Divergent polarization mechanisms during vertebrate epithelial development mediated by the Crumbs complex protein Nagie oko

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Summary
The zebrafish MAGUK protein Nagie oko is a member of the evolutionarily conserved Crumbs protein complex and functions as a scaffolding protein involved in the stabilization of multi-protein assemblies at the tight junction. During zebrafish embryogenesis, mutations in nagie oko cause defects in both epithelial polarity and cardiac morphogenesis. We used deletion constructs of Nagie oko in functional rescue experiments to define domains essential for cell polarity, maintenance of epithelial integrity and cardiac morphogenesis. Inability of Nagie oko to interact with Crumbs proteins upon deletion of the PDZ domain recreates all aspects of the nagie oko mutant phenotype. Consistent with this observation, apical localization of Nagie oko within the myocardium and neural tube is dependent on Oko meduzy/Crumbs2a. Disruption of direct interactions with Patj or Lin-7, two other members of the Crumbs protein complex, via the bipartite L27 domains produces only partial nagie oko mutant phenotypes and does not impair correct junctional localization of the truncated Nagie oko deletion protein within myocardial cells. Similarly, loss of the evolutionarily conserved region 1 domain, which mediates binding to Par6, causes only a subset of the nagie oko mutant epithelial phenotypes. Finally, deletion of the C-terminus, including the entire guanylate kinase and the SH3 domains, renders the truncated Nagie oko protein inactive and recreates all features of the nagie oko mutant phenotype when tested in functional complementation assays. Our observations reveal a previously unknown diversity of alternative multi-protein assembly compositions of the Crumbs–Nagie-oko and Par6–aPKC protein complexes that are highly dependent on the developmental context.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/121/15/2503/DC1

Key words: Nagie oko, Cell polarity, Heart, Organogenesis, aPKC, Mpp5, Pals1, Lin-7, Crumbs, Par6

Introduction
Cell polarity and maintenance of epithelial integrity are tightly linked to many developmental processes including the establishment of transepithelial diffusion barriers within gut and epidermis, as well as the morphogenesis of complex organs including the heart, retina and neural tube. Many studies have implicated proteins containing PDZ domains in cell polarization (Sheng and Sala, 2001; Bilder et al., 2003; Tanentzapf and Tepass, 2003; Hurd et al., 2003). The membrane-associated guanylate kinase (MAGUK) proteins represent an important class of these proteins and are characterized by PDZ, SH3 and the non-catalytical kinase (MAGUK) proteins represent an important class of these proteins and are characterized by PDZ, SH3 and the non-catalytical

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domain with Par6 is not essential for the maintenance of epithelial polarity within mammalian Madin Darby canine kidney (MDCK) epithelial cells (Wang et al., 2007).

In zebrafish, the nok, heart and soul (has)/prkci, and oko meduzy (ome)/crb2a mutations affect maintenance of cell polarity and epithelial integrity within the neural retina and the retinal pigmented epithelium (RPE) (Malicki and Driever, 1999; Wei and Malicki, 2002; Horne-Badovinac et al., 2001; Peterson et al., 2001; Omori and Malicki, 2006). In addition, nok and has/prkci regulate myocardial cell polarity, adhesion and cell-shape changes, which contribute to heart morphogenesis (Yelon et al., 1999; Horne-Badovinac et al., 2001; Peterson et al., 2001; Trinh et al., 2005; Rohr et al., 2006; Rohr et al., 2008). Phenotypic similarities between these mutants support the genetic and biochemical evidence from other systems for a direct interaction between the Crb-Nok and Par6-aPKC protein complexes.

Here, we functionally test the role of specific protein-protein interaction domains of Nok during zebrafish embryonic development. Tissue-specific differences in the ability of different Nok deletion proteins to rescue epithelial polarity defects suggest varying compositions and changing protein-protein interactions between components of the Crb-Nok and the Par6-aPKC complex during the embryonic development of zebrafish.

### Results

#### Polarization of different embryonic epithelia is mediated by divergent Nok protein-protein interaction domains

The complex epithelial phenotypes of nok mutants prompted us to systematically test the requirement of individual Nok protein-protein interaction domains in mediating the correct polarization and maintenance of different developing epithelia, including the squamous monolayered RPE surrounding the neural retina, the myocardium and the neural retina. To this end, we generated a series of deletion constructs that encode truncated and Myc-tagged versions of the wild-type (WT) protein (Fig. 1A; see Materials and Methods). Genetic rescue experiments were performed in the nok<sup>360</sup> or nok<sup>m520</sup> null mutant backgrounds, or in nok morpholino antisense oligonucleotide (MO<sup>360</sup>) injected embryos that were co-injected with MO<sup>552</sup> to suppress the major off-target effects of morpholinos mediated through p53 activation (Nasevicius and Ekker, 2000; Langheinrich et al., 2002; Robu et al., 2007). The nok<sup>360</sup> or nok<sup>m520</sup> mutations cause a complete loss or a C-terminal truncation of the GK domain, respectively, and only severely reduced levels of mutant protein can be detected (Wei and Malicki, 2002; Rohr et al., 2006) (Xiangyun Wei, University of Pittsburgh, PA, personal communication). To control for efficient expression of Nok deletion proteins at 32 hours post fertilization (hpf), protein levels were detected by western blotting of embryonic extracts and probing with an anti-Myc antibody (Fig. 1B).

To compare the phenotypic consequences of expressing different Nok deletion proteins in nok<sup>360</sup> or nok<sup>m520</sup> mutants compared with their expression in nok morphants, we analyzed the different mutant/morphant phenotypes and could not detect differences (see below). Therefore, neither nok<sup>360</sup> nor nok<sup>m520</sup> provide rescue activity in the context of complementation assays when injected with mRNA encoding Nok deletions.

Consistent with a function in apical-basal cell polarity, complete loss of nok causes a severe curvature of the body and disruption of central portions of the RPE which leaves most of the retina without pigmented cells (Fig. 2D). Injection of His-Myc-tagged Nok<sup>wt</sup> mRNA into nok<sup>m520</sup> mutant embryos complemented the severely disrupted and patchy RPE phenotype (n=31/31 rescued embryos) and curved body form (n=24/31 rescued embryos; Fig. 2B,H). For evaluating the efficacy of the RPE rescue, we only defined embryos as rescued in which the retina was entirely covered by pigment cells as observed in the WT.

Heart tube elongation in nok mutants and morphants is impaired, in accordance with a disruption of myocardial cell cohesion and a failure of cardiomyocytes to correctly expand in size (Fig. 3B,G) (Rohr et al., 2006). To visualize the rescue efficiency of Nok<sup>wt</sup> protein on heart development, we introduced a transgene that expresses green fluorescent protein (GFP) under the control of the cmic2 promoter [Tg(cmic2:GFP)] (Huang et al., 2003) into the nok<sup>m520</sup> mutant background. Injection of mRNA encoding His-Myc-tagged Nok<sup>wt</sup> could completely rescue heart tube elongation in a subset of mutant embryos (n=6/31 rescued embryos). The nok<sup>m520</sup> mutant genotype was verified by PCR (see Materials and Methods). Similarly, co-injection of mRNA encoding His-Myc-tagged Nok<sup>wt</sup> together with MO<sup>nok</sup> resulted in the rescue of heart tube elongation as assessed using the atrium-specific antibody S46 (n=14/39 rescued embryos; Fig. 3C,G).

To assess the organization and polarity of the neural retina and to visualize apical junctions at 34 hpf, we performed immunohistochemical stainings of retinal tissue using an antibody against ZO-1 (Fig. 4) and Rhodamine phalloidin, which has a high

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**Fig. 1.** Structure of Nok deletion mutants and their expression. (A) Nok is a MAGUK protein which contains ECR1, a bipartite L27 domain, PDZ, SH3, and GUK domains. Overview of different Nok deletions functionally tested in this study. Asterisk indicates the position of the nok<sup>m520</sup> mutation. Expected interactions (black arrows) between Nok with different members of the Crb and the Par6-aPKC protein complexes are indicated. (B) Western blot of 32 hpf tissues with Myc-tagged Nok<sup>wt</sup> (asterisk; expected size of 82 kDa), Nok<sup>ΔCrb</sup> (asterisk; expected size of 74.5 kDa), Nok<sup>ΔPar6</sup> (asterisk; expected size of 81.1 kDa), Nok<sup>ΔPatj</sup> (dot; expected size of 74.5 kDa), Nok<sup>ΔSH3</sup> (dot; expected size of 50.6 kDa), Nok<sup>ΔGUK</sup> (asterisk; expected size of 81.1 kDa), Nok<sup>ΔECR1</sup> (dot; expected size of 74.5 kDa), and Nok<sup>ΔPDZ</sup> (asterisk; expected size of 69.4 kDa). Loading control is acetylated tubulin (bottom).
Nagie oko structure-function analysis

The neural retinal tissue was counterstained with an antibody against Nok to monitor expression of His-Myc-tagged Nok protein. In comparison to the noks305 mutant phenotype (Fig. 4A), embryos injected with His-Myc-tagged nokwt mRNA showed no ectopic ZO-1-positive bundles within the neural retina, and the integrity of the tissue was maintained (n = 6/6 embryos rescued). In addition, no ectopic F-actin staining was seen. Therefore, similarly to the rescue of the RPE phenotype and myocardial development, Nokwt protein completely rescues the neural retinal polarity and integrity defects (Fig. 4B).

To evaluate the relevance of different protein-protein interaction domains of Nok for maintenance of epithelial polarity and cardiac morphogenesis, we first tested whether the interaction domain of Nok that is required for association with Par6 is essential. Interactions between murine Pals1 and the scaffolding factor Par6 are mediated via the ECR1 domain (Wang et al., 2004). We thus tested the functionality of the corresponding NokΔPar6 mutant, which lacks the ECR1 domain, in complementing different epithelial defects. Whereas the His-Myc-tagged Nokwt or (E) nokΔPar6ΔLin7 mRNA complements nok morphant heart defects, all other Nok deletions lack rescue function in this assay. The corresponding mutant cardiac phenotypes are characterized by an arrest at the heart cone stage or by a failure to extend the heart tube. (G) Summary of rescue efficiencies of the different Nok deletions expressed within the nok morphant background.

Fig. 2. Functional assays for Nok deletions. (A-G) Phenotypes of (A) wt, (D) nokm520 mutant, or (B,C,E-G) nokm520 mutants that were injected with mRNA encoding different deletions of Nok. Insets are details of the corresponding RPE phenotype. Note that the cobblestone-like appearance of this tissue is severely disrupted in nokm520 (D), nokΔCrb (F) and nokΔC (G) mutants but rescued in the other mutants and WT. (H) Summary of rescue efficiencies of the different deletion constructs in the nokm520 mutant background for two aspects of the phenotype. RPE, retinal pigment epithelium.

Fig. 3. Cardiac development in Nok deletion mutants. (A-F) Images represent reconstructions of confocal Z-stack sections of 32 hpf Tg[cmlc2:GFP] transgenic and nok morphant embryos (coinjected with MO p53 to suppress MOoff-target effects) expressing different Nok deletion proteins. Cardiac morphology is assessed using the atrium-specific antibody S46 (red). Arrows indicate the extent of atrial expansion. (C) Whereas nokwt or (E) nokΔPar6ΔLin7 mRNA complements nok morphant heart defects, all other Nok deletions lack rescue function in this assay. The corresponding mutant cardiac phenotypes are characterized by an arrest at the heart cone stage or by a failure to extend the heart tube. (G) Summary of rescue efficiencies of the different Nok deletions expressed within the nok morphant background.

affinity for submembranous F-actin (not shown). The neural retinal tissue was counterstained with an antibody against Nok to monitor expression of His-Myc-tagged Nok protein. In comparison to the noks305 mutant phenotype (Fig. 4A), embryos injected with His-Myc-tagged nokwt mRNA showed no ectopic ZO-1-positive bundles within the neural retina, and the integrity of the tissue was maintained (n = 6/6 embryos rescued). In addition, no ectopic F-actin staining was seen. Therefore, similarly to the rescue of the RPE phenotype and myocardial development, Nokwt protein completely rescues the neural retinal polarity and integrity defects (Fig. 4B).

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We next tested the requirement of the Nok interaction domain that is predicted to mediate association with Patj or Lin7 in the

Fig. 4. Neural retinal polarity in Nok deletion mutants. Optical cross sections through the neural retina at 32 hpf. Images represent reconstructions of confocal Z-stack sections. Tissues are stained for ZO-1 (red) and Nok (green). Small insets are magnifications of ZO-1 staining indicated by white arrowheads. (A) The noks305 mutant neural retina displays disruptions (white asterisks) and ectopic non-polarized clusters of junction-associated ZO-1 bundles throughout the entire tissue (white arrowhead). (B) Embryos expressing Nokwt protein within the nokm520 mutant background display WT retinal polarity. The Nok domains thought to be required for interaction with (C) Patj, (D) Par6, or (F) Crb, as well as (E) the C-terminus of Nok are essential for retinal polarity. All of these deletion mutants display the characteristic noks305 mutant phenotype suggesting that Nok functions as an important scaffold for a highly conserved assembly of Crb and Par6-aPKC complex proteins within this tissue.
polarization and maintenance of zebrafish embryonic epithelia. The murine Pals1 N-terminal bipartite L27 domains (L27N and L27C) mediate binding to Patj or Lin-7, respectively (Doerks et al., 2000; Roh et al., 2002). In zebrafish, there are three Lin-7 homologs (Wei et al., 2006), but only a single Patj homolog (N.B.-A. and S.A.-S., unpublished results), none of which has been characterized functionally. We performed a functional complementation analysis by overexpressing His-Myc-tagged Nok\(\Delta^{\text{PDZ,L}}\) or Nok\(\Delta^{\gamma}\) in nok mutants and morphants. We found that loss of the L27 domain does not affect RPE integrity (Fig. 2E,H; and data not shown) or early heart morphogenesis (Fig. 3E,G; and data not shown). By contrast, the L27 domains that mediate Nok binding to Patj or Lin-7 appeared to be essential for correct body form (Fig. 2E,H) and for neural retinal polarity and integrity (n=0/6 embryos rescued; Fig. 4C).

Association of Drosophila Sdt and murine Pals1 with the intracellular tail of the transmembrane protein Crb is mediated via their PDZ domains (Bachmann et al., 2001; Hong et al., 2001; Roh et al., 2002). We assayed the functional importance of the Nok PDZ domain by testing whether nok\(\Delta^{\text{Crb}}\) mRNA, which encodes a protein lacking the PDZ domain, could complement nok mutants or morphants. Although the truncated His-Myc-tagged protein could be detected on western blot (Fig. 1B), as well as on tissue sections of the neural retina (Fig. 4F), none of the mutant phenotypes was rescued (Fig 2F,H, Fig. 3F,G; for the retina phenotype n=0/6 embryos were rescued, Fig. 4F). Therefore, the PDZ-domain-mediated association of Nok with partner proteins is essential for the correct polarity and integrity of all epithelia analyzed.

Similarly, injection of nok\(\gamma\) mutant mRNA encoding a His-Myc-tagged C-terminal truncation of Nok that deletes the GUK, HOOK and parts of the SH3 domain, failed to rescue any of the phenotypes assayed, although Nok\(\gamma\) protein could be detected on western blot and stained tissues (Fig. 1B, Fig. 2G,H; for the retina phenotype n=0/6 embryos were rescued, Fig. 4E; and data not shown). This finding confirms that C-terminal truncation renders Nok inactive. Together, these results demonstrate that different embryonic epithelia utilize divergent Nok-mediated mechanisms for tissue polarization. Whereas correct polarization of the neural retina requires each of the Nok protein-protein interaction domains, myocardial polarity is not dependent on the two Nok L27 interaction domains that are thought to mediate association with Patj or Lin7. Moreover, integrity of the RPE does not require the Nok ECR1 domain, which is thought to mediate the association with Par6 or the two Nok L27 domains that mediate association with Patj or Lin7. Similarly, correct body form is not dependent on the expected direct interactions between Par6 and Nok via the ECR1 domain. By contrast, the Nok PDZ domain, which is thought to mediate association with Crb, is essential for polarization of all embryonic epithelia tested.

Essential interactions between Nok and Ome/Crb2a in the maintenance of myocardial apical junctions

To define domains responsible for tethering Nok to apical junctions, we analyzed the subcellular distribution of the different HisMyc-tagged Nok deletion proteins at gastrula stages (6 hpf) and within myocardial cell junctions (34 hpf) by fluorescence immunohistochemistry. During late gastrula stages, the different Nok deletion proteins colocalized with aPKC at apical junctions of the superficial enveloping layer (EVL) of WT embryos (supplementary material Fig. S1). In contrast to our finding that the PDZ-domain-mediated association of Nok with Crb is functionally essential for the polarization of epithelia, Nok\(^{\text{Acrb}}\) correctly associated with the apical membrane, which suggests that the presence of WT Nok protein stabilizes protein complexes containing this deletion protein. Therefore, in the WT, apical association is not completely disrupted in any of the deletion mutants tested at this stage.

To determine the localization of Nok deletion proteins within myocardial tissue, hearts of 34 hpf transgenic [Tg(cmlc2:GFP);nok\(^{\gamma}\)] mutant embryos were stained with anti-Nok antibody (raised against the N-terminal 200 residues, which include the ECR1 domain and parts of the L27N domain) (Bit-Avragim et al., 2008) and anti-ZO-1 antibodies. In nok\(^{\gamma}\) myocardial cells, ZO-1-positive adhesion junctions were lost and endogenous Nok protein could not be detected (Fig. 5A). As expected for Nok\(^{\text{wt}}\) and Nok\(^{\Delta^{\text{PDZ,L}}}\) proteins, which partially complement nok mutant or morphant cardiac morphogenesis defects, myocardial cells had a WT appearance and were correctly polarized, exhibiting ZO-1-positive and Nok-positive adhesion junction belts throughout the myocardial layer (Fig. 5B,C arrowheads). Therefore, association of Nok with Patj or Lin-7, which is thought to be mediated via the two L27 domains, is not necessary for apical localization of Nok and for the maintenance of apical junctions within myocardial cells. This finding is consistent with our observation that cardiac morphogenesis does not require the two L27 domains of Nok.

By contrast, and corresponding with their failure to complement heart morphogenesis defects, His-Myc-tagged Nok\(^{\Delta^{\text{PAJ,L}}}\), Nok\(^{\Delta^{\gamma}}\) and Nok\(^{\gamma}\) proteins did not localize to myocardial cell membranes but were enriched within the cytoplasm of myocardial cells (Fig. 5D-F). Similarly, ZO-1-positive junction belts were lost throughout the myocardial layer in these mutant tissues. Together, these observations suggest that stabilization of ZO-1 junctional belts within myocardial cells and localization of Nok to myocardial cell junctions requires ECR1, PDZ or C-terminal-domain-mediated protein-protein interactions.

In order to determine whether apical localization of Nok requires Crb, we analyzed the distribution of endogenous Nok protein in ome/crb2a\(^{m389}\) mutants. Whereas endogenous Nok and ZO-1 localized to apical junction belts within WT myocardial tissue (Fig. 5G), both proteins were mislocalized and not distributed in a polarized fashion in ome/crb2a\(^{m269}\) mutants (Fig. 5H). Together, these results suggest that the Nok PDZ-domain-mediated interaction with Crb proteins is essential for the correct polarization of myocardial cells and that Ome/Crb2a is required for the correct tethering or localization of Nok to apical junctions within this tissue at 34 hpf.

Apical localization of Pard6\(^{\beta}\) within the neural tube does not require association with Nok or Crb via the Par6 PDZ domain

Our observation that the Nok ECR1 domain, which is thought to mediate association with Par6 proteins, is functionally dispensable both within the RPE and for straight body form, suggests that Par6 proteins are not essential scaffolding partners for Nok within these tissues. Par6 proteins can bind murine Pals1 via the PDZ domain (Hurd et al., 2003; Wang et al., 2004). Binding of Par6 to Crb proteins is also mediated via the PDZ domain (Hurd et al., 2003; Lemmers et al., 2004; Kempkens et al., 2006).

Mutation of zebrafish pard6\(^{\beta}\) causes phenotypes similar to nok and has/pkrclc mutants within myocardial cells, the RPE and the neural tube (C.M. and D.Y.S.S., unpublished results). To test whether the Pard6\(^{\beta}\) PDZ domain is essential for apical localization,
we generated a mutant Pard6γ construct that lacks several essential residues within the PDZ-binding domain that have been shown to be essential for Par6 PDZ-domain-mediated association with other proteins (Patj ΔGUK). The subcellular localization of Pard6γΔGUK was determined by expressing N-terminal fusions of eGFP with Pard6γΔ3 or Pard6γΔGUK. H2B-mRFP mRNA co-injection was used to label nuclei (Magebon and Fraser, 2003). Similarly to Pard6γ ΔGUK, Pard6γΔGUK-GFP primarily localized to the apical surface of the neural tube in WT or pard6γ bwt Δ441 mutant embryos (Fig. 6A-C). Therefore, apical localization of Pard6γ within the neural tube does not require direct association with Crb or Nok via its PDZ domain. This finding suggests that Pard6γ is not an essential scaffolding partner for Nok within this tissue.

To test whether apical localization of Pard6γ within the neural tube is indeed independent of Nok or Ome/Crb2a, we expressed Pard6γΔGUK in nokm520 (Fig. 6E) or ome/crb2am289 mutants (Fig. 6F) and found that it failed to localize to the apical surface of the neural tube. Similarly, loss of Ome/Crb2a caused the mislocalization of Nok within the neural tube (Fig. 6H). Therefore, apical localization of Pard6γ within the neural tube is independent of PDZ-domain-mediated interactions but requires the presence of Nok or Ome/Crb2a. This observation suggests that apical tethering of Pard6γ within the neural tube is mediated by alternative binding partners.

**Discussion**

Our results provide the first systematic structure-function characterization of protein-protein interaction domains of the Crumbs complex protein Nok during development. Our analysis suggests that distinct multi-protein assemblies of Crb-Nok and Par6-aPKC function in the polarization of different zebrafish embryonic epithelia.

**The Nok PDZ domain is generally essential for epithelial integrity**

We have shown that apical junctional localization of Nok within the neural tube and myocardium requires Ome/Crb2a. Localization of the respective Nok deletion protein to apical myocardial cell junctions is impaired, which is consistent with an essential requirement for the Nok PDZ domain to mediate interactions with Crb proteins. Moreover, maintenance of the epithelial integrity of all embryonic tissues assayed in this study depends on the presence of the Nok PDZ domain. Our findings add further support to the idea that PDZ-domain-mediated interactions between Drosophila Sdt or murine Pals1 and Crb are generally essential for epithelial maintenance (Bachmann et al., 2001; Hong et al., 2001; Roh et al., 2002).

**Essential role of the Nok GUK domain in epithelial polarity**

We have shown that zebrafish NokAC localizes apically during gastrula stages, but fails to associate with the apical membrane of myocardial cells at later stages and to complement epithelial polarity defects within this tissue. Since NokAC is not functional in any of the tissues assayed, the C-terminus may be required to stabilize the protein. In Drosophila, the mutation in sdt NPK is thought to result in a premature stop codon that produces a truncated protein lacking the GUK domain, similarly to the NokAC deletion mutant used in our study (Hong et al., 2001). The truncated Drosophila Sdt protein localizes correctly within embryonic photoreceptor cells but fails to associate with the membrane in pupal and adult photoreceptor cells. In line with these findings, the C-terminus of mammalian Pals1 has been shown to be essential for tight junction formation and E-cadherin trafficking in a calcium-switch assay (Wang et al., 2007). Intramolecular conformational rearrangement caused by binding of GUK-4.1/Hook-SH3 has been predicted to
be a common feature of MAGUK proteins (Nix et al., 2000; Tavares et al., 2001). Additional members of the membrane palmitoylated protein family (Mpp) have been shown to interact with Mpp5 through the GUK domain. Mpp5 can recruit Mpp4 into the apical Crb1 complex via physical interaction between its GUK domain and the SH3 domain of Mpp4 (Kantardzhieva et al., 2005). Mpp3, another Mpp family member with potentially redundant epithelial function, is recruited to the retinal outer-limiting membrane by Mpp5 (Kantardzhieva et al., 2006). Taking these observations into account, our results suggest that the Nok GUK domain is essential for epithelial integrity.

Evidence for alternative modes of Pard6γb localization within the zebrafish neural tube and myocardium

Our results demonstrate that the localization of zebrafish Pard6γb within the neural tube depends on Ome/Crb2a and Nok. However, apical Pard6γb membrane association within this tissue is not critically dependent on its PDZ motif, which has been shown to mediate direct interactions with Crb or murine Pals1 (Hurd et al., 2003; Lemmers et al., 2004; Wang et al., 2004; Kempkens et al., 2006). This finding underlines the importance of alternative protein-protein interactions of this multi-domain linker protein. The N-terminus of Drosophila Par6 has been shown to interact with one PDZ domain of Drosophila Patj, and this interaction might also be involved in stabilizing zebrafish Pard6γb at the apical membrane of neural tube cells (Nam and Choi, 2003). Since the Pard6γb PDZ domain is not required for apical localization of the protein and

Fig. 6. Apical localization of Pard6γb within the neural tube does not require the PDZ domain. (A-C) Live 24 hpf embryos injected with H2b-mRFP and pard6γbwt-GFP or pard6γbaKPLG-GFP mRNA as indicated (dorsal views of the neurocoel between the first and sixth somite). (A,B) In WT, both pard6γbwt-GFP and pard6γbaKPLG-GFP localize along the apical ventricular surface of the neural tube (white arrowheads). (C) Within the pard6γbS441 mutant background, pard6γbaKPLG-GFP localizes correctly to apical surfaces of the neural tube suggesting alternative modes of apical localization of pard6γb independent of the PDZ-domain-mediated association with Crb or Nok. (D-F) Localization of pard6γbaKPLG-GFP within the neural tube is dependent on either (E) Nok or (F) Ome/Crb2a. (H) Apical localization of Nok requires Ome/Crb2a. Orientation: all images are apical views.

Fig. 7. Alternative multi-protein assemblies of the Crb and Par6-aPKC complexes at the tight junction. Model indicating essential protein-protein interaction partners of Nok within different tissues. Protein-binding domains that are dispensable for Nok function are white. The C-terminal GUK and SH3 domains and the PDZ domain, which is thought to mediate the interaction with Crb proteins, are generally essential within each epithelial tissue. (A) Each of the Nok interaction domains thought to be required for association with Crb, Par6, Patj or Lin-7 is essential for correct polarity of the neural retina, indicating that a highly conserved assembly of Crb and Par6-aPKC complex proteins is present within this tissue. (B) Within the neural tube, interaction with Patj via the L27(N) domain is essential for epithelial integrity. The apical localization of Par6 is independent of the Nok ECR1 domain, which suggests that Par6 associates with Patj within this tissue (see text for further discussion). (C) Association of Nok with Patj or Lin-7 via the two L27 domains is not essential for maintenance of apical ZO-1 junction belts within myocardial cells and for correct myocardial morphogenesis. Therefore, an alternative multi-protein assembly that requires interaction with Par6 via the ECR1 domain may be present within myocardial cells (see text for further discussion). (D) Within RPE cells, Nok requires the PDZ-domain, which is thought to mediate the interaction with Crb, to confer correct polarity to this tissue. Integrity of the RPE is not dependent on the presence of the Nok ECR1 domain (association with Par6) or the two L27 domains (association with Patj and Lin-7). It remains to be investigated whether alternative binding sites are utilized to tether Par6, Lin-7 or Patj to Crb-Nok or whether these proteins are not essential for the maintenance of the Crb complex within the RPE.
because the Nok L27(N) domain, which presumably interacts with Patj, is required for neural tube integrity (as indicated by the severe body curvature in mutants), Par6βγ may directly associate with Patj within neural tube cells. In turn, Patj, which thus functions as a scaffold for Par6-aPKC complex proteins, attaches to the L27(N) domain of Nok. The Nok PDZ-domain-mediated interaction with the transmembrane protein Ome/Crb2a may tether the entire multi-protein complex to the apical membrane (Fig. 7B).

In myocardial cells, deletion of the Nok bipartite L27 domains complements the nok loss-of-function phenotype and stabilizes ZO-1 junctional belts to which the respective Nok deletion protein correctly localizes. Therefore, within myocardial cells, unlike in neural tube cells, Patj (and Lin7) may not be essential to stabilize protein complexes containing Nok. This suggests that, either the attachment of Patj to Crb proteins is via a distinct physical interaction or Patj is not functional in myocardial cells. Consistent with this finding and supporting the idea of an alternative mode of protein-protein interactions, the Nok ECR1 domain, predicted to mediate direct interactions with Par6 proteins (Wang et al., 2004), is essential for myocardial tissue integrity. Thus, our results suggest that within myocardial cells, direct interactions between the Par6-aPKC complex via the Nok ECR1 domain are essential and stabilize the entire multi-protein complex at the apical membrane through the Nok PDZ-domain-mediated attachment to Ome/Crb2a (Fig. 7C). Consequently, via the Nok ECR1 domain are essential and stabilize the entire multi-protein complex within neural retinal cells (Fig. 7A).

Nok requires the ECR1 and L27 domains for retinal polarity. Members of the MAGUK protein family are ideally suited for the modulation of membrane-associated multi-protein assemblies because of the presence of multiple protein-protein interaction domains. Our structure-function analysis of Nok protein-protein interaction domains has revealed that the ECR1 domain, which is thought to mediate interactions with Par6 proteins, and the L27 domain, which may mediate interactions with Patj and Lin7, are both required for correct retinal tissue polarity. Therefore, direct interactions between Nok and Par6 proteins as well as interactions between Nok and Patj appear to be essential within this tissue. The PDZ domains of Drosophila Sdt and murine Pals1, as well as that of Par6, have been shown to directly associate with the ERL1 motif of Crb in vitro (Bachmann et al., 2001; Hong et al., 2001; Roh et al., 2002; Hurd et al., 2003; Lemmers et al., 2004). Moreover, similar calculated binding affinities between Crumbs and Sdt or Drosophila Par6 have recently been demonstrated (Kempkens et al., 2006).

DNA constructs and site-directed mutagenesis
The NokE1 (a.a. 1-703), NokE2 (a.a. 1-1429) and NokL7 (a.a. 151-265) domain were produced by PCR amplification from a full-length cDNA template, introducing *XhoI* and *HfiI* restriction sites at the 5′ and 3′, respectively, and constructs were subsequently subcloned into the pCS2 ′His-Myc expression vector (Rohr et al., 2006). For the other Nok-truncated proteins, the following residues were deleted: NokΔ293-364, NokΔ54-64, NokΔ360-477, NokΔ151-265, NokΔ293-364, NokΔ54-64, NokΔ360-477. Primer sequences are available upon request. The *h2b:mrfp* construct was kindly provided by S. G. Megason (Megason and Fraser, 2003).

Synthesis of par6βγ mutant and fusion proteins
Based on the published sequence of par6βγ (accession number NM_212563), we designed PCR primers to amplify full-length cDNA. Additional PCR primers with incorporated restriction enzyme sequences allowed us to place these full-length cDNA sequences into the pCS2 ′ vector. The KPLG mutation was introduced by site-directed mutagenesis (primer sequences are available upon request). For fusion proteins, GFP followed by a short amino acid sequence (S-G-G-G-G-S) was placed into the existing expression constructs at the 5′ end of the coding sequence.

Injections of mRNA and antisense oligonucleotide morpholinos
 Constructs were transcribed using the SP6 MessageMachine kit (Ambion). For rescue experiments, nokΔ293(ch:mgc2a:GFP) embryos were injected with 50-75 pg mRNA. For overexpression (to determine the subcellular localization patterns), 75-100 pg mRNA were used. For MO injections, the following concentrations were used: 200 μM (MO3), 150 μM (MO5), 5′-TCACGAGGGCTCAGCAGACACTGA-3′, MO3, 5′-GCCCATGGTTTGAAGAATGG-3′.

Antibodies, immunohistochemistry and sections
Antibodies were raised against amino acids 1-200 of Nok. Appropriate primers were used to amplify the cDNA encoding this portion of the protein and to clone the fragment into pET23/T7, a modified pET23a vector (Obermann et al., 1998). The His-tagged protein was expressed in *Escherichia coli* (Chumpia et al., 2003). Rabbits were immunized four times with the purified His-Nok fusion protein according to a standard protocol (Biogenes, Berlin, Germany).

**Nok**Δ293, Δ54-64, Δ360-477 behaves like a complete, or almost complete, loss-of-function allele (Rohr et al., 2006).

Materials and Methods
Fish maintenance and stocks
Zebrafish were maintained under standard conditions (Westerfield, 1994). Embryos were staged by hpf at 28.5°C (Kimmel et al., 1995). The following fish strains were used: wild-type AB, *Tg(erm-1-Z/FP) (Huang et al., 2003)*, *nokΔ293*, *nokΔ54-64* (Roh et al., 2006), *omeCΔ293* (Omori and Malicki, 2006) and *pard6Δ*mutant (C. M. and D. V.S.S., unpublished results). The nokΔ151-265 mutation causes a premature truncation of the protein that deletes the entire GUK domain (X. Wei, personal communication). Genetically, nokΔ151-265 behaves like a complete, or almost complete, loss-of-function allele (Rohr et al., 2006).

**DNA constructs and site-directed mutagenesis**
The NokE1 (a.a. 1-703), NokE2 (a.a. 1-1429) and NokL7 (a.a. 151-265) domain were produced by PCR amplification from a full-length cDNA template, introducing *XhoI* and *HfiI* restriction sites at the 5′ and 3′, respectively, and constructs were subsequently subcloned into the pCS2 ′His-Myc expression vector (Rohr et al., 2006). For the other Nok-truncated proteins, the following residues were deleted: NokΔ293-364, NokΔ54-64, NokΔ360-477, NokΔ151-265, NokΔ293-364, NokΔ54-64, NokΔ360-477. Primer sequences are available upon request. For fusion proteins, GFP followed by a short amino acid sequence (S-G-G-G-G-S) was placed into the existing expression constructs at the 5′ end of the coding sequence.

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Specific antibodies were affinity purified using Ni-NTA agarose beads (Sigma) essentially as described (Chumpia et al., 2003).

Immunohistochemistry was performed as previously described (Horne-Badovinac et al., 2001). The following antibodies were used: rabbit anti-aPKCζ (1:100, Santa Cruz Biotechnology), mouse anti-Myc (1:200, Invitrogen), 546 (1:20, Developmental Studies Hybridoma Bank), mouse anti-ZO-1 (Zymed Laboratories, Invitrogen), goat anti-rabbit Cy5 (1:200), anti-mouse FITC (1:200) (Jackson Immunoresearch). For sectioning, stained embryos were postfixed overnight at 4°C in 2% paraformaldehyde, 0.3 M sucrose. Embryos were embedded in 6 μM glycerine-HCl and purified as described (Chumpia et al., 2003). Rabbits were immunized four times with the purified recombinant protein according to a standard protocol (Biogenes, Berlin, Germany). Specific antibodies were affinity purified using Ni-NTA agarose beads (Sigma) essentially as described (Chumpia et al., 2003).

**Western blot analysis**
Protein extracts of 24 hpf zebrafish embryos were prepared according to standard protocols (Westerfield, 1994). Membranes were probed with mouse anti-Myc antibody (1:1000, Invitrogen). For loading control, membranes were stripped and tested for acetylated-tubulin (mouse anti-acetylated tubulin, 1:1000, Sigma).

**nokΔ293 genotyping**
For genotyping of rescued nokΔ293 embryos, we made use of a suitable restriction site that is deleted by the mutation and performed PCR on tail tissue followed by a Sall digest. DNA primers used for genotyping are available upon request.
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Note added in proof

Similar to our findings, Bulgakova et al. recently showed that, in the Drosophila eye, different domains of the Nagie oko orthologue Stardust are differentially required for its apical localization in pupal and adult photoreceptor cells, and that the assembly and stability of the Crb-Sdt complex relies on different regulatory processes at different developmental stages (Bulgakova et al., 2008).

References


