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ORIGINAL ARTICLE

# Redundant regulation of localization and protein stability of DmPar3

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## Abstract

Apical–basal polarity is an important characteristic of epithelia and *Drosophila* neural stem cells. The conserved Par complex, which consists of the atypical protein kinase C and the scaffold proteins Baz and Par6, is a key player in the establishment of apical–basal cell polarity. Membrane recruitment of Baz has been reported to be accomplished by several mechanisms, which might function in redundancy, to ensure the correct localization of the complex. However, none of the described interactions was sufficient to displace the protein from the apical junctions. Here, we dissected the role of the oligomerization domain and the lipid-binding motif of Baz in vivo in the *Drosophila* embryo. We found that these domains function in redundancy to ensure the apical junctional localization of Baz: inactivation of only one domain is not sufficient to disrupt the function of Baz during apical–basal polarization of epithelial cells and neural stem cells. In contrast, mutation of both domains results in a strongly impaired protein stability and a phenotype characterized by embryonic lethality and an impaired apical–basal polarity in the embryonic epithelium and neural stem cells, resembling a *baz*-loss of function allele. Strikingly, the binding of Baz to the transmembrane proteins E-Cadherin, Echinoid, and Starry Night was not affected in this mutant protein. Our findings reveal a redundant function of the oligomerization and the lipid-binding domain, which is required for protein stability, correct subcellular localization, and apical–basal cell polarization.

**Keywords** Cell polarity · Par3 · Adherens junctions · *Drosophila* · Lipid binding

## Introduction

Apical–basal polarity is a hallmark of epithelial tissues and is essential during development and tissue homeostasis. In monolayered epithelial cells, the apical plasma membrane domain faces towards the outer environment or a lumen and the basal domain contacts the basement membrane. This polarity is achieved by the distinct position of conserved protein complexes along the apical–basal axis: The Crumbs complex (consisting of the transmembrane protein Crumbs and the adaptor proteins PATJ and Stardust) and the Par complex determine the apical domain identity, whereas the Scribble/Lethal giant larvae/Discs large complexes counterbalance their activity at the baso-lateral domain [1, 2]. Both apical junctional complexes, the Crumbs and the Par complex, overlap in the so-called subapical region and at the adherens junctions (AJs) [3–6].

Bazooka (Baz), the *Drosophila* homolog of *C. elegans* and vertebrate Par3, is a scaffold protein and forms together with Par6 and aPKC, the Par complex [7–9]. Moreover, binding of the small GTPase CdcC42 to Par6 is essential for the apical accumulation of Baz and the formation of the AJs in the *Drosophila* epidermis [10]. In *C. elegans*, Par6 and aPKC shuttle between Par3 and Cdc42 to form distinct complexes, where aPKC is inactive in the Par3 complex, but becomes active in complex with Cdc42 to polarize the embryo [11, 12]. In line with these observations, Baz localizes subjacent to Par6 and aPKC at the AJs in *Drosophila* and binding of Cdc42 to Par6 promotes the segregation of Par6 and aPKC towards the sub apical membrane in photoreceptor cells [3, 13–17]. Par3/Baz acts as an apical cue to establish the AJ by positioning *Drosophila* E-Cadherin (DE-Cad) and mediates the formation of the tight junctions in cultured mammalian cells [18–21]. Furthermore, Par3 acts

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as an exocyst receptor to regulate the delivery of membrane proteins [22, 23].

In addition, Baz is required to establish apical–basal polarity and correct spindle orientation of *Drosophila* neural stem cells (neuroblasts, NBs), which is essential for their asymmetric cell division [24–26]. NBs originate from the embryonic neuroectoderm and initially inherit their apical–basal polarization. During their asymmetric cell divisions, the Par complex localizes to the apical cortex of the NB, which maintains its stem cell identity after asymmetric division, whereas proteins of the basal domain are segregated into the second daughter cell, the ganglion mother cell, which further differentiates into two neurons or glia cells [27–29].

In epithelial cells, the kinase Par1 inhibits the formation of the Par complex in the baso-lateral region by phosphorylating Baz at two conserved residues (Ser151 and Ser1085). Binding of 14-3-3 proteins to these phosphorylated residues prevents the oligomerization and association with aPKC [30, 31]. In NBs, the protein phosphatase PP2A counteracts the Par1 phosphorylation and thereby promotes the maturation of the Par complex [32]. Vice versa, at least in mammalian cells, Par1 is excluded at the apical domain by aPKC-mediated phosphorylation [33]. Furthermore, aPKC phosphorylates and inhibits Lgl at the apical cortex of NBs, which leads to a release of Par6 and aPKC from Lgl to promote the formation of the mature Par complex and the asymmetric localization of Numb and Miranda to the basal region [34, 35]. The mutual antagonism of basal and apical protein complexes maintains a border between both regions in epithelia and neural stem cells.

Within the Par complex, Par6 activates aPKC by replacing its pseudosubstrate domain [36], whereas the aPKC-binding region of Baz inhibits aPKC kinase activity [37]. The phosphorylation of Ser980 of Baz by aPKC leads to the dissociation of Baz/PAR-3 and aPKC, whereupon Crumbs outcompetes phosphorylated Baz for binding to aPKC [3, 16, 17, 38]. Furthermore, Baz recruits the Crumbs adaptor Stardust during the early embryogenesis, which is released upon aPKC-mediated phosphorylation of Baz [4, 5]. Thus, Baz/Par3 functions as an important polarity cue, recruiting the Par complex to the apical junctions.

In the *Drosophila* epithelium, the small GTPase Rap1 and Canoe are essential to regulate the apical positioning of Baz during cellularization [39]. Vice versa, Baz and aPKC also contribute to the localization of Canoe [39]. Notably, how exactly Baz/Par3 itself localizes to the plasma membrane is still not fully clarified: Baz/PAR-3 contains an oligomerization domain (OD) at its N-terminus [40–44] and three PDZ (PSD-95, Disc Large, ZO-1) domains, which interact with the cell adhesion molecule Echinoid (Ed) and Armadillo (Arm), the *Drosophila* homologue of β-catenin, which in turn stabilizes DE-Cad and thereby localizes Baz to the AJs [45]. The PDZ domains of Baz/Par3 have been suggested to directly bind to phospholipids of the plasma membrane [46, 47] and we have demonstrated previously, that a C-terminal phosphoinositide lipid-binding (LB) domain of Baz directly binds to $PtdIns_{(4,5)}P_2$ (PIP2) and $PtdIns_{(3,4,5)}P_3$ (PIP3) to tether Baz to the cell cortex [48], which was confirmed independently in *Drosophila* and for mammalian Par3, too [49, 50]. Furthermore, the OD and the PDZ domains have been described to redundantly contribute to the apical localization of Baz in an overexpression system [51]. However, deletion of none of these domains in Baz/Par3 on its own or in combination disrupts the localization of the protein [4, 47, 48, 50, 51].

In this study, we report that impaired oligomerization of Baz enhances degradation of the protein, but affects only mildly the rescue capacity of the mutant protein. We confirm that the OD and LB motifs mediate the correct localization of the protein in redundancy. Consequently, loss of both domains results in Baz degradation, which leads to the disruption of apical–basal cell polarity in epithelial cells of the embryonic epidermis and embryonic NBs and consequently embryonic lethality.

## Materials and methods

### DNA and constructs

Cloning of Baz pENTR was described before [32]. For expression plasmids, we recloned Baz pENTR variants into UGW, UWS, and PWG vectors (modified from UGW, UWG, and PWG, which were obtained from the *Drosophila* Genomic Resource Center as described before [52]) using the gateway technology (Life Technologies). The following primers were used to introduce the mutants in Baz pENTR:

Baz1-968: 5′-ACAAACTCGGGCTGAGGATCCGGAGGTCACGCCTCCAAGGTG-3′
BazV14D-F: 5′-GGCGACGTTCGCATTCTG GAT CCCTGTGGTTCCGGC-3′
BazD68K-F: 5′-GTCCGCGACGTGGCC AAA GATCGGGAGCAGATATTG-3′
BazK1173K1174A-F: 5′-AAGTCGTCGCGGGCCGCGGCGCCAAGCATACTGCGC-3′

### Fly stocks and genetics

In all experiments, we used the $baz^{815\text{-}8}$ allele, which is a null allele. *baz* germline clones were generated with $baz^{815\text{-}8}$ FRT19A using the dominant female sterile technique [53]. Homozygous mutant embryos were identified by loss of mCherry signal (from *FM7-sqh::mCherry*) in Western

Blots. For immunofluorescence of germline clones, male embryos were selected by the absence of Sxl staining.

Ubi::GFP-Baz, Ubi::Baz-One-Strep, and UASp::Baz-GFP transgenes were generated using phiC31-mediated germline transformation; and attP40 was used as landing site [54]. For overexpression of Baz during the early embryogenesis, we used mat-tub::GAL4 (#6356) (obtained from the Bloomington *Drosophila* Stock Center).

## Lethality test

To test the lethality of embryos, 100 embryos of each genotype were tested in three biological replicates. Embryos derived from germline clones were selected against mCherry. The embryos were kept at 25 °C on apple juice agarose plates; and the amount of dead embryos, larval stage 1/2 (L1/2), larval stage 3 (L3), pupae, and survivors was counted.

## Cuticle preparation

Cuticle preparations were done as described previously [55]. The cuticle phenotypes were classified into the four categories: wild type, shrunken with holes, holes, and cuticle rest.

## Real-time PCR analysis

Embryos from overnight apple juice agar plates were used to isolate total RNA with TRIzol (Life Technologies) according to the manufacturer's instructions. To convert the RNA into cDNA, 1 µg total RNA was used for reverse transcription with the qScript cDNA Synthesis Kit (Quantabio). Real-time PCR was performed using the SensiFAST™ SYBR No-ROX Kit (Bioline) and the LightCycler 480 II (Roche). Relative expression levels of genes of interest were calculated as $\Delta C_t$ values normalized to the rp49 control. The following primers were used: Baz qPCR F 5′-GTCCGTTTGTGACGC AGGTG-3′, Baz qPCR R 5′-GGTCGGCGCGCCCACCCT TC-3′, rp49 F 5′-GCGGGTGCGCTTGTTCGATCC-3′, rp49 R 5′-CCAAGGACTTCATCCGCCACC-3′.

## Cell culture

*Drosophila* S2R cells were kept at 25 °C in *Drosophila* Schneider medium supplemented with 10% FCS and 1% penicillin and streptomycin and passaged every 3–4 days.

Cells were transfected with FuGene HD (Promega) according to the manufacturer's instructions and allowed to grow for additional 3–4 days after transfection.

## Antibody production

To produce sera against the N-terminus of Baz, a rabbit and a guinea pig were immunized with the recombinant GST-Baz1-318 (Eurogentec Inc.).

## Immunofluorescence

Embryos were fixed in 4% formaldehyde, phosphate buffer pH 7.4 as previously described [32]. The following primary antibodies were used for immunofluorescence: chicken anti GFP (1:2.000, Aves Labs Inc., #GFP-1020), mouse anti Dlg (1:25, DSHB, #4F3), rat anti DE-Cad (1:5, DSHB, #DCAD2), rabbit anti Baz (1:1.000, this study), Gp anti Baz (1:500, this study), rat anti Mir (1:1.000, gift from A. Wodarz), rabbit anti aPKC (1:500, Santa Cruz, #sc-216), and mouse anti Sxl (1:25, DSHB, #M114). Secondary antibodies conjugated with Alexa 488, Alexa 568, and Alexa 647 (Life Technologies) were used at 1:400. Images were taken with a Zeiss LSM710 and processed and analyzed with FIJI [56]. To quantify the co-localization of Baz with aPKC or DE-Cad, the Coloc 2 plugin was used. Epithelia of three representative embryos per genotype were analyzed and the Pearson correlation coefficient is shown.

## Embryonic lysates, immunoprecipitation, and western blotting

For embryonic lysates, embryos from overnight apple juice agar plates were collected and dechorionated in 50% bleach. The embryos were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM Tris–HCl, pH 7.5) supplemented with protease inhibitors. After incubation for 20 min at 4 °C, the lysates were centrifuged and SDS sample buffer was added before boiling at 95 °C for 5 min.

For immunoprecipitation, transfected S2R+ cells were lysed in lysis buffer supplemented with protease inhibitors. After centrifugation, cell lysates were added to StrepTactin beads for precipitation of One-Strep tagged Baz proteins for 45 min at 4 °C. The beads were washed three times in lysis buffer and 2× SDS sample buffer was added before boiling at 95 °C for 5 min followed by western blotting. Western blotting was performed according to the standard protocols. The following primary antibodies were used in this study: mouse anti GFP (1:500, Santa Cruz #sc-9996), rabbit anti GST (1:5000, Sigma #G7781), mouse anti c-Myc (1:100, DSHB, #9E10), mouse anti actin (1:1000, Santa Cruz #sc-47778), and rabbit anti Baz (1:1000, a gift from A. Wodarz). Quantification of Western blots was done with FIJI [56] of

three biological replicates. For Baz blots, the whole lane was quantified and normalized towards actin.

### In vitro crosslinking

The first 81 amino acids of wt or BazV14D D68K were fused to GST and purified from *E. coli* (strain BL21*) using glutathione beads (Macherey-Nagel). For in vitro crosslinking experiments, 5 µM recombinant protein was incubated in PBS for 1 h on ice. Subsequently, formaldehyde was added to a final concentration of 2% and incubated for 10 min at room temperature. The reaction was quenched by adding Tris to a final concentration of 250 mM. Then, 5× SDS sample buffer was added before boiling at 65 °C for 5 min. The crosslinked proteins were analyzed by western blotting.

### Statistical analysis

Data were analyzed using one-way ANOVA followed by Turkey's post hoc test with Graphpad Prism 6. All plots are expressed as the mean ± standard deviation (SD).

## Results

### Oligomerization and lipid binding promote Baz localization redundantly

Baz functions on the top of a hierarchy regulating apical–basal polarization in the epidermis of the developing *Drosophila* embryo. However, it is still not fully understood, how Baz itself is recruited to the membrane. To analyze the contribution of structural domains of Baz to its localization in the *Drosophila* embryonic epithelium, we generated transgenic flies that express GFP-tagged *baz* transgenes (Fig. 1a). The *baz* constructs were expressed with the *ubiquitin* promoter, which resulted in a weak overexpression of the full-length protein (Fig. 1b). GFP-Baz was expected to run at ~ 190 kDa, but we have observed that the endogenous as well as the ectopically expressed protein display a higher molecular mass in western blot, which might result from posttranslational modifications such as phosphorylation by aPKC, Par1, or Rho-kinase [16, 30, 50]. Moreover, we always detected several smaller specific Baz fragments (Fig. 1a, c, d) [4, 32], which presumably result from (proteolytic) processing of Baz.

GFP-Baz co-localizes with the AJ marker DE-Cad at the AJs (Fig. 2b), similar to endogenous Baz (Fig. 2a) and can fully rescue the embryonic lethality of the $baz^{815-8}$ null allele (91.4 ± 2.8% hatched L1 larvae) (Fig. 1a).

To analyze the function of the N-terminal oligomerization domain, we generated a oligomerization-deficient version of Baz, Baz V14D D68K (hereafter BazΔOD), which has been reported to abolish the self-association of two N-terminal monomers in rat Par3 [42]. Indeed, BazΔOD had a strongly attenuated capacity to self-associate in vivo and in vitro (Fig. 1c, d). Surprisingly, in lethality tests with $baz^{815-8}$ germ line clones (GLCs), which are deprived of maternal Baz mRNA and protein, GFP-BazΔOD rescued the embryonic lethality of the $baz^{815-8}$ null allele almost as efficient as wild-type Baz (61.0 ± 0.6%) (Fig. 1a). Furthermore, GFP-BazΔOD localizes to the AJs in epithelial cells of the embryonic epithelium indistinguishable from its wild-type counterpart (Fig. 2c compared to Fig. 2a, b).

Baz is capable of binding to the phospholipids PIP2 and PIP3 [48]. However, mutation of the lipid-binding domain of Baz (GFP-BazK1173-1174A = BazΔLB, Fig. 6b) did not attenuate the apical junctional localization of GFP-BazΔLB or its rescue capacity (95.3 ± 5.3%) (Figs. 1a, 2d). In contrast, a variant of Baz which cannot oligomerize or bind to phospholipids (GFP-BazΔODΔLB) displayed a cytoplasmic localization and failed to rescue the $baz^{815-8}$ mutant (Figs. 1a, 2e). Thus, Baz oligomerization and binding to phospholipids function in redundancy to target the protein to the apical junctions and ensure its function. This is confirmed by the finding that a Baz variant encoding the first 968 amino acids (GFP-Baz1-968), which includes the oligomerization domain, is localized to the apical junctions with a slight baso-lateral mislocalization (Suppl. Figure 1). Mutation of the oligomerization domain in GFP-Baz1-968 (GFP-Baz1-968ΔOD) abolishes its cortical localization (Suppl. Figure 1). Notably, GFP-Baz1-968 did not rescue the embryonic lethality of $baz^{815-8}$, most likely due to a lack of the C-terminal part of the protein, which includes the aPKC-binding region.

Taken together, the N-terminal oligomerization domain and the C-terminal LB motif of Baz contribute redundantly to the localization of the protein. Based on the rescue capacity of the Baz variants, we found that neither the oligomerization nor the binding to phospholipids is essential for viability.

### Binding to phospholipids is not sufficient for the function of Baz

To further investigate the role of the LB motif regarding the localization and function of Baz, we substituted the C-terminus including the intrinsic LB motif of GFP-BazΔOD by the Pleckstrin homology (PH) domains of either PLCδ (BazΔODΔ1106-1464-PHP) or Akt1 (BazΔODΔ1106-1464-PHA) (Fig. 1a). The PH domain of PLCδ specifically binds to $PtdIns_{(4,5)}P_2$ (PIP2) [57], whereas the PH domain of Akt1 binds to $PtdIns_{(3,4,5)}P_3$ (PIP3) [58]. Baz itself binds both, PIP2 and PIP3 in vitro [48].

In the epithelium of transgenic embryos, both GFP-BazΔODΔ1106-1464-PHP and

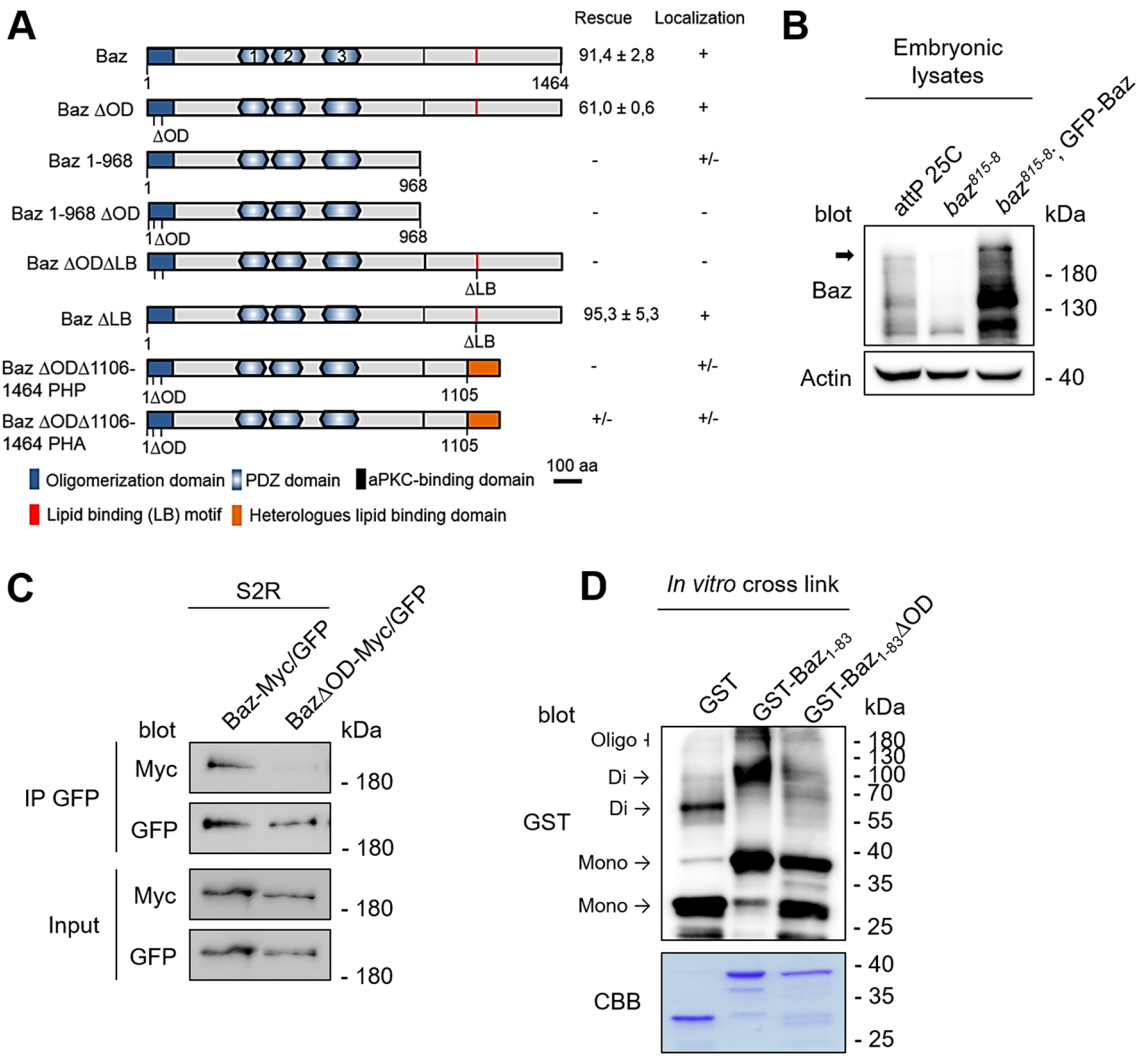


**Fig. 1** Oligomerization of Baz is dispensable for viability. **a** Schematic representation of different Baz deletion constructs. All constructs were expressed from the same genomic locus (attP40) with an N-terminal GFP-tag under the control of the *ubiquitin* promotor. The ΔOD mutation (V14DD68K) prevents oligomerization and the ΔLB mutation (K1173-74A) abolishes membrane binding. The ability to rescue the embryonic lethality of $baz^{815-8}$ germ lines clones was quantified and the localization determined, where "+" indicates the wild-type situation, "+/−" indicates a lateral cortical localization and "−" indicates a cytoplasmic localization. **b** Expression of GFP-Baz in the $baz^{815-8}$ mutant background results in a weak overexpression compared towards endogenous Baz. Full-length Baz is indicated with an arrow. **c** Co-immunoprecipitation of GFP- and Myc-tagged Baz or BazΔOD in S2R cells. Mutations of the OD domain strongly attenuate the capacity of Baz to self-associate. **d** In vitro crosslinking of the Baz OD domain (aa 1–83). Mutations of the OD domain (V14DD68K) strongly impair the formation of oligomers in vitro

GFP-BazΔODΔ1106-1464-PHA had a cortical localization and punctual enrichments at the AJ where they co-localized with DE-Cad (Suppl. Figure 1). However, only GFP-BazΔODΔ1106-1464-PHA rescued occasionally the zygotic $baz^{815-8}$ allele (< 1%), but not embryos, which have been depleted for the maternal and zygotic protein expression (GLCs). Moreover, expression of both variants together, GFP-BazΔODΔ1106-1464-PHP and GFP-BazΔODΔ1106-1464-PHA, in a *baz*-mutant background did not produce surviving animals, indicating that either simultaneous binding of one Baz molecule to PIP2 and PIP3 is essential or that the C-terminus (aa 1106–1464) is essential for Baz' function in our experimental setup, which differs from the previous studies, which used proteins overexpressed by the UAS/GAL4 system [48, 51].

## BazΔODΔLB fails to polarize the epithelium of the embryonic epidermis

To better understand why BazΔODΔLB failed to rescue $baz^{815-8}$ mutant embryos, we analyzed the epithelium of GLCs. To exclude the possibility that the N-terminal

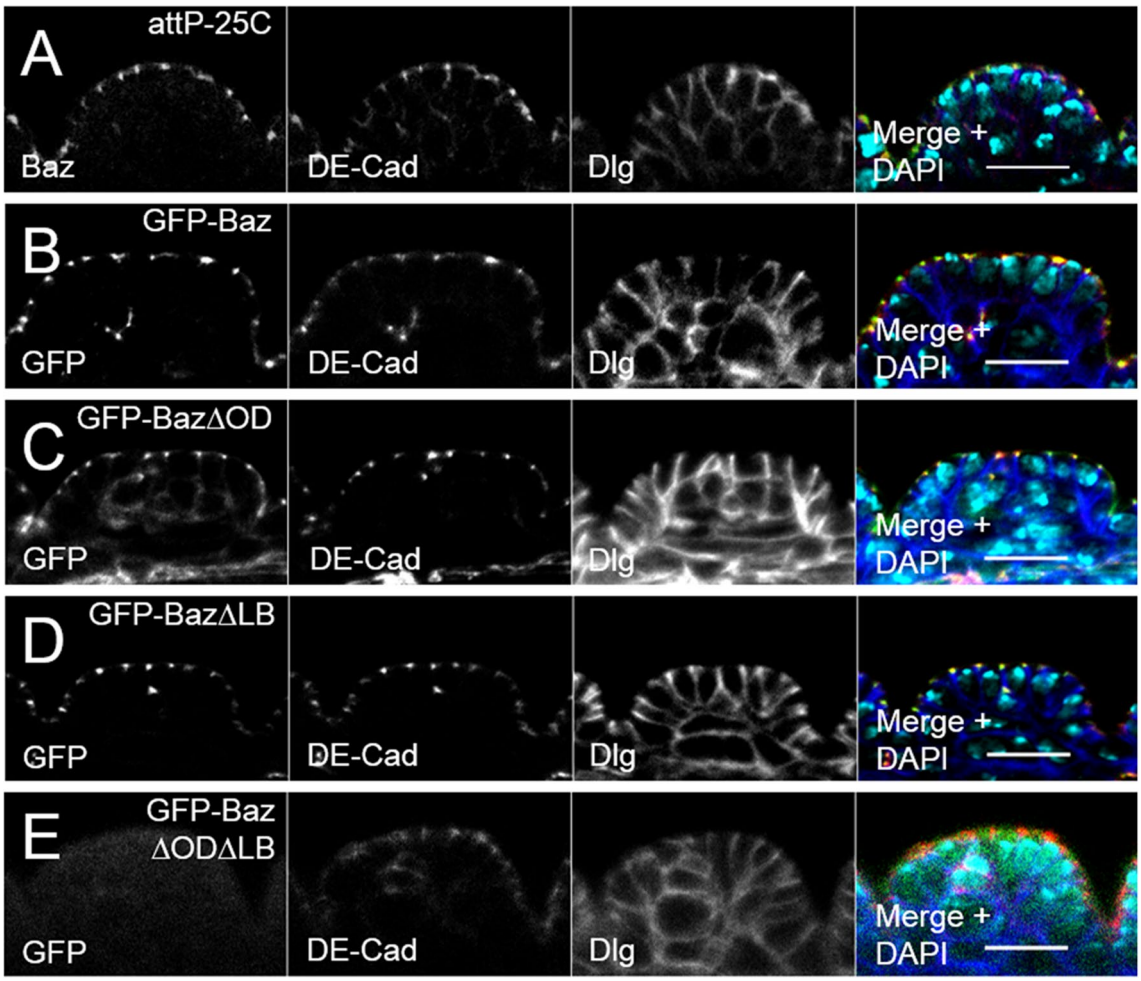


**Fig. 2** Oligomerization and lipid-binding promote Baz localization redundantly. **a** In immunostainings of the *Drosophila* embryonic epithelium, endogenous Baz (green) co-localizes with DE Cadherin (DE-Cad, red) at the apical junctions. Disc large (Dlg, blue) was stained as a lateral marker. **b** Localization of GFP-Baz is indistinguishable from the endogenous protein. **c** Oligomerization-deficient GFP-BazΔOD displays an accumulation at the AJ, overlapping with DE-Cad. **d** The localization of a lipid-binding deficient GFP-BazΔLB protein is similar to wild-type Baz. **e** GFP-BazΔODΔLB double mutant is absent from the cell cortex and displays a diffuse cytoplasmic localization. All transgenes were expressed in a wild-type background. Scale bars are 10 µm

GFP-tag interferes with the function of Baz, we created transgenic flies, which express Baz transgenes fused with a small One-Strep-tag (OneS) at the C-terminus under the control of the *ubiquitin* promotor. As the GFP variants, Baz-OneS displayed a strong rescue capacity of the embryonic lethality in $baz^{815\text{-}8}$ GLCs, whereas BazΔODΔLB-OneS displayed a complete embryonic lethality (Fig. 3). To test if the overexpression of BazΔODΔLB might help to overcome the embryonic lethality of $baz^{815\text{-}8}$ GLCs, we expressed UASp::Baz-GFP and UASp::BazΔODΔLB-GFP with mat-Tub::Gal4 in $baz^{815\text{-}8}$ GLCs (Fig. 5a). Baz-GFP rescues the embryonic lethality of GLCs to the same extend as Baz-OneS (87.3 ± 8.5 and 89.5 ± 1.8%, respectively), whereas BazΔODΔLB-GFP expressing GLCs failed to escape embryonic lethality (Fig. 3).

Next, we evaluated the phenotypes of embryonic cuticles of *baz* GLCs expressing the different rescue constructs. The Cuticle is secreted from the epidermis and allows drawing a conclusion of its integrity, in particular the formation of a function apical domain. We divided the observed phenotypes in five groups [hatched (= normal cuticle), wild type (= dead but cuticle without obvious defects), cuticle rest, holes and shrunken with holes]. As expected, the $baz^{815\text{-}8}$ mutant displayed large cuticle hole or some cuticle rest (Suppl. Figure 2). In contrast to the null allele, most animals of Baz-OneS hatch and the dead embryos had either a wild-type or shrunken cuticle phenotype (4.8 or 5.7%, respectively, Suppl. Figure 2). BazΔODΔLB-OneS partially rescued the $baz^{815\text{-}8}$ phenotype, because some embryos developed further and

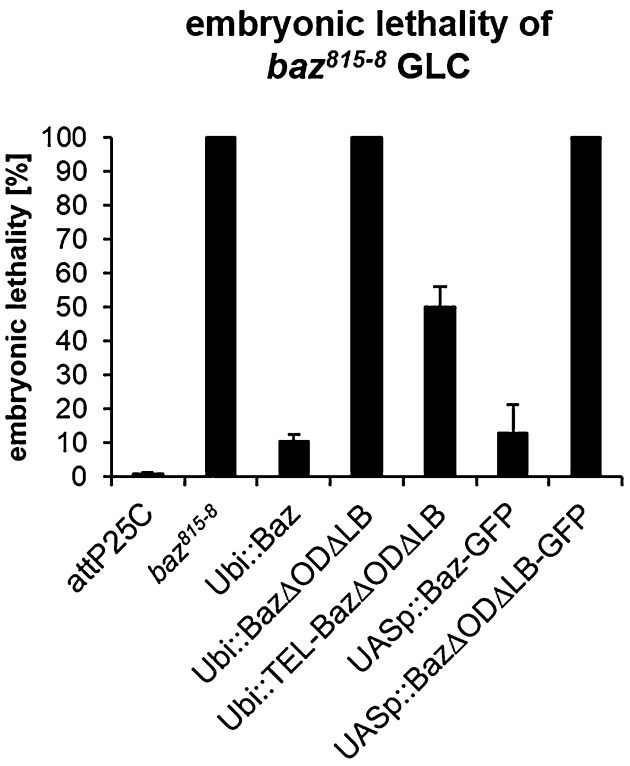


**Fig. 3** Loss of oligomerization and lipid-binding causes embryonic lethality. Baz variants that carry a C-terminal One-Strep (OneS) tag were expressed under the *ubiquitin* promoter and tested for their capacity to rescue $baz^{818-8}$ germ line clones. Baz-OneS efficiently rescues the lethality of the $baz^{818-8}$ allele (10.5 ± 1.8% embryonic lethality). BazΔODΔLB-OneS failed to rescue the embryonic lethality. Fusion of the oligomerization domain of the human TEL protein (aa 45–115) to the N-terminus of Baz restores its function and rescues embryonic lethality of $baz^{815-8}$ to a large extent (50.0 ± 6.1% embryonic lethality). Similarly, wild-type Baz-GFP overexpressed with mat-Tub::Gal4 using the Gal4/UAS-system had comparable efficiencies as the constitutively expressed variant (12.7 ± 8.5%), whereas overexpression of BazΔODΔLB-GFP failed to rescue (100% embryonic lethality). Bars represent the mean ± SD, $n = 300$ each

had either a wt or shrunken with holes phenotype (2.3 or 23.3%, respectively, Suppl. Figure 2).

Interestingly, fusion of the heterologous oligomerization domain of the human TEL protein (residues 45–115 [59]) to the N-terminus of BazΔODΔLB-OneS (TEL-BazΔODΔLB-OneS) partly restores the rescue capacity of the mutant Baz protein. Furthermore, TEL-BazΔODΔLB-OneS embryos had a milder phenotype, as most dead embryos had a wild-type or shrunken cuticle phenotype (21.9 and 17.9%, respectively, Suppl. Figure 2).

Next, we scored for the localization of polarity markers in the embryonic epithelium by immunostainings. Hemizygous mutant embryos derived from GLC were identified by the lack of Sex lethal (Sxl) staining. As expected, in the epithelium of $baz^{815-8}$ GLCs, we did not detect a signal for Baz, whereas aPKC exhibited a cytoplasmic localization (Fig. 4b). Baz-OneS and TEL-BazΔODΔLB-OneS both had a robust apical localization and recruited aPKC to the apical junctions, similar to endogenous Baz (Fig. 4a, c, e; Suppl. Figure 3B, D, F). In contrast, BazΔODΔLB-OneS showed a cytoplasmic mislocalization (Fig. 4d). Nevertheless, BazΔODΔLB-OneS managed to recruit some aPKC to the apical junctions, but the majority of the aPKC protein still accumulates in the cytoplasm (Fig. 4d). The amount of aPKC which co-localized with BazΔODΔLB-OneS was significantly reduced, but was to a large extent rescued by TEL-BazΔODΔLB-OneS (Suppl. Figure 3L). In general, the overall structure of the epithelium was disrupted in BazΔODΔLB-OneS embryos.

Then, we examined the assembly of intact AJ by scoring for the localization of DE-Cad in $baz^{815-8}$ GLCs (Fig. 4f–j). Baz-OneS and TEL-BazΔODΔLB-OneS both showed an apical junctional targeting of DE-Cad comparable to the wild-type control (Fig. 4f, h, j; Suppl. Figure 3G, I, K). Unlike as for aPKC, we did not observe a localization of DE-Cad in the apical region or at the plasma membrane in BazΔODΔLB-OneS expressing $baz^{815-8}$ GLCs (Fig. 4i; Suppl. Figure 3L).

In summary, BazΔODΔLB-OneS has only a weak capability to polarize the embryonic epithelium and displayed strong cuticle defects, as well as an impaired function, since aPKC was inefficiently and DE-Cad not at all recruited to the apical junctions. Restoring the oligomerization capacity of Baz by fusing the oligomerization domain of TEL to BazΔODΔLB-OneS restores its functionality to a large extent.

## Apical–basal polarity of embryonic NBs is disrupted in BazΔODΔLB embryos

Similar to polarization of the epithelium of the embryonic epidermis, Baz is also required for the establishment of apical–basal polarity of dividing NBs. Hence, we investigated if BazΔODΔLB-OneS had similar phenotypes in embryonic NBs as in the epithelium. Baz accumulates at the apical cortex, recruiting aPKC and Par6, whereas the scaffold protein Miranda (Mir) is restricted to the basal region of metaphase NBs (Fig. 5a). Basal segregation of Mir depends on the apical formation of the Par complex [60, 61]. Therefore, we analyzed the localization of Baz variants and Mir in NBs of $baz^{815-8}$ GLCs. We found that BazΔODΔLB-OneS phenocopied the *baz* null allele, as Mir is not restricted to the basal region of NBs in both genotypes but can be found more or less all around the cortex (Fig. 5b, d). BazΔODΔLB-OneS also failed to localize to the apical membrane of NBs, but rather displayed a weak cytoplasmic localization (Fig. 5d). Like in the epithelium, Baz-OneS and TEL-BazΔODΔLB-OneS rescued the asymmetric distribution of Mir and

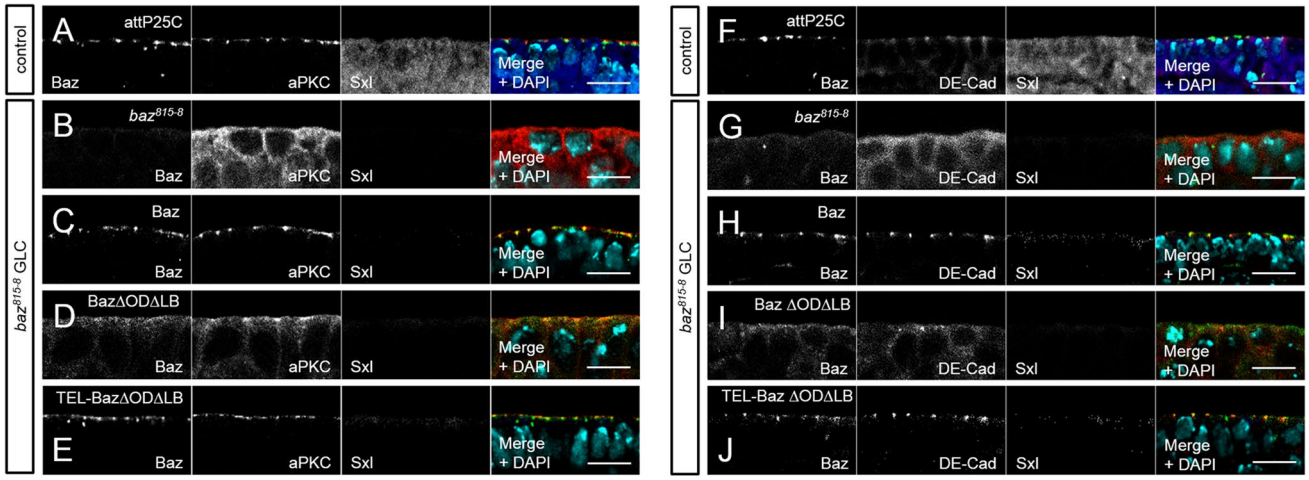


**Fig. 4** Epithelial polarization requires the functional redundancy of the OD and LB domains. **a** Immunostaining of endogenous Baz (green), aPKC (red), and Sxl (blue) in the embryonic epidermis. **b–e** Immunostaining of Baz variants and endogenous aPKC in the embryonic epidermis of *baz*$^{818-8}$ germ line clones. Hemizygous mutant embryos were identified by the absence of Sxl staining. **b** Loss of Baz in the embryonic epidermis of disrupts epithelial polarization and aPKC accumulates in the cytoplasm. **c** Baz-OneS efficiently recruits aPKC to the apical junctions, such as endogenous Baz, whereas BazΔODΔLB-OneS displays a cytoplasmic mislocalization. **d** BazΔODΔLB-OneS rescues targets some aPKC to the apical junctions, but the majority of the protein still accumulates in the cytoplasm. **e** TEL-BazΔODΔLB-OneS localizes at the apical junctions and is capable of recruiting aPKC to rescue the defects of BazΔODΔLB-OneS. **f–j** Immunostaining of Baz (green), DE-Cad (red), and Sxl (blue) in the embryonic epidermis demonstrates a loss of AJ in *baz*$^{815-8}$ germ line clones, which can be rescued by wild-type Baz (**h**) and TEL-BazΔODΔLB-OneS (**j**) but not by BazΔODΔLB-OneS (**i**). Scale bars are 10 µm

localized to the apical membrane in metaphase NBs, such as the control (Fig. 5a, c, e).

In contrast to the epithelium where aPKC displayed at least a minimal polarization in BazΔODΔLB-OneS GLC, its localization in NBs is cytoplasmic, similar to the *baz* null allele (Fig. 5g, i). As expected, Baz-OneS and TEL-BazΔODΔLB-OneS recruit aPKC to the apical cortex comparable to endogenous protein (Fig. 5f, h, j).

Thus, the functional redundancy of the OD and the LB motif are essential to polarize embryonic NBs, since neither Mir nor aPKC exhibited a correct localization in BazΔODΔLB-OneS GLC.

## BazΔODΔLB is still recruited to the cortex by transmembrane proteins

To elucidate the mechanism why BazΔODΔLB-OneS fails to accumulate at the apical junctions and why it is non-functional, we tested whether the membrane recruitment by its described interacting transmembrane proteins is disturbed. Therefore, we used *Drosophila* S2R cells as a model system, because they do not exhibit a polarity or detectable amounts of polarity proteins [48]. In S2R cells, Baz-GFP clearly localizes to the cell cortex (Fig. 6a), whereas BazΔLB-GFP appears in cytoplasmic aggregates (Fig. 6b), indicating that in S2R cells, cortical localization of Baz depends exclusively on the binding to phospholipids. Co-transfection of BazΔLB-GFP with DE-Cad-RFP or Ed-RFP led to the formation of cell–cell contacts of transfected cells. Both proteins recruited BazΔLB-GFP to the artificial cell–cell contacts, but not RFP alone (Fig. 6c–e). In addition, the intracellular domain of the atypical cadherin Starry night (Stan) fused to the extracellular domain of DE-Cad also recruited BazΔLB-GFP to ectopic cell–cell contacts (Fig. 6f). By contrast, the Stan isoform Flamingo, which lacks the C-terminal PDZ-binding motif, did not recruit lipid-binding deficient Baz (data not shown). Expression of BazΔODΔLB-GFP alone or together with DE-Cad, Ed, or Stan showed that BazΔODΔLB-GFP was recruited to the cell–cell contacts, such as its oligomerizing counterpart (Fig. 6g–k). Surprisingly, deleting the PDZ domains of GFP-BazΔLB, which facilitate binding to Stan, Ed, and Arm/DE-Cad, does not disturb its localization at the apical junctions in the embryonic epidermis (Fig. 6l).

## Cortical targeting protects Baz from degradation

Despite its capacity to interact with transmembrane proteins, BazΔODΔLB-OneS is non-functional and does not localize to the apical junctions. Therefore, we tested, whether simultaneous loss of oligomerization and lipid-binding affects the protein stability, we blotted *baz*$^{815-8}$ GLC rescued with GFP-Baz and Baz-OneS variants to detect the exogenous Baz protein. Indeed, we observed that loss of the oligomerization domain caused a reduced amount of Baz protein in the embryo, which is further enhanced upon the loss of the

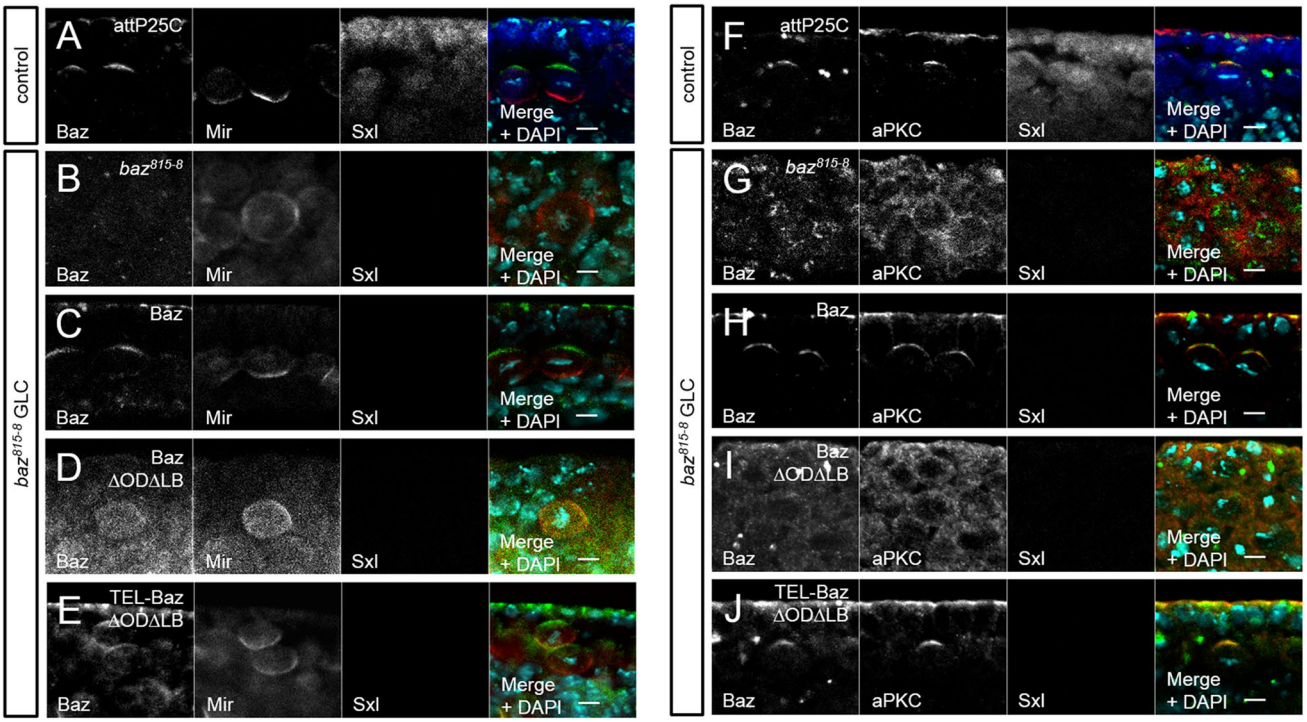


**Fig. 5** Neuroblast polarity requires Baz's capacity to either self-associate or to bind lipids. **a** Immunostaining of endogenous Baz (green), Mir (red), and Sxl (blue) in embryonic metaphase NBs. Baz localizes at the apical cortex, whereas Mir accumulates basally. **b–e** Immunostaining of Baz variants (green) and endogenous Mir (red) in embryonic NBs of $baz^{818-8}$ germ line clones during metaphase. Hemizygous mutant embryos were identified by the absence of Sxl staining. **b** Loss of Baz in $baz^{818-8}$ germ line clones disrupts NB polarity and Mir localization. **c** Baz-OneS rescues NB polarity, whereas BazΔODΔLB-OneS does not localize at the cortex and fails to polarize NBs (**d**). **e** TEL-BazΔODΔLB-OneS restores apical–basal polarity in metaphase NBs, such as wild-type Baz. **f** Immunostaining of endogenous Baz (green), aPKC (red), and Sxl (blue) in embryonic metaphase NBs. Baz recruits aPKC to the apical pole of metaphase NBs. **g** aPKC accumulates in the cytoplasm in metaphase NBs of $baz^{818-8}$ germ line clones. **h**, **j** Baz-OneS and TEL-BazΔODΔLB-OneS both show a comparable localization and recruit aPKC to the apical pole. **i** In contrast, BazΔODΔLB-OneS localizes in the cytoplasm, such as endogenous aPKC. Scale bars are 5 µm

LB motif (Fig. 7a, b, d, e). In contrast, mutation of the LB motif alone did not reduce the amount of protein, but rather seemed to elevate it (Fig. 7d, e), whereas the amount of BazΔODΔLB-OneS protein is drastically reduced in lysates of GLC (Fig. 7a, b). Notably, introduction of an ectopic oligomerization capacity in (TEL-BazΔODΔLB-OneS) restored protein stability (Fig. 7a, b). The reduced amount of BazΔODΔLB-OneS was not due to impaired gene expression, because all transgenes were expressed from the same promoter and genomic location and exhibited comparable mRNA levels with no significant differences (Fig. 7c).

Finally, we tested whether the phenotypes observed in BazΔODΔLB rescued embryos are only due to protein degradation of the mutant protein. Strikingly, overexpression of wild-type Baz-GFP but not BazΔODΔLB-GFP rescued the embryonic lethality of $baz^{815-8}$ (Fig. 3), although both proteins are expressed at comparable levels (Suppl. Figure 4A). Moreover, BazΔODΔLB-GFP is still cytoplasmic, whereas its wild-type counterpart localizes to the apical junctions (Suppl. Figure 4C in comparison to B). These data suggest that BazΔODΔLB fails to localize to the apical junctions, accumulates in the cytoplasm, and is degraded.

## Discussion

Taken together, the oligomerization domain of Baz is not essential for viability of the *Drosophila* embryo, but contributes to the stability of the protein; and the functional redundancy of the oligomerization domain and the LB motif are indispensable for the function of Baz during *Drosophila* embryogenesis (Fig. 7f).

The previous studies reported either an important role of the OD for Baz/Par3 localization in *Drosophila* and mammalian cells [40, 41] or found only a minor influence of the OD on Baz localization [47, 51], which we could confirm. This discrepancy might be explained by the different setups: In contrast to the previous studies [40, 48, 51] using overexpressed proteins with the UAS/GAL4 system in rescue experiments, we used a constitutive expression, which

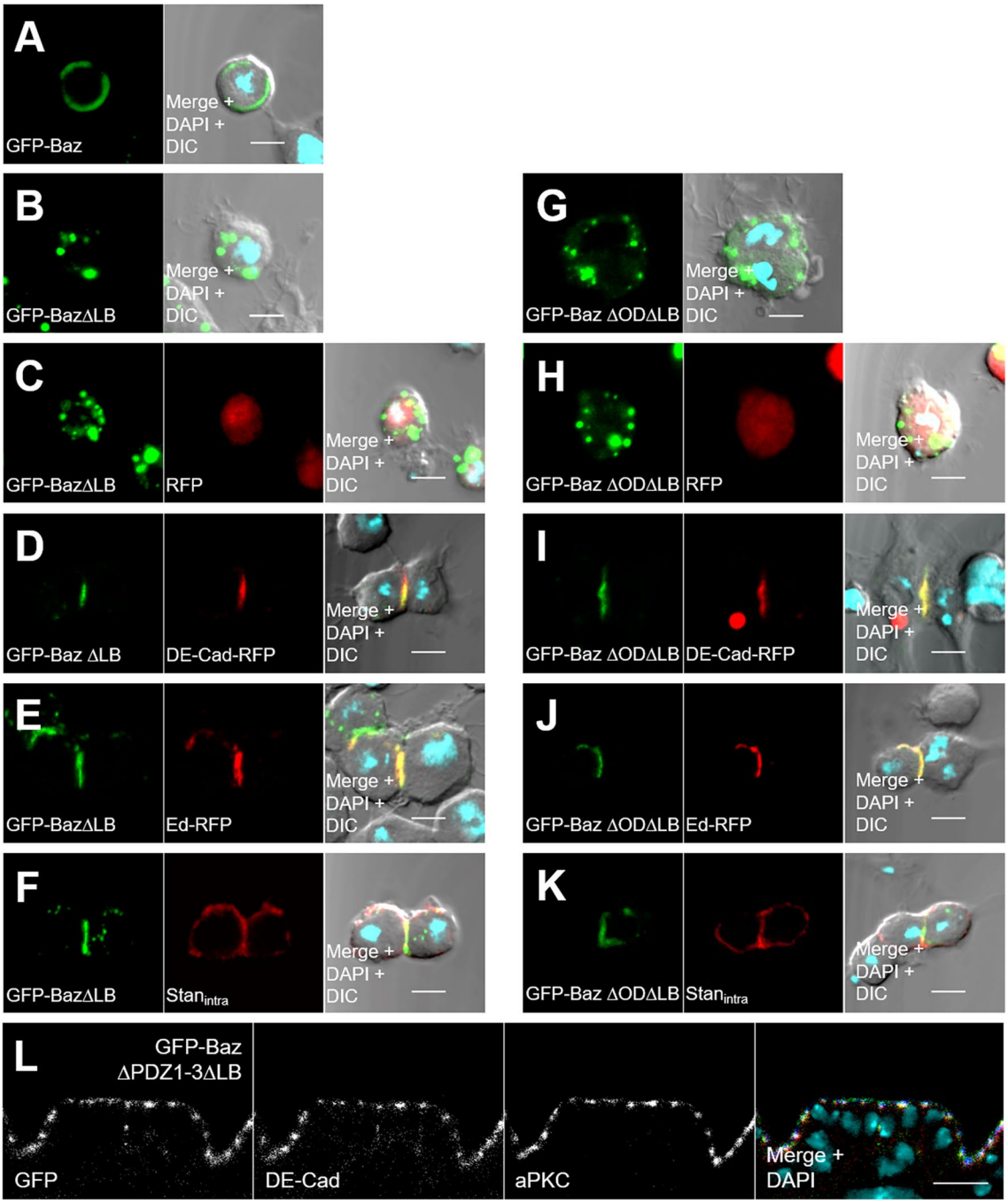


**Fig. 6** Recruitment of Baz by DE-Cad, Ed, and Stan does not depend on self-association or lipid-binding of Baz. **a** Wild-type Baz-GFP was expressed with the *ubiquitin* promoter in S2R cells and localizes at the plasma membrane. **b** BazΔLB-GFP accumulates in cytoplasmic aggregates. **c** RFP displays a diffuse cytoplasmic localization and cannot recruit BazΔLB-GFP to the cortex. **d**, **e** DE-Cad-RFP and Ed-RFP recruit BazΔLB-GFP to artificial cell–cell contacts. **f** Similar, the intracellular domain of Stan fused to the extracellular and transmembrane domain of DE-Cad (DE-CadΔintra-StanΔextra) targets BazΔLB-GFP to cell–cell contacts. DE-CadΔintra-StanΔextra was detected with an anti DE-Cad antibody, which recognizes the extracellular domain of DE-Cad. **g**–**k** In the same experimental setup with BazΔODΔLB-GFP, the double mutant was efficiently recruited by DE-Cad, Ed, and Stan without apparent differences. **l** Deletion of all three PDZ domains in BazΔLB does not affect the localization of the mutant protein at the apical junctions (green = GFP-BazΔPDZ1-3 ΔLB, red = DE-Cad, blue = aPKC). *DIC* differential interference contrast. Scale bars are 5 µm in **a**–**k** and 10 µm in **l**

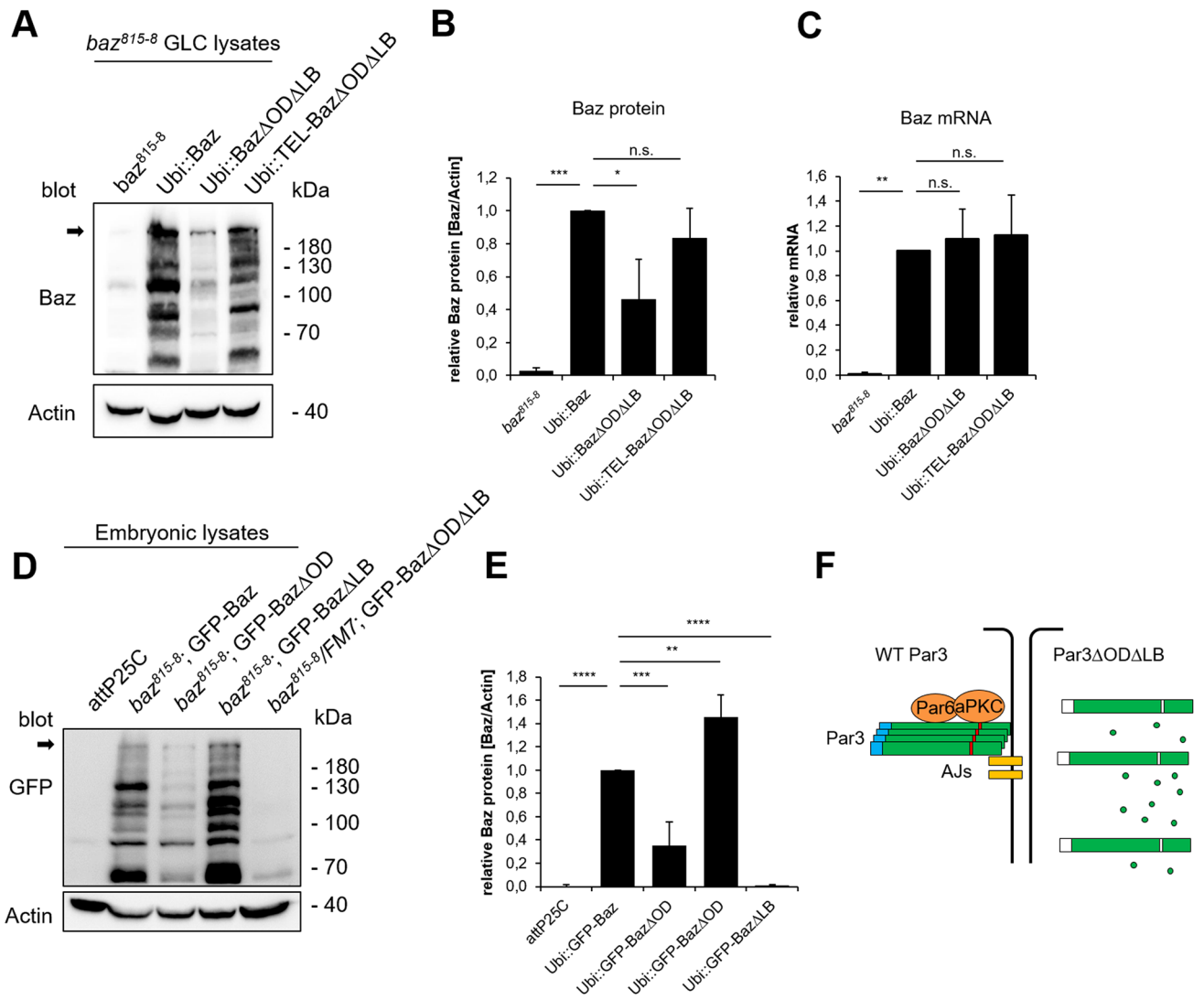


**Fig. 7** Oligomerization and lipid binding are crucial for Baz' stabilization. **a** Lysates of *baz*$^{818\text{-}8}$ germ line clones that express different Baz variants were blotted against Baz. Actin was used as loading control. Full-length Baz is indicated with an arrow. Loss of Baz oligomerization and lipid binding (BazΔODΔLB) strongly decreases the amount of Baz protein. TEL-BazΔODΔLB-OneS rescues the degradation of BazΔODΔLB-OneS. **b** Quantification of Baz-OneS variants in *baz*$^{818\text{-}8}$ germ line clones. The whole Baz lanes were quantified and normalized towards actin from three biological replicates. **c** qPCR of total RNA from the *baz*$^{818\text{-}8}$ germ line clones shows that all transgenes were expressed without significant differences. **d** Embryonic lysates of different GFP-Baz variants in a *baz*$^{818\text{-}8}$ genetic background were blotted against GFP. Full-length Baz is indicated with an arrow. Actin was used as loading control. Loss of oligomerization reduces the stability of GFP-BazΔOD, which is drastically enhanced upon the mutation of the lipid-binding motif in GFP-BazΔODΔLB. However, mutation of the lipid-binding motif alone (GFP-BazΔLB) does not affect the protein stability. **e** Quantification of GFP-Baz variants. The whole GFP lanes were quantified and normalized towards actin from three biological replicates. **f** Scheme of the functional redundancy between the OD and LB domains. Bars represent the mean ± SD. Statistics were one-way ANOVA followed by Tukey's post hoc test, n.s. $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

resulted in a rather mild overexpression. Nonetheless, the slightly reduced rescue capacity (61% of BazΔOD in contrast to 91% for wild-type Baz) and the redundant function of the OD underline the importance of Baz self-association. The fact that the heterologous oligomerization domain of TEL can rescue the defects of BazΔODΔLB suggests that the OD promotes indeed self-association instead of interaction with other binding partners.

In line with the previous results [48, 51], we found that the lipid-binding domain of Baz/Par3 is dispensable for the localization and function of the protein—however, it can, to a far extent, compensate the loss of the OD. Notably, in contrast to McKinley et al. [51], we found that deletion of the PDZ domains does not affect the localization of GFP-Baz (or other apical determinants), which might be explained by the fact that, in that study, the authors used the UAS/

GAL4-System to (over)express a C-terminal-tagged Baz (in which the GFP might be cleaved due to processing of the C-terminus, which can be frequently observed) in both, live-imaging and fixed samples, whereas we utilized a constitutively expressed transgene and an N-terminal tag only in fixed tissues. Although the PDZ domains as well as the aPKC-binding region certainly contribute to the fine-tuning of Baz localization, our data demonstrate that the OD and the LB domain are the crucial two domains which regulate the membrane-tethering of the protein. We further demonstrate here that protein stability of Baz depends on membrane localization, as BazΔODΔLB is degraded, whereas fusion of heterologous lipid-binding domains or an oligomerization domain rescues the protein stability (Suppl. Figure 1E; Fig. 7a, b). A reduced amount of Baz has been reported to localize at the apical domains upon deletion of the OD [51], which might be explained by our observation of the decreased protein stability of BazΔOD.

Finally, one important question remains: How does the OD contribute to the localization of Baz? One likely possibility is that Baz forms oligomers, which are then recruited to the plasma membrane by a transmembrane- or membrane-associated protein. The previous studies have identified three transmembrane proteins (DE-Cad (via Arm), Ed, Stan, and Canoe) as interaction partners of Baz, which might be capable of recruiting the protein to the membrane [39, 45, 62]. The interaction of Baz with Arm, Ed, and Stan has been described to be mediated by the PDZ domains of Baz [45, 51, 62]. However, deletion of all three PDZ domains together with the lipid-binding motif does not substantially affect the apical–junctional localization of the mutant protein (Fig. 6l). Given that BazΔODΔLB does not display an apical accumulation, the functional redundancy of the OD and LB motif might be essential for the initial localization to the plasma membrane. Here, it seems to be not important, whether Baz binds to PIP2 or PIP3, as both chimeric rescue constructs restore protein stability and (at least to some extent) the localization of BazΔODΔLB (Supplementary Fig. 1C–E). Work from Harris and Peifer nicely demonstrated that Baz functions upstream of at least DE cadherin in the polarization of the embryonic epidermis [19]. However, it is still unclear, if other Baz-binding proteins, such as Ed, Stan, or Canoe, could contribute to the initial recruitment of Baz to the plasma membrane of epithelial cells during the early embryonic development [39, 45, 62]. Therefore, we tried to abolish the expression of DE Cadherin, Ed, and Stan in the early embryos using triple GLCs, which unfortunately did not produce any eggs (data not shown), indicating that these three genes are involved in oogenesis, too. Nonetheless, we observed that BazΔODΔLB is still able to interact with DE-Cad, Ed and Stan (Fig. 6). This is surprising as in vivo, none of these interaction partners seem to be capable of targeting the mutant protein to the apical junctions, although they are all expressed in the embryonic epidermis. Thus, we can exclude DE-Cad, Ed, and Stan to be involved in the initial recruitment of Baz. Although Canoe is important for the apical positioning of Baz, loss of Canoe does not prevent the membrane association of Baz [39]. Moreover, the fact that deletion of all three PDZ domains in BazΔLB does not disturb the correct apical junctional localization of the mutant protein (Fig. 6l) suggests that another domain is essential for the recruitment of Baz oligomers. A possible model for the correct recruitment of Baz could be that in the absence of lipid binding, oligomerized Baz is targeted to the apical junctions by several weak mechanisms and interactions with proteins, independently (or redundantly) of the PDZ domains. The multiplicity of backup mechanisms for Baz localization further underlines the critical role of the localization of this polarity regulator in establishing apical–basal polarity in the embryonic epidermis.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no competing interests.

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