recruit Prospero to the cortex. In association with the cortical cytoskeleton (Betschinger et al., 2003; Plant et al., 2003; Yamanaka et al., 2003; Betschinger et al., 2005). Cortical Lgl in turn is required for the cortical localization of the adapter protein Miranda, which recruits Prospero to the cortex. In embryos mutant for any of the four hypomorphic aPKC alleles Miranda was mislocalized in metaphase NBs, but not to the same extent and with the same penetrance as in embryos mutant for the null allele aPKC<sup>650401</sup>. These findings show that the kinase activity of aPKC is important but may not be absolutely essential for inactivation of Lgl.

The four hypomorphic alleles of aPKC are fully functional in oocyte determination

The hypomorphic nature of the four new aPKC alleles was very obvious during oogenesis. Germ-line clones of the null allele aPKC<sup>650401</sup> failed to form an oocyte in 72% of the cases analyzed. By contrast, oocyte determination failed in only 6% of germ-line clones for any of the four new alleles, demonstrating that neither the loss of the kinase activity of aPKC nor the inability of aPKC to bind PAR-6 affect oocyte determination.

There are controversial reports about the involvement of aPKC and its phosphorylation targets in polarization of the oocyte at stages 9 and 10. Two papers claim that neither Lgl nor Crb are important for germline development (Benton and St Johnston, 2003; Doerflinger et al., 2006), but a recent paper reported that the localization of Staufen is abnormal in 47% of aPKC<sup>650403</sup> mutant oocytes at stages 9-10 and that these defects are most probably caused by the failure to phosphorylate Lgl (Tian and Deng, 2008). In our experiments, Staufen and Gurken localized normally in all oocytes mutant for any of the four hypomorphic aPKC alleles and in the vast majority of aPKC<sup>650403</sup> mutant oocytes at stage 9-10, which would suggest that there is no central function for aPKC in polarization of the oocyte at later stages of oogenesis.

Conclusions

We have described the molecular and biochemical properties and the mutant phenotypes of four hypomorphic alleles of aPKC encoding proteins that either fail to bind to PAR-6 or lack kinase activity. The reduced function of these alleles causes severe defects in apical-basal polarity in embryonic epithelia and neuroblasts, whereas oocyte determination and polarity of the follicle epithelium are nearly unaffected. Together, these data point to fundamental differences in the functioning of aPKC in these different tissues and they furthermore show that some functions of aPKC are independent of its kinase activity and of its ability to bind PAR-6.

### Materials and Methods

#### Fly stocks and genetics

2R-69-30, 2R-141-24, 2R-265-2 and 2R-417-13 alleles were derived from a mosaic screen for maternal effect embryonic lethal mutations that were induced on a chromosome carrying the P[FRT(w<sup>r</sup>)]G13 (42B) FRT element (Luschnig et al., 2004) and were renamed aPKC<sup>6504</sup>, aPKC<sup>65041</sup>, aPKC<sup>65042</sup> and aPKC<sup>65043</sup> in this work after demonstrating that they are hypomorphic alleles of aPKC. Mutations in aPKC<sup>6504</sup> flies (Luschnig et al., 2004) were identified by sequencing of PCR fragments encompassing the whole coding region of aPKC from genomic DNA of homozygous mutant or heterozygous flies. We furthermore used the null allele aPKC<sup>650401</sup> (Wodarz et al., 2000; Rolls et al., 2003) recombined onto the P[FRT(w<sup>r</sup>)]G13 (42B) chromosome and the null allele par-6<sup>6592</sup> (Petronczki and Knoblich, 2001) recombined onto the P[FRT(w<sup>r</sup>)]G13 (18E) chromosome. Df(2R)Jp1-CyO (Bloomington Drosophila Stock Center #5518) takes out the whole aPKC locus. For generation of germ-line clone embryos with the FLP-DHS technique, the appropriate ovo<sup>D1</sup> stocks were used (Chou and Perrimon, 1992; Chou and Perrimon, 1996). For generation of genetically marked germ-line clones and follicle cell clones, appropriate FRT stocks carrying transgenes encoding green fluorescent protein (GFP) under control of the ubiquitin promoter were used.

#### Generation of antibodies against PAR-6 and Miranda

Antibodies against PAR-6 and Miranda were raised by injection of the keyhole limpet hemocyanin (KLH)-conjugated peptides HHHQNASNASTIMASDVKDGVLHL (PAR-6) and CSPPKQVKLKARNI (Miranda) into guinea pigs (Eurogentec, Herstal, Belgium).

#### Immunohistochemistry, TUNEL assay, electron microscopy and cuticle preparations

Embryos and ovaries were fixed in 4% formaldehyde and phosphate buffer pH 7.4. The primary antibodies used were rabbit anti-PKC<sup>z</sup> C20 (Santa Cruz Biotechnology) 1:1000, rabbit anti-Baz (Wodarz et al., 1999) 1:1000, guinea-pig anti-PAR-6 1:1000, guinea-pig anti-Mira 1:1000, rabbit anti-Insc (Kraut and Campos-Ortega, 1996) 1:1000, mouse anti-Not BP106 (DSHB) 1:5, rat anti-DE-Cadherin, DCD-2 (Uemura et al., 1996) (DSHB) 1:20, rat anti-Crb (U. Tepass) 1:500, rabbit anti-Dig (Woods and Bryant, 1991) 1:1000, mouse anti-Gfkl 1D12 (DSHB), rabbit anti-Staufen (St Johnston et al., 1991) 1:500, mouse anti-Orb 4H8 (DSHB) 1:20, rat anti-Vasa (Tomancak et al., 1998) 1:5000, rabbit anti-active DRICE (Yoo et al., 2002) 1:5000. DNA was stained with YOYO-1 (Invitrogen). Secondary antibodies conjugated to Cy3, Cy 2, Cy 5 (Jackson Laboratories) and Alexa-Fluor-647 (Invitrogen) were used at 1:400. Immunoreactions were taken on a Zeiss LSM 510 Meta confocal microscope and processed using Adobe Photoshop. TUNEL assays, for detection of cell death in situ, were performed as described previously (Wang et al., 1999). Transmission electron microscopy was performed as described previously (Tepass and Hartenstein, 1994). Cuticle preparations were done as described previously (Wieschaus and Nüsslein-Volhard, 1986).

#### Western blots and immunoprecipitation

Methods were as described by Wodarz (Wodarz, 2008). For western analysis, the antibodies used were rabbit anti-CAJ C20 (Santa Cruz Biotechnology) 1:2000, rabbit anti- phospho-PKC<sup>z</sup> T410 (Santa Cruz Biotechnology) 1:1000, rabbit anti-Baz/8980 (Krah et al., 2009) 1:100, rabbit anti-GST (Sigma #G7781) 1:1000, guinea-pig anti-PAR-6 1:2000. For immunoprecipitations, 2 µl of rabbit anti-GFP (Molecular Probes A11122), 2 µl of guinea pig anti-PAR-6, 2 µl of the corresponding preimmune serum, 10 µl of rabbit anti-β-galactosidase (MP Biomedicals #55976) or 2 µl of rat anti-HA 3F10 (Roche) were added to cell lysate containing 500 µg of total protein from S2 cells in TNT (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl pH 7.5) supplemented with protease inhibitors. Immune complexes were harvested using protein A/G-conjugated agarose (Roche), washed three times in TNT and boiled in 2× SDS sample buffer before SDS-PAGE and western blotting.

#### In vitro kinase assays

In vitro kinase assays were essentially performed as described previously (Lin et al., 2000). In brief, GFP-aPKC was immunoprecipitated from lysates of transfected S2 cells as described above. The beads were washed once in washing buffer (150 mM NaCl and 50 mM Tris-HCl pH 7.5) and twice in reaction buffer (20 mM Hepes, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol). Immunoprecipitates were incubated for 1 hour at 30°C in reaction buffer containing 2 µg affinity purified GST-Baz, 250 µM ATP, 0.5 µM [γ-<sup>32</sup>P]ATP, 0.1 mM cantharidin and protease inhibitors. The reaction was terminated by addition of SDS sample buffer and samples were subjected to
Generation of aPKC and Baz expression constructs

Details will be provided upon request.

Molecular dynamics simulations

ATP-binding free energy double differences (\(G^{\text{Mut} - \text{WT}}\) and \(G^{\text{WT} - \text{Mut}}\)) and folding free energy differences (\(G^{\text{Mut} - \text{WT}}\) and \(G^{\text{WT} - \text{Mut}}\)) have been calculated from the thermodynamic cycle shown in Fig. 6B via the mutation free energy calculations were carried out with the Crooks' Gaussian Intersection (CGI) scheme (Goette and Grubmüller, 2009) and a soft-core potential (Beutler et al., 1994) for all Lennard-Jones interactions of two polarity complexes implicated in epithelial tight junction assembly.

Starch interacts with Crumbs to control polarity of epithelia but not neuroblasts.

We would like to thank Marion Müller-Borg and Alexandra Grimm for technical assistance. We thank William Chia, Chris Doe, Anne Bello, B., Reichert, H. and Hirth, F. (2006). aPKC controls microtubule organization to balance cell polarity establishment in Drosophila. Nature 447, 455-465.

References


Kinase independent function of aPKC


Table S1. aPKC localization in metaphase neuroblasts

<table>
<thead>
<tr>
<th>aPKC localization</th>
<th>wild type ((n=57))</th>
<th>(aPKC^{psu69}) ((n=46))</th>
<th>(aPKC^{psu141}) ((n=48))</th>
<th>(aPKC^{psu265}) ((n=54))</th>
<th>(aPKC^{psu417}) ((n=38))</th>
<th>(aPKC^{k06403}) ((n=164))</th>
<th>(par6^{A226}) ((n=45))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
<td>49.0</td>
<td>23.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Weak apical</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>5.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Weak punctate apical</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>29.4</td>
<td>23.7</td>
<td>0.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Total apical</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
<td>78.4</td>
<td>52.7</td>
<td>0.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Diffuse / no staining</td>
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<td>100.0</td>
<td>100.0</td>
<td>21.6</td>
<td>47.3</td>
<td>100.0</td>
<td>89.0</td>
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Values are % of the total number of neuroblasts counted.
Table S2. PAR-6 localization in metaphase neuroblasts

<table>
<thead>
<tr>
<th>PAR-6 localization</th>
<th>wild type (n=52)</th>
<th>aPKC_{psi69} (n=38)</th>
<th>aPKC_{psi141} (n=75)</th>
<th>aPKC_{psi265} (n=69)</th>
<th>aPKC_{psi417} (n=71)</th>
<th>aPKC_{k06403} (n=125)</th>
<th>par-6_{Δ226} (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical</td>
<td>100.0</td>
<td>18.4</td>
<td>32.0</td>
<td>32.0</td>
<td>52.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Weak apical</td>
<td>0.0</td>
<td>13.2</td>
<td>18.7</td>
<td>30.0</td>
<td>9.9</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Total apical</td>
<td>100.0</td>
<td>31.6</td>
<td>50.7</td>
<td>62.0</td>
<td>62.0</td>
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<td>0.0</td>
</tr>
<tr>
<td>Diffuse / no staining</td>
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<td>68.4</td>
<td>49.3</td>
<td>38.0</td>
<td>38.0</td>
<td>100.0</td>
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Values are % of the total number of neuroblasts counted.
<table>
<thead>
<tr>
<th>Bazooka localization</th>
<th>wild type (n=156)</th>
<th>aPKC&lt;sup&gt;psu69&lt;/sup&gt; (n=98)</th>
<th>aPKC&lt;sup&gt;psu141&lt;/sup&gt; (n=56)</th>
<th>aPKC&lt;sup&gt;psu265&lt;/sup&gt; (n=85)</th>
<th>aPKC&lt;sup&gt;psu417&lt;/sup&gt; (n=102)</th>
<th>aPKC&lt;sup&gt;k06403&lt;/sup&gt; (n=172)</th>
<th>par-6&lt;sup&gt;Δ226&lt;/sup&gt; (n=72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical</td>
<td>100.0</td>
<td>90.0</td>
<td>80.4</td>
<td>76.4</td>
<td>68.6</td>
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<td>52.9</td>
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<tr>
<td>Weak apical</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<td>3.4</td>
<td>13.8</td>
</tr>
<tr>
<td>Total apical</td>
<td>100.0</td>
<td>90.0</td>
<td>80.4</td>
<td>76.4</td>
<td>90.2</td>
<td>29.7</td>
<td>66.7</td>
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<tr>
<td>Cortical</td>
<td>0.0</td>
<td>1.0</td>
<td>1.8</td>
<td>1.2</td>
<td>0.0</td>
<td>8.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Diffuse / no staining</td>
<td>0.0</td>
<td>9.0</td>
<td>17.8</td>
<td>22.4</td>
<td>9.8</td>
<td>61.8</td>
<td>33.3</td>
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Values are % of the total number of neuroblasts counted.
Table S4. Mira localization in metaphase neuroblasts

<table>
<thead>
<tr>
<th>Miranda localization</th>
<th>wild type ($n=273$)</th>
<th>aPKC$^{psu69}$ ($n=164$)</th>
<th>aPKC$^{psu141}$ ($n=131$)</th>
<th>aPKC$^{psu265}$ ($n=61$)</th>
<th>aPKC$^{psu417}$ ($n=188$)</th>
<th>aPKC$^{k06403}$ ($n=211$)</th>
<th>par-6$^{a226}$ ($n=42$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>100.0</td>
<td>40.9</td>
<td>64.9</td>
<td>75.4</td>
<td>25.0</td>
<td>0.5</td>
<td>23.8</td>
</tr>
<tr>
<td>Basal and weak apical</td>
<td>0.0</td>
<td>6.7</td>
<td>6.1</td>
<td>16.4</td>
<td>5.3</td>
<td>1.4</td>
<td>4.8</td>
</tr>
<tr>
<td>Cortical</td>
<td>0.0</td>
<td>22.6</td>
<td>9.9</td>
<td>4.9</td>
<td>46.8</td>
<td>19.4</td>
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</tr>
<tr>
<td>Apical</td>
<td>0.0</td>
<td>3.7</td>
<td>2.3</td>
<td>0.0</td>
<td>8.5</td>
<td>21.3</td>
<td>11.9</td>
</tr>
<tr>
<td>Apical and weak basal</td>
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<td>0.0</td>
<td>0.8</td>
<td>0.0</td>
<td>10.0</td>
<td>5.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Lateral, one side only</td>
<td>0.0</td>
<td>0.6</td>
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<td>3.3</td>
<td>1.0</td>
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<td>7.1</td>
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<tr>
<td>Lateral, on both sides</td>
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<td>18.3</td>
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<td>1.4</td>
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<tr>
<td>Diffuse / no staining</td>
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<td>1.2</td>
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<td>1.0</td>
<td>50.7</td>
<td>16.7</td>
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Values are % of the total number of neuroblasts counted.